Changes of endoplasmic reticulum chaperone complexes, redox state, and impaired protein disulfide reductase activity in misfolding α1-antitrypsin transgenic mice

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We wanted to elucidate how stress proteins of the endoplasmic reticulum (ER), their complexes, and function change during chronic stress, such as protein aggregation using a transgenic mouse model, which overexpresses the mutant human α1-antitrypsin (PiZ) protein forming insoluble aggregates in the ER of the liver cells.

PRINCIPAL FINDINGS

1. Examining the stress response in the ER, we found that calnexin, Grp78, Grp94, and PDI were at the same concentration in PiZ transgenic and in age- and gender-matched control mice, which had the same genetic background but did not express the mutant protein.

2. We found an elevated concentration of the cytoplasmic chaperones, Hsp70 and Hsp90, and the antioxidant enzyme, thioredoxin in PiZ animals compared to control mice.

3. Immunoprecipitation with AAT antibodies showed that most of the ER chaperones (such as Grp78, Grp94, calnexin, and the protein disulfide isomerase family member ERp72) did not bind to the PiZ variant of AAT.

4. The same set of experiments showed that the 58 kDa protein disulfide isomerase protein (PDI), the most abundant disulfide isomerase and chaperone of the ER coprecipitated with the PiZ protein even after the reduction of the samples. During PDI immunoprecipitation, a high amount PDI remained unbound to the immunoprecipitational construct in PiZ transgenic mice, and remained in the supernatant.

5. PDI and Grp94 immunoprecipitation experiments showed that the Grp94-PDI complex, which is a typical component of the protein folding machinery, is formed in lower amount in PiZ transgenic mice.

6. Because oxidative stress often prevails in folding diseases, we checked the redox parameters of both the ER and the cytoplasm. We found more reduced proteins in the ER of the PiZ transgenic mice, which was accompanied with a higher glutathione/oxidized glutathione (GSH/GSSG) ratio, and an increased total GSH amount.

7. In contrast, the cytoplasmic redox parameters did not show the signs of abnormality in the concentration of protein thiols. The GSH/GSSG balance showed a slight, but not significant shift toward the more oxidizing state, despite the elevated concentration of total GSH.

8. With the help of a redox-sensitive dye, AMS, we proved that PDI is in a more reduced state in PiZ transgenic mice than in control animals, while the redox state of other, disulfide containing chaperones of the ER (such as ERp72, Grp94, and calnexin) remained unchanged.

9. To get functional data about the efficiency of the microsomal redox folding machinery, we prepared a fluorescent substrate for PDIs, difluorescrein-thiocarbamyl-insulin (di-FiTZ insulin). Our measurements showed that the protein disulfide reductase activity (PDR) of the ER is significantly reduced in PiZ transgenic mice than in control animals (Fig. 2).

CONCLUSIONS AND SIGNIFICANCE

The results of our investigations show, that chronic protein aggregation does not provoke the elevation

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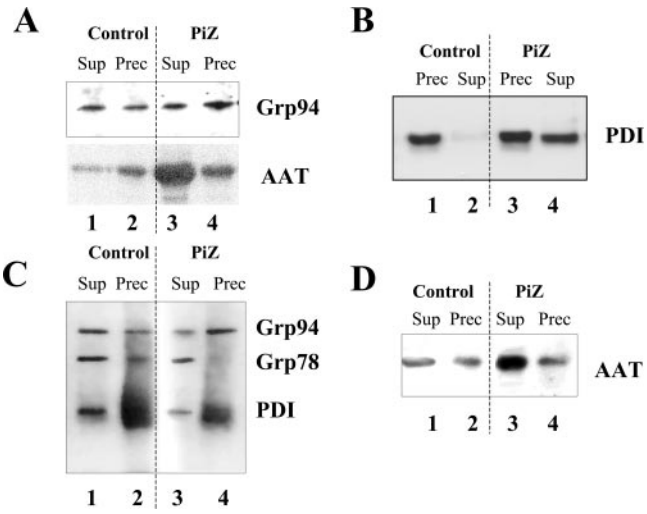

Figure 1. Immunoprecipitation with chaperone antibodies. Immunoprecipitation of 80 μg of microsomal proteins from control (Control) and PiZ transgenic (PiZ) mice and PAGE of the samples were performed as described in Materials and Methods using PDI antibody (Ab)-covered *(A* and *B)* or Grp94 Ab-covered *(C* and *D)* Sepharose beads. "Sup" refers to the supernatant (not bound proteins) of the immunoprecipitates, whereas "Prec" denotes the proteins remained in the pellet of the immunoprecipitates. Western blot membranes were developed with specific antibodies against the proteins indicated on the right side *(A, B,* and *D)* or with a specific Ab against the KDEL sequence *(C).* Representatives of three independent experiments are presented.

of the major stress proteins participating in unfolded protein response (UPR) in PiZ transgenic mice. This is in good correlation with previous findings on PiZ mice, cell lines, and also on human patients suffering from PiZ aggregation. In cell lines, Grp94 and Grp78, as well as calnexin were found to bind PiZ, but these complexes gradually disappeared with passage of time after PiZ transfection. Because in our model, the aggregation is more chronic, it is not surprising, that the detachment of these early-phase foldases from PiZ became already complete in the approximately 5-mo-old transgenic animals that we examined. PDI, which was not investigated earlier, was found attached to the PiZ complexes. This is in good agreement with the appearance of PDI in lysosyme aggregates. Because PDI is the chaperone, which holds misfolded proteins until their reverse transport and degradation, it is not surprising, that this is the protein, which remained bound to the PiZ complexes, probably helping their unfolding and reverse transport toward the proteasomal degradation system. Because the protein disulfide reductase (PDR) activity is provided predominantly by PDI in the ER, its decrease in PiZ transgenic mice may reflect a decreased availability of PDI, probably due to the sequestration of PDI by the protein aggregates. In addition, a redox-dependent switch of PDI activity has been proposed, that is, PDI acts as a chaperone rather than a disulfide isomerase in its reduced state. Thus the shift of ER redox potential toward a reduced state and the reduction of PDI disulfides, as well as the decreased availability of PDI, may explain the PDR deficiency of PiZ transgenic mice. Whether the higher amount of reduced proteins in the ER and the shift in the GSH/GSSG ratio found in PiZ transgenic mice is a prerequisite to keep PDI in reduced form or whether it is a consequence of the decreased oxidizing power of the redox foldase machinery remains an open question. The fact that in chronic diseases, in which the accumulation of proteins does not occur, such as diabetes, where similar redox shift was found in the ER of the liver cells, supports the idea that the onset of the more reducing environment is a beneficial, protecting mechanism rather than a simple consequence. This is supported further by the finding, that cells with hypo-oxidizing ER can avoid apoptosis better than normal ones.

Hsp90 and Hsp70 are both usually induced during the ERAD process, to protect the proteasome and avoid oxidative stress and aggregation of denatured proteins. The proteasome plays an important role in PiZ degradation. This may explain the elevation of Hsp90 and Hsp70 in PiZ transgenic mice. Both the lower efficiency of the ER-folding machinery and PiZ protein degradation can lead to an increased cytoplasmic oxidative stress. This may explain the increase in the amount of the antioxidant thioredoxin in the cytoplasm.

As a conclusion, our data suggest that in chronic ER stress, such as in PiZ transgenic mice, different protective pathways are activated than in short-term ER stress. The redox changes, the decreased PDR

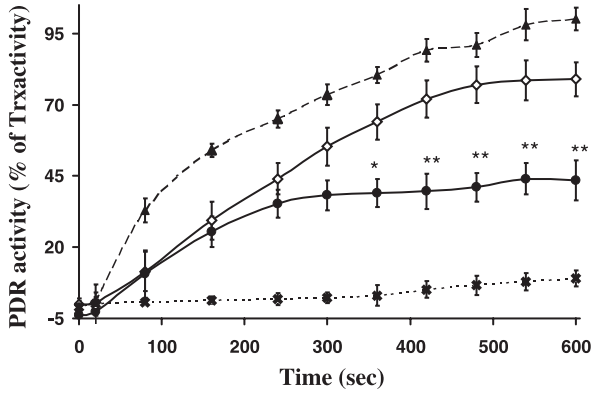

Figure 2. Protein disulfide reductase (PDR) activity of the endoplasmic reticulum. For protein disulfide reductase (PDR) activity measurements 75 μg microsomal proteins were added to 1 μM di-FiTZ insulin solution in 5 mM Tris·HCl buffer in the presence of a 5:1 GSH/GSSG redox buffer (final GSH concentration 2 mM), pH 7.2 and the enhancement of fluorescence was measured as described in the Materials and Methods. Open diamonds or filled circles represent microsomal samples from control or PiZ transgenic mice, respectively. Dashed line represents the positive control in the presence of 5 mM DTT; the dotted line represents the negative control with buffer only. All data are expressed as a percentage of fluorescence reached by thioredoxin (Trx). Means ± SD of 8 independent experiments with 6 mice per data set are presented. *Significant change compared with control, $P < 0.05$. **Significant change compared to control, $P < 0.01$.

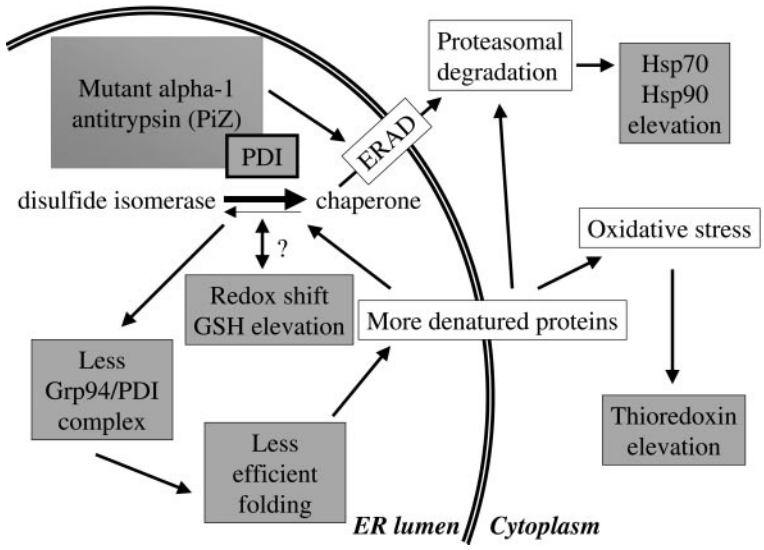

Figure 3. Scheme detailing the long-term adaptive response in the livers of mutant human α1-antitrypsin (PiZ) transgenic mice. In the endoplasmic reticulum (ER) the excess of the aggregating PiZ protein induces a shift in the function of the protein disulfide isomerase (PDI), decreasing the protein disulfide reductase activity. This is accompanied with a shift in the redox balance of the ER toward a more reduced state, a change in the ER chaperone complexes, and signs of cytoplasmic stress and antioxidant response.

activity and the differences in chaperone complexes in the ER, as well as chaperone and antioxidant enzyme induction in the cytoplasm suggest a long-term adaptive response, which sacrifices efficient protein folding for the sake of long-term survival (**Fig. 3**). Our results shed a novel light in the molecular background of the pathophysiology of folding diseases in a complex model of transgenic animals. FJ

Changes of endoplasmic reticulum chaperone complexes, redox state, and impaired protein disulfide reductase activity in misfolding α1-antitrypsin transgenic mice

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ABSTRACT **α1-antitrypsin (AAT) deficiency is characterized by the accumulation of the misfolded mutant, Z form of α1-antitrypsin (PiZ) inside the lumen of the hepatic endoplasmic reticulum (ER). Both human patients and PiZ transgenic mice have similar symptoms of hepatic failure culminating in cirrhosis and hepatocellular carcinoma. The involvement of molecular chaperones, as well as the relevance of oxidative stress in this disease is not characterized well yet. Here, we show that, in the PiZ transgenic mice, the 58-kDa protein disulfide isomerase (PDI), the most important oxidoreductase and chaperone of the endoplasmic reticulum, is in a complex with PiZ, which is accompanied by a decrease of protein disulfide reductase activity of the ER. PiZ transgenic mice have a shift toward a more reduced ER environment and an elevation of cytoplasmic chaperones and antioxidant enzymes. Our data suggest that lower availability of PDI and a decreased protein disulfide reductase activity of the ER along with a cytoplasmic stress may contribute to the toxic effects of PiZ aggregation.—Papp E., Száraz P., Korcsmáros T., and Csermely P. Changes of endoplasmic reticulum chaperone complexes, redox state, and impaired protein disulfide reductase activity in misfolding α1-antitrypsin transgenic mice. *FASEB J.* 20, E235–E244 (2006)**

Key Words: glucose regulated proteins · heat shock proteins · oxidative stress · protein aggregation · redox imbalance

α1-ANTITRYPSIN (AAT) DEFICIENCY is one of the common causes of neonatal liver disease and emphysema (1). The genetic background of this conformational disease is a point mutation in the gene of AAT at position 342 resulting in a change of the amino acid glutamate to lysine, which induces a loop-sheet polymerization of the protein inside the liver cells (2). This mutation is often referred as Hong Kong-type or Z-type mutation (PiZ). Both human patients and transgenic mice carrying the mutant human AAT (PiZ transgenic mice) are suffering from liver cirrhosis, sterile hepatitis, and are more prone to get hepatocellular carcinoma (3–5). PiZ transgenic mice have a similar life span to the healthy ones but are more sensitive to different interventions, such as fasting (6). The toxicity of this pathological state is well known; however, its mechanism is poorly understood (3).

PiZ accumulates within the endoplasmic reticulum of the liver cells (2). Because this organelle is the site of the folding of many membrane, and secretory proteins, including several major proteins of the plasma, the state of the hepatic endoplasmic reticulum is a key question of the overall fitness of the cell and eventually of the whole organism.

Molecular chaperones assist protein folding. The relationship between PiZ accumulation and molecular chaperone action was recently addressed (7,8). In one of the studies no up-regulation of the major stress proteins of the endoplasmic reticulum, Grp78 and Grp94, was detected using PiZ-transfected CHO or HEK293 cell lines. It is worthwhile to mention that these PiZ-expressing cells were highly sensitive to heat and ER stress, if compared to the nontransfected cells, as well as cells transfected with normal AAT (7). When ER chaperone complexes were examined in PiZ-expressing fibroblasts, Grp78 and Grp94 were found in a complex of the soluble fraction of PiZ (8). In contrast, Grp78 was present in neither the PiZ aggregates of a PiZ transgenic mouse model (9), nor those of human patients (10). The interaction of calnexin with PiZ during the early steps of synthesis was demonstrated (11). Other stress proteins, such as Grp170 and uridine diphosphate (UDP)-glucose: glycoprotein glucosyltransferase in different cell lines were also interacting with a small fraction of PiZ (8). Signs of the unfolded protein response were found neither in a stably transfected cell line, nor in transgenic mice as was published by Hidvegi et al. recently (12).

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Links between redox changes and protein folding defects in various diseases are receiving a revived attention these days. In diabetes the change toward a more oxidizing state regarding the whole organism is accompanied with a shift toward a reducing state in the lumen of the endoplasmic reticulum (13). In many conformational diseases, the relevance of the oxidative stress has already been proven. Oxidative changes are common features of Alzheimer disease, Parkinson disease (14,15), diabetic neuropathy (16), and in many other diseases that have been also found to be related to protein aggregation (16).

Inside the ER, the major oxidant of the proteins is the 58-kDa protein disulfide isomerase, PDI (17). PDI mediates disulfide formation, isomerization, or reduction depending on its redox state (18). Besides its redox foldase function, PDI serves as a chaperone, and it is suggested that the ratio of redox foldase and chaperone activities changes as a function of its redox state (19). PDI is needed for the ER protein degradation mechanism, the ERAD (20). Cysteine-free proteins also need PDI for their secretion, showing its importance as a bona fide chaperone (21).

ER chaperones fulfill multiple roles depending on the actual state of the cell. A Grp94-centered chaperone complex is formed during protein synthesis from Grp78, PDI, ERp72, and other components (22). The elimination of the misfolded proteins depends on the ERAD process, in which calnexin and other lectins, as well as Grp78, PDI, and other chaperones participate, depending on the misfolded protein of question (23).

In the ER the redox balance is in a very sensitive and carefully maintained equilibrium (24). The relatively oxidizing environment is needed for the secretory proteins to gain their native structure before getting out into the extracellular space. The most important ER redox buffer is the glutathione (GSH)/oxidized GSH (GSSG) pair. The GSH:GSSG ratio in the ER is between 1:1 and 1:3 (25). The different PDI activities, like oxidization, isomerization, reduction and bona fide chaperone activity go parallel in the ER and are probably regulated by the redox state of the ER or its respective subcompartment (18).

Despite recent investigations using *in vitro* models, the question remained open, whether chaperones are binding to PiZ in *in vivo* model systems. It is also an important question, whether oxidative stress, which is present in many conformational diseases, accompanies PiZ aggregation acting as a factor contributing to its pathomechanism.

We found no induction of molecular chaperones in the endoplasmic reticulum of PiZ transgenic mice and observed that the most important redox foldase PDI protein disulfide isomerase is bound to PiZ to a great extent. We found a major change in the ability of PDI to bind Grp94, as well as a change in its redox state accompanied with a general change in the redox state of the ER in PiZ transgenic mice compared to control animals. The cytoplasm did not show the signs of oxidative imbalance, but antioxidant enzymes and chaperones were induced in PiZ transgenic mice reflecting their involvement in the pathomechanism of PiZ aggregation. Our data suggest an adaptation mechanism, in which a change of the ER redox environment helps the survival of cells imposed to long-term stress.

MATERIALS AND METHODS

Materials

AMS was purchased from Molecular Probes (Eugene OR, USA). Polyvinylidene difluoride (PVDF) membranes and electrophoresis reagents were from Bio-Rad (Hercules, Mississauga, ON, Canada). PDI (SPA-891), ERp72 (SPA-720), Grp78 (SPA-827), Grp94 (SPA-850), calnexin (SPA-860), Hsp70 (SPA-810) and thioredoxin (MSA-150E) antibodies were purchased from StressGen (Victoria, BC, Canada). KDEL antibody (Ab) (PA1–013), Hsp90 Ab (PA1–013) and Hsp90 Ab (PA3–012) were from Affinity Bioreagents (Golden, CO, USA). Peroxidase-conjugated goat IGG fraction to hAAT (55236) was purchased from ICN (Aurora OH, USA), The MsrA Ab was a kind gift of Bertrand Friguet, from Denis Diderot University, Paris, France (25). Enhanced chemiluminescence (ECL) kit was from the Amersham Pharmacia Biotech (Piscataway, NJ, USA). DTNB and other chemicals were purchased from Sigma (St. Louis, MO, USA).

Animals

Pathogen-free H2k/B6 mice (on C57BL/6 genetic background) carrying the human mutant AAT gene in a heterozygous form (27) were kind gifts from John Rodgers, (Baylor College, Houston TX, USA). Littermates were separated by gender at the age of 14 days, and their genetic status was examined at the age of 2 mo from tail tissue pieces with polymerase chain reaction (PCR) using forward and reverse primers specific to human AAT following the instructions given by John Rodgers Laboratory. All animal experiments were performed according to the rules and regulations of the NIH "Principles of Laboratory Animal Care" and the Hungarian Animal Protection Law under the license no. 25–135/3/2002. For all experiments, age- and gender-matched mice littermates carrying no human AAT were used as controls. Animals aged 5 mo were used for the experiments, as in this age transgenic mice already show marked symptoms (7), but still rarely develop malignant transformations that would change the protein expression patterns and give misleading results.

Microsome isolation

Liver microsomes were prepared from the mice using the method used by Varsanyi et al. (28). Intactness of microsomal vesicles was checked by measuring the change in light scattering after the addition of the nonpermeable compound, sucrose (29). To avoid uncontrolled oxidation, we used fresh samples and degassed, oxygen-free buffers. The samples were kept on ice during the procedures, and parallel samples were used. The protein content of the microsomes was measured following the Bradford method (30) using BSA as a standard.

Measurement of the thiol content of proteins

Subcellular fractions (containing 2.5 mg protein/ml) were diluted in 50 mM Tris·HCl, pH 7.2, and precipitated by 10%

trichloroacetic acid, washed 3 times by 70% acetone, and resuspended in 50 mM Tris·HCl, buffer, pH 6.8, containing 8 M urea and 2% sodium dodecyl sulfate. Thiol content of the resuspended protein fraction was measured by the Ellman method (31) using a Hitachi U-1500 spectrophotometer and applying a molar extinction coefficient of 14,150 for 2-nitro-5-thiobenzoic acid.

Gel electrophoresis and immunoblot

Microsomal or cell lysates containing 40 μg of proteins were dissolved in 0.1 M Tris·HCl, pH 7.2. Proteins were precipitated by 5% trichloroacetic acid, and after centrifugation, the pellet was washed 3 times with 70% acetone and once with washing buffer (20 mM Tris·HCl, pH 7.6). After washing, proteins were dissolved in 50 mM Tris·HCl, pH 6.8 containing 8 M urea and 2% sodium dodecyl sulfate. Proteins were separated on polyacrylamide gel (at acrylamide concentrations indicated at the specific experiments). Membranes were blocked overnight with washing buffer containing 5% nonfat milk powder and then probed with primary antibodies against various antibodies diluted in washing buffer for 1 h at room temperature. After three washes, horseradish peroxidase-conjugated secondary antibodies were added for 30 min. After four additional washes, labeled protein bands were detected using the ECL technique according to the manufacturer's instructions.

Measurement of the redox state of PDI

Microsomal proteins were processed as detailed above. After dissolving the proteins in the urea-SDS containing buffer, samples were treated with 20 mM 4-acetamido-4′maleimidyl-stilbene 2,2′-disulphonic acid (AMS) for 30 min at 37°C using a method based on Frand and Kaiser (32). In brief, AMS binds to the free thiol groups of cysteine residues, resulting in retained run of the proteins during SDS-PAGE. In each experiment, we used the reducing agent and a thiol oxidizer, diamide (5 mM each), to check the efficiency of AMS labeling and the position of maximally reduced and oxidized state of proteins. AMS-labeled proteins (15 μg) were separated by electrophoresis on nonreducing 9% sodium dodecyl sulfate polyacrylamide gels, electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes, and processed for immunoblot as described above.

Immunoprecipitation experiments

Microsomal samples were incubated with an equal volume of Sepharose-protein A for 30 min at 4°C to remove mouse IgG from the samples. Twenty microliters of protein-A Sepharose and 1 to 2 μl of the Ab of interest were incubated in 100 μl of the immunoprecipitation buffer (20 mM Tris·HCl, pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40 with a protease inhibitor mixture) for 60 min, on 4°C. Fifty micrograms microsomal proteins were incubated with the Sepharose-antibody for 2 h to capture the Ab-binding protein complexes. After removing the supernatant (referred as sup. in the experiments), the precipitate was washed with the immunoprecipitating buffer 4 times. Both the supernatant and the immunoprecipitate was mixed with denaturing reducing loading buffer and used for SDS-PAGE and immunoblot following the methods described above. In all experiments Ab-free beads and Ab in itself were used as controls to check unspecific binding. All blotting membranes were stained with Ponceau stain to check equal protein loading. Only those experiments were processed for data evaluation, where loading differences were below detection limit.

Determination of total GSH levels

One-hundred microgram proteins of samples were incubated together with 0.024 ml of 50 mM Tris·HCl (pH 7.2), 2 μl NADPH solution (416.7 μg in 5 μl Tris·HCl), and 1 U of glutathione reductase (diluted with Tris buffer before use, if necessary) for 30 min at 37°C. After exactly 30 min, the reaction was stopped by addition of 0.075 ml 5% trichloroacetic acid; then the mixture was centrifuged at 3,000 *g* for 3 min. One milliliter of the supernatant was transferred into a 10-mM cuvette and mixed with 200 μl of 400 mM Tris·HCl (pH 7.2) and 5 μl Ellmann's reagent solution (20 mM DTNB in 25% DMSO, 75% distilled water). After 5 min, total glutathione (in the form of GSH) was measured at 412 nM with an UV-VIS spectrophotometer.

Determination of reduced glutathione content

Forty-microgram proteins of the respective microsomal or cytoplasmic samples were mixed with 0.04 ml 5% trichloroacetic acid and were centrifuged at 3,000 *g* for 3 min. A 200-μl aliquot of the supernatant was processed for GSH determination following the method described above.

Measurement of microsomal protein disulfide reductase activity

Difluorescein-thiocarbamyl-insulin (di-FiTZ-insulin) was synthesized according to the method of Heuck and Wolosiuk (33). Seventy-five micrograms microsomal proteins were added to the solution of 1 μM di-FiTZ-insulin (in 500 μl 5 μM Tris buffer, pH 7.2) in the presence of a GSH/GSSG buffer using 5:1 ratio and maintaining a final GSH content of 2 mM. Fluorescence was monitored at the excitation/emission wavelengths of 495/520 nM, respectively, using a Hitachi F-4500 fluorescence spectrophotometer for the times indicated. 5 mM DTT and buffer were added to positive and negative controls, respectively.

Statistical analysis

Data are presented as means ± SD of at least three independent experiments unless otherwise indicated, and were analyzed with unpaired Student's *t* test. $P < 0.05$ was accepted as indicating a statistically significant difference compared with controls.

RESULTS

Chaperone and antioxidant protein levels in PiZ transgenic mice

Our first question was, whether the ER chaperones became induced in the liver of PiZ transgenic mice. As shown in **Fig. 1*A***, where a KDEL antipeptide-antibody was used to examine Grp94, Grp78, and PDI levels, which all contain this ER retention signal, no change in the expression of these proteins were detected. Similarly, calnexin, a key chaperone in glycoprotein folding was not induced in PiZ transgenic mice, if compared to

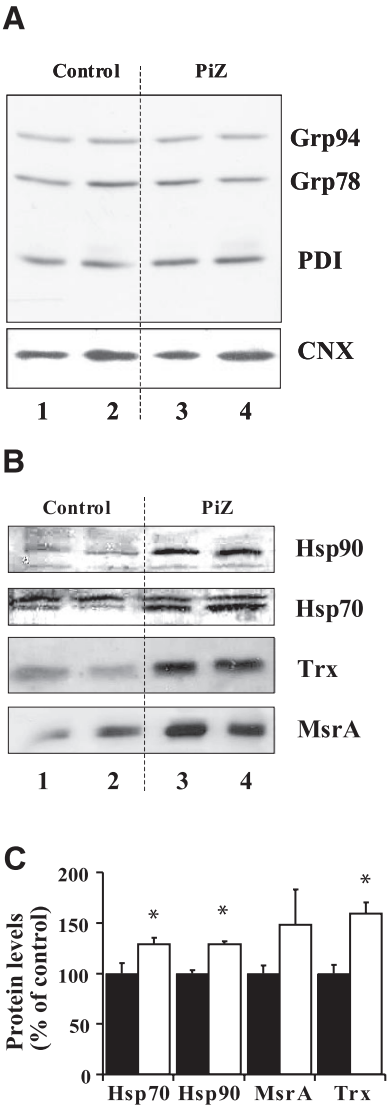

Figure 1. Levels of the chaperones in PiZ transgenic mice. Samples were prepared from microsomes containing 40 μg of proteins (*A*) or from cytoplasmic fractions of the same samples containing 20 μg of proteins (*B*), and were subjected to gel electrophoresis as described in Materials and Methods. In all experiments, 9% polyacrylamide gels were used except thioredoxin (Trx), where a 12% gel was used. Lanes 1 and 2 ("Control") show samples from control littermates of the PiZ transgenic mice. In lanes 3 and 4 ("PiZ"), samples from PiZ transgenic mice are shown. Western blot membranes were developed with specific antibodies against the chaperones indicated as described in Materials and Methods. *A)* Chaperones of the endoplasmic reticulum. *B)* Cytoplasmic proteins. Representatives of at least 3 independent experiments are presented. *C)* Quantitated results of experiments shown in *B* are shown. Data are means ± SD of 3 to 5 independent experiments. Asterisks denote significant ($P<0.05$) difference between control and PiZ transgenic samples.

control mice. PiZ transgenic mice in all the experiments were 5 ± 1 mo old and contained 50% males and 50% females. Control mice here and in all experiments were gender- and age-matched littermates of transgenic mice, that is, had the same genetic background but did not overexpress the mutant form of human AAT.

To our surprise, the levels of the major cytoplasmic chaperones, Hsp90, and Hsp70 were elevated in the case of PiZ transgenic mice (Fig. 1*B*, lanes 3 and 4 compared to lanes 1 and 2). The upper band of the Hsp90 blots is the inducible isoform, Hsp90-alpha, while the lower band is the mostly constitutive Hsp90-beta (34). The upper and lower bands of the Hsp70 blot represent the constitutive form and the inducible form of Hsp70, respectively. As it is shown on Fig. 1*B*, only the inducible Hsp70 and Hsp90 were elevated in PiZ transgenic mice, if compared to control mice. Similar results were obtained using antibodies against the two key antioxidant enzymes of the cytoplasm, thioredoxin (Trx) and methionin sulphoxide reductase A (MsrA). The PiZ transgenic mice had an elevated concentration of both antioxidant proteins (Fig. 1*B*, lanes 3 and 4 compared to lanes 1 and 2). We evaluated the induction of these proteins by densitometric analysis. The results are presented on Fig. 1*C*. While Hsp90, Hsp70 and thioredoxin showed significant elevation in transgenic mice comparing to their control littermates, the MsrA concentration showed variance from mouse to mouse, so, despite the pronounced change in the mean, the increase is not significant in this case.

AAT complexes of PiZ transgenic mice

Our next question was, whether the misfolded AAT, PiZ can trap certain chaperones, as has been demonstrated in PiZ-transfected cells (8). To explore this possibility, we designed immunoprecipitation experiments. The first set of experiments was performed using Sepharose beads decorated with an antiserum against human AAT. Our pilot experiments showed that this Ab recognized the endogenous mouse AAT (data not shown), so this method could be used to compare the AAT complexes of healthy and PiZ transgenic mice. In these experiments the supernatant contained all those proteins that were not able to bind to the Ab-decorated beads, and are referred in the figures as "Sup", and the proteins that remained bound to the beads, also referred as immunoprecipitated ones, are marked as "Prec". In all experiments control samples were used to check unspecific binding of proteins to the beads or cross-reaction of the Ab used for immunoprecipitation and the Ab used for detection of proteins. These controls were always negative (data not shown) showing a negligible concentration of nonspecific binding, as well as no cross-reactivity.

The PDI family member redox chaperones ERp72 and calnexin remained in the supernatant fraction of the samples from both control and PiZ transgenic mice, showing that they were not attached to AAT strongly enough to be fished out by the anti-AAT Ab (**Fig. 2*A***).

Fig. 2*B* shows the result of the AAT immunoprecipitation developed by KDEL Ab. While Grp78 and Grp94 were present only in the supernatant of the samples from both control and PiZ transgenic mice, the immunoprecipitated pellet contained significantly more PDI in PiZ transgenic mice compared to samples from

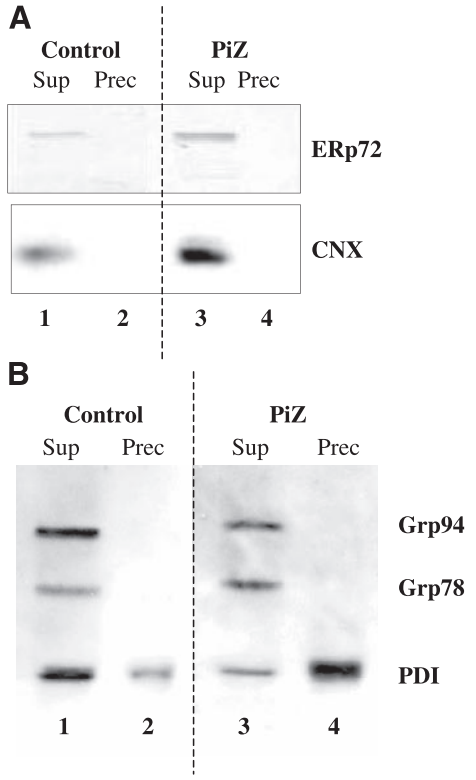

Figure 2. Chaperones pulled out with immunoprecipitation using an AAT Ab. Immunoprecipitation of 80 μg of microsomal proteins from control (Control) and PiZ transgenic (PiZ) mice and PAGE of the samples were performed as described in Materials and Methods using AAT Ab-covered Sepharose beads. "Sup" refers to the supernatant (not bound proteins) of the immunoprecipitates, while "Prec" denotes the proteins remained in the pellet of the immunoprecipitates. Western blot membranes were developed with specific antibodies against the proteins indicated on the right side (*A*) or with a specific Ab against the KDEL ER retention sequence (*B*). Representatives of at least 4 independent experiments are presented.

control mice. The supernatant fractions also contained PDI, which demonstrated that a certain fraction of PDI is not in a complex with AAT. In the case of control mice, this fraction is larger than the immunoprecipitated fraction, while in PiZ transgenic mice, the amount of PDI forming a complex with AAT is comparable to the fraction of PDI remained in the supernatant. It is worthwhile to mention that treating the samples with the reducing agent DTT before immunoprecipitation had no effect on the binding of PDI to PiZ (data not shown).

PDI and Grp94 complexes of PiZ transgenic mice

These results showed that PDI is attached strongly to the PiZ form of AAT. As a logical next step, we tried to detect the partners of PDI and the possible changes of the PDI-related chaperone complexes. However, only Grp94 was detectable in PDI immunoprecipitates. Grp94 was equally pulled by PDI from the control and the PiZ transgenic samples (**Fig. 3*A***). This may reflect that Grp94 binds only the endogenous mouse AAT present in all the samples. Grp78 and calnexin were not present in detectable amounts in the pellet of these samples, remained completely in the supernatant (data not shown). Similar amounts of AAT were in PDI immunoprecipitates from both control and PiZ transgenic mice (Fig. 3*A*), representing most probably the endogenous mouse AAT in both cases.

Analyzing the efficiency of PDI immunoprecipitation with PDI Ab showed an equal amount of immunoprecipitated PDI from the control and the PiZ transgenic samples (Fig. 3*B*). However, there was a great difference between the amount of PDI remaining in the supernatant. The high concentration of PDI in the supernatant of PiZ transgenic samples indicates a high amount of PDI inaccessible for immunoprecipitation in these animals (Fig. 3*B*, lane 4).

To explore the ER chaperone complexes further, we decorated the Sepharose beads with Grp94 Ab, and the composition of the immunoprecipitated complex was analyzed on Western blots using the KDEL Ab. The results of Fig. 3*C* show that in control mice a huge amount of PDI was attached to Grp94 (Fig. 3*C*, lane 2), while in PiZ transgenic samples the amount of PDI attached to the Grp94 was much less pronounced (Fig. 3*C*, lane 4). This finding agrees with the previous result, showing the difference between transgenic and control mice in the availability of PDI (Fig. 3*B*). Interestingly, the amount of Grp94-attached Grp78 was also diminished in PiZ transgenic samples, if compared to control.

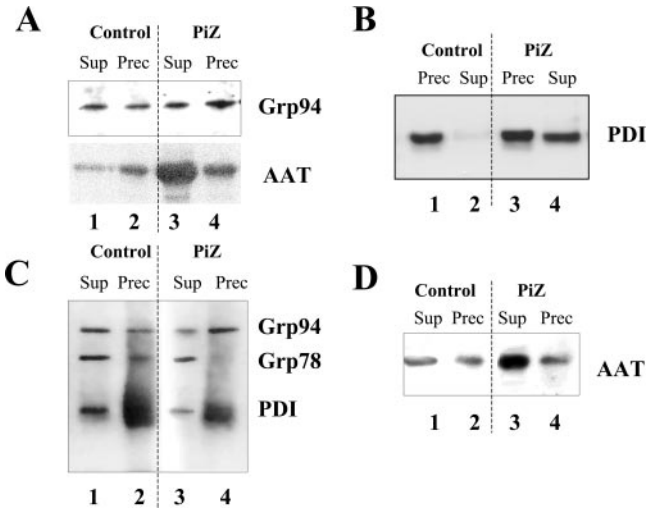

Figure 3. Immunoprecipitation with chaperone antibodies. Immunoprecipitation of 80 μg of microsomal proteins from control (Control) and PiZ transgenic (PiZ) mice and PAGE of the samples were performed as described in Materials and Methods using PDI Ab-covered (*A* and *B*) or Grp94 Ab-covered (*C* and *D*) Sepharose beads. "Sup" refers to the supernatant (not bound proteins) of the immunoprecipitates, while "Prec" denotes the proteins remained in the pellet of the immunoprecipitates. Western blot membranes were developed with specific antibodies against the proteins indicated on the right side (*A*, *B*, and *D*) or with a specific Ab against the KDEL sequence (*C*). Representatives of three independent experiments are presented.

The same set of samples was developed with an AAT Ab. Our results showed that AAT was pulled out from the samples the same extent in both control and PiZ transgenic mice, suggesting that Grp94 binds only to the healthy, foldable AAT (Fig. 3*D*).

Redox status of the ER and the cytoplasm in PiZ transgenic mice

To explore the possible contributors of the changes of PDI complexes found in PiZ transgenic mice, we performed measurements to check the redox state of the cells. Results of **Table 1** show that the redox state of the endoplasmic reticulum differs in control and PiZ transgenic mice; namely, we found a more reducing state in the ER of PiZ transgenic mice. The amount of the reduced protein thiol groups, as well as the total GSH, are both significantly larger in PiZ transgenic mice, which is in agreement with the reductive shift experienced in the GSH/GSSG ratio. The experiments carried out on the cytoplasmic fraction (**Table 2**) of the same samples showed that the redox changes did not affect the cytoplasm. All the measured cytoplasmic redox parameters were about the same in the control and PiZ transgenic mice, with the exception of a slight, but (at $P=0.05$) not significant decrease in the reduced GSH concentration (GSH/GSSG ratio is 72 vs. 62).

Redox status of PDI of the PiZ transgenic mice

Our next question was whether the changes found in the redox balance of the endoplasmic reticulum are correlated to the changes of the redox state of PDI, as we found earlier in diabetic rats (13). For this purpose, we labeled the proteins with AMS, which binds to the free thiol groups of the proteins, causing their retention in SDS-PAGE, using nonreducing conditions (32). In these experiments, we used control samples treated with DTT (Supplemental Fig. 1., lane 5) as a reducing agent, and diamide (Supplemental Fig. 1., lane 6) as a thiol-specific oxidizer to detect the effectiveness of AMS labeling. The experiments consistently showed that PDI was in a more reduced state in the liver of PiZ transgenic mice than that of the control mice (Supplemental Fig. 1, lanes 1 and 2 representing control mice, and lanes 3 and 4 representing PiZ transgenic samples). Other chaperones were also investigated with the same method, such as the PDI family member, ERp72, Grp94, and calnexin, which all contain disulfide bridges, but we could not detect any difference in their redox states (data not shown).

Table 1. *Changes in the redox status of the ER in PiZ transgenic mice*

	Control	PiZ
	nmol/mg protein	
protein thiol	122 ± 17.7	156 ± 34.6
GSH	16.3 ± 2.5	32 ± 1.5*
GSH/GSSG	1.22 ± 0.075	3.85 ± 0.1*

For thiol content, proteins (containing 2.5 mg protein/ml) were diluted in 50 mM Tris·HCl, pH 7.2, and immunoprecipitated by 10% TCA, washed 3 times by 70% acetone, and resuspended in 50 mM Tris.HCl, pH 6.8, containing 8 M urea, and 2% SDS. Thiol content was measured by the Ellman method (31) as described in Materials and Methods. GSH level was determined using 100 μg protein of the samples incubated with 0.024 ml of 50 mM Tris·HCl (pH 7.2), 2 μl NADPH solution and 1 U of glutathione for 30 min at 37°C. The reaction was stopped by 0.075 ml 5% TCA, then the mixture was centrifuged at 3,000 *g* for 3 min. One milliliter of the supernatant was measured according to Ellman's method as described in the Materials and Methods. GSH/GSSG ratio was calculated from the difference of the reduced and total GSH content. Means ± SD of 3 independent experiments with 3 mice per data point are presented. The asterisk represents a significant difference at $P < 0.05$.

Table 2. *Changes in the redox status of the cytoplasm in PiZ transgenic mice*

	Control	PiZ
	nmol/mg protein	
Protein thiol	122 ± 12.5	118 ± 19.7
GSH	103 ± 17.7	114 ± 34.6
GSH/GSSG	72 ± 22	62 ± 14

Experiments were carried out similarly than in the case of endoplasmic reticulum (see the legend of Table 1), following the methods described in Materials and Methods. Means ± SD of 3 independent experiments with 3 mice per data point are presented.

Protein disulfide reductase (PDR) activity of the PiZ transgenic mice

To obtain insight into whether the redox activity of PDI is affected by the changes found, we measured protein disulfide reductase (PDR) activity of PDI. For this we synthesized a fluorescent substrate of PDI, the di-FiTZ-insulin, following the method of Heuck and Wolosiuk (33). In this method, reduction and subsequent aggregation of the di-FiTZ-labeled insulin results in the elevation of its fluorescence, allowing the measurement of PDR activity in complex and turbid samples such as the microsomal vesicles (13). The experiments showed that the microsomes derived from the liver of PiZ transgenic mice (**Fig. 4**, solid circles) have a significantly decreased ability to reduce the substrate, if compared to the same values from control mice (Fig. 4, open diamonds). As a positive control, 5 mM DTT was used (Fig. 4, dashed line) and buffer alone was measured to check the spontaneous aggregation of the substrate (Fig. 4, dotted line). The experiments shown in Fig. 4 were performed in a redox buffer containing GSH:GSSG at a ratio of 5:1 (with a final GSH concentration of 2 mM). We found the same, significant difference between the PDR activity of control and PiZ transgenic mice, when the experiments were performed without an addition of GSH/GSSG buffer, showing that in the original redox milieu, the difference of PDR activity still persists (data not shown).

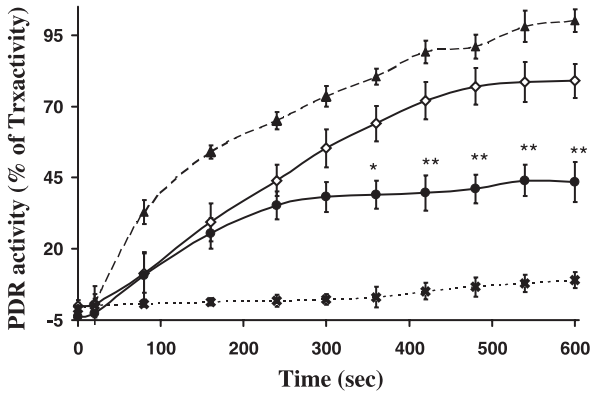

Figure 4. Protein disulfide reductase (PDR) activity of the endoplasmic reticulum. For PDR activity measurements, 75 μg microsomal proteins were added to 1 μM di-FiTZ insulin solution in 5 mM Tris·HCl buffer in the presence of a 5:1 GSH/GSSG redox buffer (with a final GSH concentration of 2 mM), with pH 7.2, and the fluorescence was measured as described in Materials and Methods. Open diamonds or filled circles represent microsomal samples from control or PiZ transgenic mice, respectively. Dashed line represents the positive control in the presence of 5 mM DTT; the dotted line represents the negative control with buffer only. All data are expressed as a percentage of fluorescence reached by thioredoxin (Trx). Means ± SD of 8 independent experiments with 6 mice per data set are presented. *Significant change compared with control, $P < 0.05$. **Significant change compared to control, $P < 0.01$.

DISCUSSION

α1-antitrypsin deficiency is a well-known folding disease, in which the mutant protein accumulates inside the lumen of the endoplasmic reticulum of the liver cells. This aggregation has severe consequences, involving hepatitis, cirrhosis, and elevated risk for hepatocellular carcinoma. Until recently, the details of the pathomechanism of these symptoms were not known (3). Since the mutant protein, PiZ is aggregated inside the lumen of the endoplasmic reticulum, the question arises, whether the function of the endoplasmic reticulum is affected. An important role of the endoplasmic reticulum is the folding of the secretory and membrane proteins. The question we asked was whether the chaperones of the folding machinery are involved in the pathomechanism of the disease and whether the oxidative stress, which is often accompanied with chronic protein aggregation plays a role in the onset of the symptoms.

Our model for the experiments was the PiZ overexpressing transgenic mouse model. In PiZ-overexpressing animals, the folding incompetent mutant human protein accumulates and forms aggregates in the ER and causes the same symptoms in PiZ over-expressing mouse and human patients with the mutant protein (3–5). It is important to mention that similar to the PiZ overexpressing transgenic mice, healthy α1-antitrypsin (PiM) synthesizing mice also exist (35). These PiM mice, despite the high copy-number of the PiM gene and a strong expression (and secretion) of the PiM protein, showed none of the symptoms that were observed in the PiZ mice and human patients (35). This proves that not the expression, but the misfolding and aggregation of the protein, is the reason for most of the pathophysiological changes experienced in PiZ transgenic mice.

To address the role of ER chaperones in PiZ transgenic mice, first, we examined the induction of the major stress-inducible ER chaperones. No induction of the Grp94, Grp78, PDI, or calnexin was found, in agreement with the findings on PiZ transgenic mice (9,12) and on transfected cell lines (7,10,12).

Examining the chaperones bound to AAT, we found no detectable amounts of calnexin, Grp78, or Grp94 attached to AAT. These results are different from the findings obtained in cell culture studies (8,11). However, these differences seem to be only apparent. In the cell culture experiments, a sequential participation of the chaperones was described, in which first calnexin, the basic glycoprotein foldase became bound to the newly synthesized PiZ protein, but it was released gradually after the first 3 to 4 h (11). The next actor was Grp78; however, the participation of Grp78 was also diminished with time and with the growing size of the PiZ aggregates (8,11). In the PiZ transgenic mice, where protein accumulation becomes chronic, these chaperones appear to be released from the mutant AAT, PiZ. The results of our immunoprecipitation experiments are not sufficient to conclude whether the ER chaperones were trapped in PiZ aggregates or were attached to PiZ mono- or oligomers. However, on the basis of the extensive aggregation of PiZ, we may assume that most of our immunoprecipitation experiments, in fact, reported PiZ aggregate-attached ER chaperones.

We found a great amount of PDI, the most important redox foldase of the ER bound to PiZ, which is in good correlation with the fact, that PDI is a key element of the ERAD process, holding the misfolded proteins until their proteasomal cleavage (19,20). Treating the samples with the reducing agent, DTT before immunoprecipitation had no effect on the binding of PDI to PiZ (data not shown). This suggests that on the contrary to the PDI/Ero1 complex, where PDI is bound to Ero1 via a covalent, disulfide bridge, PDI is not bound to PiZ via disulfide bridges.

A decrease in the Grp94-PDI complex was detected in samples from PiZ transgenic mice. The PDI-Grp94 complex is characteristic to the assistance of protein folding in the ER. Furthermore, Grp94 seems to bind to the advanced folding products rather than completely misfolded proteins, according to our experiments and others (8 and data not shown). During immunoprecipitation with PDI Ab, AAT and Grp94 were fished out equally from PiZ transgenic and control mice, while immunoprecipitation with Grp94 Ab captured much more PDI from control mice than from PiZ transgenic mice. The explanation for this apparent contradiction may be that the PDI Ab could reach only that population of the PDI pool, which is soluble, participates in N

protein folding, and binds to Grp94 and substrate proteins, such as to the mouse AAT. The immunoprecipitation with Grp94 Ab revealed that the PDI-Grp94 complex was much more abundant in control than in PiZ transgenic mice. This may be the consequence of "trapping" PDI in the large PiZ aggregates, as it has been shown in the case of denatured lysozyme (36).

In addition, microsomes of PiZ transgenic mice produced less protein disulfide reductase (PDR) activity than that observed in control mice. The changes of PDR activity were accompanied with a change in the redox state of the endoplasmic reticulum, which was shifted toward a more reducing state. This feature may be an adaptation process of the endoplasmic reticulum, as was found in other models of long-term stress, such as in diabetes (13). This explanation is supported by the finding, that in a CHOP knockout cell line, which misses a transcription factor initiating the endoplasmic reticulum-driven apoptosis, the ER became more reducing, and this coincided with a higher resistance toward apoptosis (37). It is also known, that in a reduced environment, PDI, which has an oxidoreductase, as well as a chaperone function, tend to act as a chaperone rather than an oxidoreductase (18). This data altogether may reveal a rescuing mechanism activated on long-term, nonlethal stress, during which a less productive, but more protective steady state of the endoplasmic reticulum is maintained, in which a more reducing environment protects the ER from oxidative stress and apoptosis and regulates PDI to act as a chaperone rather than an oxidoreductase.

Although there are no signs of oxidative imbalance in the cytoplasm of the PiZ transgenic mice, the induction of cytoplasmic chaperones and antioxidant enzymes shows that the cytoplasm may experience stress in these PiZ transgenic mice. In agreement with our data, Hsp90 and Hsp70 were also found to be induced in human patients with α1-antitrypsin-related liver disease (38). This may be the result of a greater occupancy of proteasomes by PiZ, as PiZ is degraded partially by the proteasome (39). Additionally, a competition between targets of glycoprotein folding and quality control was found in the case of α1-antitrypsin and another glycoprotein, transferrin (40). This suggests that in the liver cells of PiZ transgenic mice a higher ratio of the newly synthesized proteins are misfolded and directed to degradation via the ERAD process. This compromises proteasomal activity further. As it has been demonstrated before (41), the decrease in proteasomal activity leads to the induction of Hsp90.

The decline of proteasomal activity leads to the accumulation of oxidized proteins within the cytoplasm (25,42). Because mitochondria were found to be damaged in human patients with the PiZ form of hAAT, in PiZ transgenic mice and also in PiZ-transfected cell lines (43), it is probable that mitochondrial damage is an additional source of the cytoplasmic oxidative stress.

Induction of Hsp70 is a response to oxidative stress both generally (24) and if derived from mitochondria (44). MsrA was found to be induced during oxidative injury (45), and both thioredoxine and GSH were proven to play an important role in maintaining the redox balance and avoiding oxidative damage of the cytoplasm (46). Thus, the elevated levels of Hsp70 and thioredoxin found in PiZ transgenic mice, along with the increased amount of glutathione, represent a stronger antioxidant and redox buffer capacity for the cytoplasm. This can be the explanation for the unchanged redox state of the cytoplasm of PiZ transgenic mice, despite the signs of oxidative stress.

Damaged mitochondria can be responsible for the caspase-9 activation found in PiZ-expressing PiZ transgenic mice (47). Signs of ER-initiated apoptosis were examined by caspase-12 activation, but no changes of caspase-12 levels were found, when compared to the control mice (data not shown), which contradicts earlier findings (12). This can be explained by the difference between the models used, because in the study by Hidvegi et al. (12), protein levels were assessed maximum 22 days after switching on the synthesis of PiZ protein, whereas in our case, the PiZ synthesis starts from the embryonic stage of mice. However, other ways of ER-mediated apoptosis cannot be excluded, since cross-talk between the different apoptotic cascades was recently proven (48).

Our most important findings are that PDI forms a complex with the PiZ protein, which goes parallel with a decrease in protein disulfide reductase activity and a redox shift in the ER. The amount of the Grp94-PDI foldase complex is decreased in PiZ transgenic mice. These data support a hypothesis by which the adaptation to chronic stress diverts the endoplasmic reticulum toward a more protective, but less effective, state, which represents a higher load of stressors to the cytoplasm. This transition may decrease the harmful effects of the disease, since the cytoplasm with the highly inducible chaperones, antioxidant enzymes, and a stronger redox buffer capacity can probably fight against the stress more effectively than the ER.

The elucidation of the relationship between the changes of the ER chaperone complexes, the experienced cytoplasmic stress response, mitochondrial damage, and PiZ aggregation needs further investigations. Our results may open the way for further studies on situations imposing a chronic stress, which focus on the changed availability and activity of molecular chaperones, as well as on the possible adaptation mechanism by which cells suffering from a long-term stress may rescue their key functions and may survive. FJ

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