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# Effects of Unfolded Protein Accumulation on the Redox State of the Endoplasmic Reticulum

Eszter PAPP, Gábor NARDAI, A.S. SREEDHAR, Tamás KORCSMÁROS and Péter CSERMELY
*Semmelweis University, Dept. of Medical Chemistry, Budapest, Hungary*

**Abstract.** Alpha1-antitrypsine (AAT) is a serum protein synthesized by the liver cells. 4% of the humans is carrying its folding deficient mutant, the Z form (or PiZ). This protein can neither be folded, nor degraded by the proteasome although it is partially transported back to the cytoplasm. Only 15% of PiZ is released into the bloodstream. The remaining proteins accumulate mainly in the endoplasmic reticulum forming insoluble aggregates inside the lumen. Protein aggregates are also present in the cytoplasm. The resulting AAT deficiency is a well-characterized disease causing emphysema, hepatic injury, liver cirrhosis and a higher risk for hepatocellular carcinoma in human patients.

Our animal model, the PiZ transgenic mouse is expressing the Z form of AAT protein in its liver, having similar symptoms than human patients have. The only difference of the animal model is that transgenic mice do not develop emphysema, because they have the normal, mouse AAT gene also.

In the hepatic endoplasmic reticulum of AAT mice we found an elevated level of protein thiols, and an elevation in both the GSH/GSSG ratio and total GSH. There were no significant changes in the levels of the most important lumenal stress proteins, like Grp94, Grp78, PDI or calnexin. In contrast, the cytoplasm did not show any marked change in the redox state, but we found a marked induction of Hsp70 as well as Hsp90. The higher lipid peroxidation found in red blood cells suggests a higher oxidative damage regarding the whole organism.

Our results are reminiscent to the redox changes after the onset of diabetes in STZ-mice showing a gross disturbance of folding homeostasis in the endoplasmic reticulum. Retrograde transport of unfolded proteins and their accumulation in the cytoplasm may explain the induction of major heat shock proteins in this cellular compartment. Mutant AAT, which can neither be folded nor degraded may remain bound to the chaperones causing a chaperone overload and a relative shortage of chaperones. These phenomena may lead to a general defect in protein folding of the affected livers, and may be a clue to the higher rate of hepatic tumors in PiZ mice.

## Introduction

Links between redox changes and protein folding defects in various diseases receive a revived attention in these days. In diabetes the common 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 [1]. It is known that oxidative stress is one of the reasons of the pathogenesis in Parkinson's disease, since oxidized alpha-synuclein is more prone for aggregation than the unmodified one [2]. Several pieces of evidence show that in Alzheimer's disease the neurotoxicity of the _-amyloid is related to the oxidative state of a methionine [3]. Although we have a plenty of data about the oxidative stress as a consequence of various pathological states, the changes of redox protein folding are not

well characterized yet. The endoplasmic reticulum (ER) deserves a particular attention, since this organelle is the place of folding – and oxidative folding – of secretory proteins, hence changes in the redox balance can heavily influence the efficiency of protein folding and secretion in the ER.

### *Chaperones*

*Inside the ER*, the major oxidant of the secretory proteins is the protein disulfide isomerase, PDI. PDI has four domains, in an αββ'α' arrangement. The domains have a thioredoxine-like fold with the motif "CxxC". This motif is responsible for the isomerising activity of PDI. PDI mediates disulfide formation, isomerisation or reduction depending on its redox state [4]. There is a recent report about ERp44, a member of the PDI family showing the involvement of ERp44 in the retention of the misfolded proteins in the ER lumen. The partner of ERp44 is PDI, which reduces the misfolded proteins. This process is needed for the ER protein degradation mechanism, the ERAD [5]. These data show that PDI is also involved in cases, where higher degradation activity is needed.

*In the cytoplasm*, the majority of the chaperones are heat shock proteins, Hsp-s. The most abundant one is Hsp90, the 90 kDa heat shock protein, which is involved in many of the intracellular events, such as signalling, protein degradation and protein synthesis. Cytoplasmic chaperones have a key role in stress survival. The inducible form of Hsp90 is synthesized when heat, osmotic, or oxidative stress occurs [6,7]. Hsp70 is also an abundant stress protein in eukaryotic cells acting alone as well as part of the Hsp90-centered chaperone machinery, the foldosome [8]. Both Hsp90 and Hsp70 become oxidized early in oxidative stress [6,9]. Interestingly, the activity of these chaperones remains unchanged in their oxidized form. It is very likely that both Hsp90 and Hsp70 also serve as oxidative scavengers of the cell.

### *Redox Balance*

*In the ER* the redox balance is a very sensitive and carefully maintained system. The relatively oxidizing environment is needed for the secretory proteins to gain their native structure before getting out into the extracellular space. The different PDI activities, like oxidization, isomerization and reduction go parallel in the ER. It has been proposed that the ER may have local differences in its redox state [10,11]. The whole mechanism for the maintenance of redox balance in the ER is not known yet, but many of the elements have been already cleared.

The most important buffering system is the glutathione(GSH)/oxidized glutathione(GSSG) pair. GSH, this small tripeptide can be oxidized easily, and subsequently forms a dimer having a disulfide bridge. This oxidized form is often referred as GSSG. The GSH:GSSG ratio in the ER is between 1:1 to 1:3. It was shown that in diabetes, where oxidative stress is general, this ratio could be as high as 1:10, i.e. a "reducing stress" occurs [1]. Besides the GSH/GSSG system, the ascorbate/dehydroascorbate, the vitamin K, vitamin E, and many other systems are also involved in the regulation of ER redox balance [1].

*In the cytoplasm,* the regulation of the redox balance is also based on the GSH/GSSG system. Here the GSH:GSSG ratio is usually around 1:100, which is highly reducing. The ratio is changed during pathological states, which are related to oxidative stress.



Oxidative damage is repaired by several enzymatic systems. Here we would like to highlight only the two most important systems, the thioredoxins and the methionine sulfoxide reductases [12-14]. *Thioredoxine* can reduce selected proteins. The oxidized thioredoxine is reduced back by the thioredoxine reductase, which uses NADPH as a cofactor [12]. Thioredoxine is not only a redox enzyme. This 13 kDa protein can bind to other proteins and protect them from the oxidation or change their original function. Thioredoxine may take part in extracellular signaling, since a truncated form was found to be secreted [13].

*Methionine sulfoxide reductases* (MSRs) play a role only when an oxidative damage prevailed [14]. The methionine, as other sulfur containing amino acids, is very sensitive to oxidation. MSRs can reduce back the oxidized methionine protecting the host protein from structural changes. Interestingly, an MSR isoform, MSRa contains many methionines on its surface. These methionines can be oxidized without influencing MSRa enzymatic activity. This suggests a passive protective mechanism working parallel to the enzymatic activity mentioned above [9,15].

### *The Chaperone Overload Theory*

Although chaperones are abundant and inducible proteins, their activity has its limitations. When the stress is higher than the protecting capacity of available chaperones, protein damage prevails. During stress chaperones capture oxidized, denatured or covalently modified proteins, and hold them in an ATP-independent process. When the stress is over, and normal cellular life is started again, chaperones help the refolding of damaged proteins or direct them toward the degradation machinery, predominantly to the proteasome. All these latter processes require ATP.

But what happens when the stress never ends? Chronic diseases, such as diabetes impose a long-term, continuous stress. Moreover, there are certain diseases when a mutant protein is synthesized in the cell. In even more unlucky cases these proteins aggregate, and can neither be degraded nor folded properly. Parkinson's disease, cystic fibrosis and alpha1-antitrypsine deficiency are well-known examples of these processes. Since chaperones bind to damaged proteins, it is conceivable that in the above, chronic diseases growing portions of chaperones are helplessly bound to the misfolded, aggregated proteins. Indeed, chaperones were found in the aggregates of alpha-synuclein aggregates as well as in the alpha-1 antitrypsine deposits [2].

For a transient stress the inducibility of chaperones is usually enough to cover the increased need of chaperone capacity. However, in chronic stress chaperone inducibility becomes usually exhausted, and finally there will be a shortage in chaperone activity. As a consequence of this shortage, a higher amount of misfolded proteins accumulates leading to a higher load of the protein degradation system. Moreover, both chaperones and the protein degradation machinery, the proteasome are highly sensitive to stress, themselves. This aggravates the damage further.

Chaperone shortage leads to another consequence: hidden mutations of the genome become uncovered [16]. Mutations occur in all living cells. Single nucleotide polymorphisms may affect as many as 30,000 amino acids in human cells. Most of these mutations are harmless, many of them do not cause a major change in the structure of mutated proteins. In this mild case chaperones may help to re-gain the native structure of these proteins. If chaperone activity decreases, this function also suffers. In this case, the effect of the mutation is revealed in the phenotype, which may contribute to a higher probability of polygenic diseases, like diabetes or cancer [17]. Supporting this idea, in the

case of cystic fibrosis as well as in alpha-1 antitrypsine deficiency, a higher rate of tumor incidence were detected [18,19].

*The Alpha1-Antitrypsine (AAT) Model*

To examine the possible consequences of chaperone overload, we selected a model animal, the alpha-1 antitrypsine (AAT) transgenic mice [20]. These mice have an additional gene in their genome. This gene codes a mutant form of the human alpha1-antitrypsine protein, the Z type AAT. This mutation is well known from human patients, since 0.2 percent of humans are carrying this mutation. In the Z type AAT a point mutation results in a break of a salt bridge causing a major change in the secondary structure of the protein. Because of this change, the protein cannot be folded, aggregates, and avoids degradation.

Homozygous human patients have severe problems, partly because of the lack of functional AAT, which is an important protease inhibitor, and partly because of the damages of the synthesizing organ, the liver. These patients are born with neonatal hepatitis, which leads to cirrhotic changes and higher sensitivity to tumors in later years. The AAT(Z) transgenic mice does not have to face AAT-deficiency, since they have their wild-type, endogenous alpha1-antitrypsine synthesized in normal rates in their liver. However, the misfolded transgenic human AAT(Z) aggregates in their liver forming similar deposits in the endoplasmic reticulum than in the case of the human patients [20].

In general, these mice are born with a lower body weight and remain smaller than their normal littermates [21]. Although there is no significant change in the lifespan of transgenic AAT mice, they seem to be very sensitive to stress: a 72 hour-long starving is 100% lethal for them, while normal mice can easily survive this fasting period [22]. AAT transgenic mice are shown to be much more sensitive to the tumorigenic SV40 virus than their non-transgenic counterparts [19]. The sterile hepatitis is also present in these animals but there was no significant change found in the synthesis of albumin and transferrin, the two most abundant and important hepatic secretory proteins [20].

Our aim was to examine the involvement of chaperones in the protein folding of AAT(Z) transgenic mice as well as to characterize the redox changes of both the ER and the cytoplasm of liver cells from these animals.

## 1. Materials and Methods

*1.1. Preparation of Liver Subcellular Fractions*

Microsomes and cytoplasmic fractions were isolated by the method of Lambert and Freedman [20]. All steps were performed quickly, keeping the samples on ice to minimize sample damage. Livers were minced by scissors, were washed in ice cold TKM buffer (50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.0) and were homogenized in two volumes of S-TKM buffer (TKM buffer + 250 mM sucrose, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) using 5 strokes of the Potter homogenizer. Homogenates were filtered through a cheesecloth, and were centrifuged at 700 x g for 10 min at 4°C. Supernatants were centrifuged at 12,000 x g for 10 min at 4 °C. The supernatant was ultracentrifuged at 100,000 x g for 60 min at 4°C. The resulting supernatant (cytosolic fraction) was quickly frozen in liquid nitrogen, and stored at –80°C under nitrogen. The pellet was taken up in S-TKM buffer and re-centrifuged at 100,000 x g for 60 min. The final pellet (microsomal fraction) was re-suspended in 150 mM Tris-HCl (pH 7.2) buffer, and was used freshly or rapidly frozen in liquid nitrogen and stored at –80°C. The protein content of the obtained



samples was measured using the Bradford method with bovine serum albumin as a standard. Intactness of the microsomal vesicles was checked by detection of the light scattering signal upon the addition of the nonpermeant compound, sucrose, which indicates vesicle leakyness by monitoring the reduced shrinking of vesicles [1].

*1.2. Assessment of the Levels of Microsomal Stress and Redox Proteins*

Dot blot and Western blot was used to estimate stress and redox protein levels. Dot blot was made using 10 μg protein per well transferred to nitrocellulose membranes. The procedure afterwards is identical with the Western blot method. On Western blots equal amounts (10 to 30 μg) of proteins were analyzed after an SDS-PAGE using 9% and 12% gels. After semi-dry blotting to nitrocellulose membranes, blots were blocked by 1% Tween 40 and visualized by antibodies, using the ECL chemiluminescence kit (Bio-Rad, Richmond, CA, USA).

*1.3. Determination of GSH/GSSG Ratio*

For the determination of total glutathione levels, 0.025 ml 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 minutes at 37 °C. After exactly 30 minutes, the reaction was stopped by addition of 0.075 ml 5% trichloroacetic acid, then the mixture was centrifuged at 3,000 x g for 3 minutes. 1 ml 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 minutes, total glutathione (in the form of GSH) was measured at 412 nm with an UV-VIS spectrophotometer. For the determination of reduced glutathione (GSH) content, 0.01 ml samples were mixed with 0.04 ml 5% trichloroacetic acid, and were centrifuged at 3,000 x g for 3 minutes. A 0.05 ml-aliquot of the supernatant was transferred into a 10 mm cuvette and 200 μl of 400 mM Tris-HCl (pH 7.2) as well as 5 μl Ellmann's reagent solution were added. After 5 minutes, GSH content was measured at 412 nm with an UV-VIS spectrophotometer.

*1.4. Determination of Total and Protein Thiol Contents*

Total microsomal thiol content was measured from detergent-permeabilized vesicles by the Ellman's method using sodium-deoxycholate in 0.5% concentration. Proteins were isolated by 10% trichloro-acetic acid precipitation followed by a centrifugation at 1,200 x g for 10 min at 4°C. Samples were washed twice with 70% acetone, and were resuspended in a buffer of 100 mM Tris-HCl (pH 7.2), 2% SDS and 8 mol/l urea using an Eppendorf tube homogenizer. The thiol content of the protein fraction was determined using the Ellman's method.

*1.5. Determination of the Redox State of Microsomal Stress and Redox Proteins*

Protein redox state was determined using AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid), a specific thiol labeling agent, which decreases the mobility of proteins

with free sulfhydryl groups on nonreducing SDS polyacrylamide gels. Microsomes were suspended in 50 mM Tris-HCl (pH 7.0), their protein fraction was precipitated by 10% trichloro-acetic acid, washed twice with 70% acetone, and resuspended in 100 mM Tris-HCl buffer (pH 7.2) containing 8 M urea and 2% SDS using an Eppendorf tube homogenizer. Samples were centrifuged and the supernatant incubated with 20 mM AMS for 15 min on ice and 15 min at 37°C. Nonreducing Laemmli sample buffer was added, samples were resolved on a discontinous 9% SDS-polyacrylamide gel, and analyzed by the standard Western blot procedure as described above.

### *1.6. Determination of Malondialdehyde Content*

Blood samples were colleted into heparinised ice-cold Eppendorf tubes. 0.1 ml of blood, 0.4 ml 0.9% NaCl solution and 0.5 ml 3% SDS was mixed. 2 ml of a mixture containing 15% TCA, 0.375 % TBA and 0.25 M HCl was added subsequently. The solution was kept on 95°C for 30'. After cooled down to room temperature, 3 ml of *n*-butanol was added and centrifuged with 3000 rpm for 15 min. From the supernatant the pink conjugate of MDA and TBA was detected at 532 nm using an UV-VIS spectrophotometer.

## 2. Results

The aim of the first investigations was to get information about the state of the most general chaperones in the hepatic cells. We estimated the chaperone protein levels using the dot blot method. The chaperones we checked were Hsp70, Hsp90, MSRa, thioredoxin, calnexin, PDI and the 94 kDa glucose regulated protein, Grp94. Interestingly, we found marked elevation in the case of all cytoplasmic chaperones, both the Hsps and the antioxidant enzymes seem to be induced. As another surprising set of data we found no elevation in the level of endoplasmic reticulum resident proteins, although the major accumulation of alpha1-antitrypsine is found in this cell compartment.

Because of the known cases when a protein aggregation-related disease shows the signs of oxidative changes and because of the findings of our lab considering diabetes [1], we proposed that the stress response found in the alpha1-antitypsine transgenic animals may be accompanied by redox changes. To get more subtle information about the stress on the organellar level, we made new liver preparations and separated cytoplasmic and microsomal fraction by ultracentrifugation. The fractions were checked for gross redox state and for the redox state of proteins only. We examined the GSH/GSSG ratio in these systems as well as the total amount of GSH.

The results were surprising again, since we could not detect any change in the redox state or in the GSH amount of the cytoplasm, which showed induced chaperone levels suggesting a massive stress in this cell compartment. Conversely, we found a reductive shift in the endoplasmic reticulum supported by a changed GSH/GSSG ratio, a more reduced state of proteins. We found higher level of total glutathione also in the ER.

These results brought up new questions: are those ER chaperones, which take part in the redox folding of newly synthesized proteins compromised by the redox changes or can they save their original features? The most important ER chaperone involved in redox folding is PDI. Since we found no gross changes in PDI level before, we examined the chaperone by a gel-electroforetic method, which is used to make redox state visible (see Materials and Methods). Using this procedure, we found that PDI is much more reduced in the transgenic animals than in their normal littermates.



These results show that these animals are not adapted perfectly to the inceased load of aggregating proteins. We found differences in the levels of chaperones as well as in the redox state of the liver cells. The liver has a central role in the metabolism of the whole organism. We were curious whether there are effects concerning the animal in general as a consequence of the disturbances found in the liver. We have chosen a widely used method measuring lipid peroxidation to evaluate the general oxidative status of transgenic mice. We checked blood samples of 8 animals and found higher malondialdehide levels in all of them. This difference shows that the disturbances found in the liver have effect on the whole organism.

## 3. Discussion

Our first results, the marked changes in the levels of the cytoplasmic chaperones, Hsp70 and Hsp90 show that the accumulation of unfolded proteins in the endoplasmic reticulum generates a stress response in the cytoplasm. Since two of the major antioxidant enzymes, thioredoxin and MSRa was also induced, we expected an extensive oxidative disturbance in the cytoplasm. But, surprisingly, the measurements done concerning the redox status of the cytoplasm, showed no differences between the transgenic and normal animals. The level of the GSH, as well as the ratio of glutathione and its oxidized partner (GSSG) remained unchanged. We did not find any changes in the availability of -SH groups nor in the case of the gross peptides of the cytoplasm, neither examining only the precipitated proteins. These negative results indicate that the stress signs found in the cytoplasm are not accompanied by oxidative stress, or, more likely, the induced level of chaperones successfully diminishes these oxidative changes.

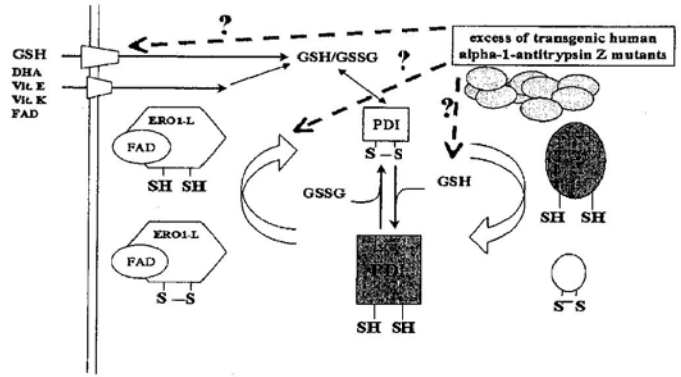


**Figure 1.** Redox Changes in the ER

Starting from the left upper corner: GSH and other redox mediators are transported into the ER lumen to maintain redox balance (expressed with the help of GSH/GSSG ratio). The redox balance is also influenced by the ratio of the reduced and oxidized portion of PDI. PDI oxidization is done by ERO1-L, a membrane anchored protein, which uses FAD as a cofactor. The reducing ability of PDI is dependent of these processes. The overwhelming amounts of AAT cause a shift towards a more reduced state. We do not know whether the transport of GSH or other redox buffering molecule is changed or the electron transport between ERO1-L and PDI is damaged, or the electron exchange between PDI and its client proteins is less efficient.

The picture is quite the opposite in the endoplasmic reticulum. PDI, which is usually synthesized in higher amounts during stress, as well as the stress proteins Grp94 and calnexine were synthesized only at basic levels in transgenic animals, if compared to levels of these chaperones in wild type mice. However, the ER had another response to the

aggregating alpha1-antitrypsin: oxidative changes. All the examined parameters showed a shift towards a more reducing state of the ER. In addition, we found a higher level of the total ER GSH in transgenic mice, which may represent a defensive mechanism against the oxidative damage.

The mechanism of this redox change is not clear yet. On Figure 1. we summarize the basic structure of the redox system in the ER. AAT accumulation can have more possible targets: it may influence the transport of GSH or that of other redox substances, the electron transmission between ERO-1L and PDI or between PDI and its client proteins. There are more possible explanations for these results. Since the mice examined were 5 month old, old enough to become exhausted considering the stress protein synthesis, we suppose that after an early stress response the system works only at basic level, and the redox changes become more and more extensive with the age. Another possibility is that there is no need for the induction of ER resident chaperones and the reductive shift can be also a helpful tool: it can maintain the redox state of the PDI in reduced form in higher ratio, supposed that it can help the retrograde transport and degradation of unfolded proteins.

We do not know whether the changes we found influence the liver function in general, but the increase of the oxidized end-product of lipids, malone dialdehyde from blood shows that the disease has effects outside the liver also, and generates disturbances in the whole organism.

These data are far from giving a complete picture about the consequences of protein aggregation and chaperone overload, but the differences found in the alpha1-antitrypsine transgenic mice are useful starting points to get a more generalized picture about the diseases characterized by accumulating non-degradable proteins, such as Parkinson's, Alzheimer's disease, cystic fibrosis or the human disease alpha1-antitypsin deficiency itself.

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# Endoplasmic Reticulum: A Metabolic Compartment

Edited by

Angelo Benedetti

*Dipartimento di Fisiopatologia e Medicina Sperimental, Università di Siena, Siena, Italy*

Gábor Bánhegyi

*Department of Medical Chemistry, Pathobiochemistry and Molecular Biology, Semmelweis University, Budapest, Hungary*

and

Ann Burchell

*Division of Maternal and Child Health Sciences, Ninewells Hospital and Medical School, University of Dundee, Dundee, United Kingdom*

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