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Diabetic changes in the redox status of the microsomal protein folding machinery

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Abstract

Changes in assisted protein folding are largely unexplored in diabetes. In the present studies, we have identified a reductive shift in the redox status of rat liver microsomes after 4 weeks of streptozotocin-induced diabetes. This change was reflected by a significant increase in the total- and protein-sulfhydryl content, as well as in the free sulfhydryl groups of the major protein disulfide isomerases (PDIs), the 58 kDa PDI and the 57 kDa ERp57 but not other chaperones. A parallel decrease of the protein-disulfide oxidoreductase activity was detected in the microsomal fraction of diabetic livers. The oxidant of PDI, Ero1-Lα showed a more oxidized status in diabetic rats. Our results reveal major changes in the redox status of the endoplasmic reticulum and its redox chaperones in diabetic rats, which may contribute to the defective protein secretion of the diabetic liver.

Keywords: Diabetes; Endoplasmic reticulum; Ero1-Lα; Glucose regulated proteins; Heat shock proteins; Molecular chaperones; Protein disulfide isomerase; Rat liver; Redox status; Streptozotocin

Chaperones of the endoplasmic reticulum (ER) lumen assist in the quality control of protein secretion and aid the gradual oxidation of both secreted and plasma membrane proteins [1–3]. The major chaperone of the oxidative protein folding, protein disulfide isomerase (PDI), has been discovered more than 40 years ago simultaneously by Christian Anfinsen's group [4] as well as by Pál Venetianer and Bruno Straub [5]. However, other members of the oxidative protein folding machinery and their interactions have been uncovered only recently. In the meantime, besides the original 58 kDa-PDI (PDI-58), a number of other PDIs were identified, such as ERp57, which contributes selectively to the folding of the glycoproteins [6], and others with usually unknown function [7]. Additionally, there is a set of proteins, such as ERp28, which show a sequence similar to PDI-58, but lack the thioredoxin-box (-Cys-X-X-Cys-), the redox catalytic site [8]. The exact function of these latter proteins is currently unknown.

An elegant set of studies identified the Ero1 proteins as the protein family helping the re-oxidation of PDIs [9,10]. Although some important elements are still missing (e.g., what is the exact mechanism of Ero1 re-oxidation and how small redox molecules influence the process), we begin to have a gross picture about chaperone assistance in oxidative protein folding, which nicely extends our rather voluminous knowledge on other chaperones of the ER, such as calnexin, the 78 kDa glucose regulated protein (Grp78 or BiP) and the 94 kDa glucose regulated protein, Grp94, which all help the folding and quality control of secreted or plasma membrane proteins [1,2].

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Changes in assisted protein folding in various pathological conditions, and especially in diabetes are largely unexplored. Recent studies identified a decrease in the 70 kDa heat shock protein, Hsp70 in diabetic patients [11] as well as in the livers of streptozotocin (STZ)-diabetic rats [12]. Concerning the ER, our earlier data showed no change in protein levels of Grp78 [13] and Grp94 [14] in the livers of STZ-rats. There are controversial data on the changes of PDI-58 level, but a slight decrease is likely in long-term diabetes [15]. An altered chaperone activity of glycated α-crystallin was detected [16], however, the functional characterization of molecular chaperones in diabetes is missing.

The present studies were prompted by our earlier observations, which showed a surprising increase in ER sulfhydryl (–SH) levels in short-term (3 days) hyperglycemia [17]. Besides the analysis of the redox status of rat liver microsomes after 4 weeks of STZ-induced diabetes, we wanted to analyze the redox status of all major redox ER chaperones in diabetic liver, such as PDI-58 and another major PDI, ERp57, as well as their oxidant protein, Ero1-Lα. In case we found any changes, we wanted to study if these changes can be reverted in vitro by dehydroascorbic acid (DHA), oxidized glutathione (GSSG) or FAD. With these studies we would get the first information on functional status of ER molecular chaperones in diabetes, which may contribute to the explanation of the defective protein secretion of the diabetic liver. Besides, these data could clarify the possible contribution of ascorbate/dehydroascorbate and other luminal redox systems to the oxidative protein folding process.

Materials and methods

Chemicals. Fructosamine kit was a Roche product (Basel, Switzerland; Cat.No. 1930010), the thiol-group labeling agent, AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) was purchased from Molecular Probes (Eugene, OR, USA; Cat.No. A-485), the reagents used for SDS–PAGE and protein determination were from Bio-Rad (Richmond, CA, USA). Difluorescein-thiocarbamyl-insulin (di-FTC-insulin) was a kind gift of Ricardo A. Wolosiuk (Instituto Leloir, Buenos Aires, Argentina [18]). Protein A beads for immunoprecipitation were from Pierce (Rockford, IL, USA). Streptozotocin (STZ), thiobarbituric acid (TBA), glutathione-reductase, and other materials were obtained from Sigma Chemicals (St. Louis, MO, USA).

Antibodies. The sources of various antibodies used were as follows: PDI-58, anti-bovine PDI polyclonal antibody (StressGen, Victoria, BC, Canada; Cat. No. SPA-890); ERp57/Grp58, anti-rat Grp58 polyclonal antibody (StressGen, Cat. No. SPA-580); Ero1-Lα, (A29) anti-human Ero1-Lα C-terminal polyclonal antipeptide antibody for Western blots and D5 antiserum for immunoprecipitations (special gifts from Ineke Braakman [10,19]); Grp94, anti-rat Grp94 polyclonal antibody [20]; ERp28, anti-ERp28 polyclonal antipeptide IgG (special gift from David Ferrari [8]); calnexin, anti-canine calnexin C-terminal polyclonal antibody (StressGen, Cat. No. SPA-860); and Grp78, anti-KDEL polyclonal antipeptide antibody (Affinity BioReagents, Golden, CO, USA; Cat. No. PA1-013).

Animals, characterization of diabetes model. Male Wistar rats (weights: 200–230 g) were obtained from Charles River (Hungary). Animals were fed ad libitum and housed in the controlled animal room of our department under identical conditions. Twenty healthy rats were used as controls. Diabetes was induced by a single injection of 65 mg/kg STZ into the tail veins of another 40 rats. Serum glucose level was checked on the 3rd day after STZ injection by the LifeScan, One Touch II-blood glucose monitoring system Thirty-three animals having blood glucose level higher than 20 mM were used as the diabetic group. The diabetic state was maintained for 28 days. At the end of this period body weights, serum glucose, and fructosamine levels and lipid peroxidation were measured. Fructosamine level was determined by standard clinical laboratory procedure using the Roche fructosamine kit and a Hitachi 901 spectrophotometer. Lipid peroxidation was characterized as elevated malondialdehyde levels in the serum and the determination was based on the measurement of the thiobarbituric acid reactive substances (TBARS, [21]). Briefly, 0.5 mg serum protein (diluted in 0.5 ml of 75 mM NaCl, 1.5% SDS) was incubated in 95 °C for 30 min with 1.0 ml TBA reagent (0.375 w/v% thiobarbituric acid (TBA), 15 w/v% TCA in 0.25 N HCl). After incubation, samples were cooled on ice, centrifuged (2000*g*, 10 min), and supernatants were measured in Hitachi 901 spectrophotometer at 532 nm. Animals were sacrificed to isolate liver subcellular fractions. 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.

Preparation of liver subcellular fractions. Microsomes were isolated by the method of Lambert and Freedman [22]. All steps were performed quickly, keeping the samples on ice to minimize sample damage. Rat livers were minced by scissors, were washed in ice cold TKM buffer (50 mM Tris–HCl, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.0), and were homogenized in two volumes of S-TKM buffer (TKM buffer + 250 mM sucrose, 10–10 μg/ml aprotinin and leupeptin, and 1 mM PMSF, pH 7.0) using 5 strokes of the Potter homogenizer. Homogenates were filtered through cheesecloth and were centrifuged at 700*g* for 10 min at 4 °C. Supernatants were centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was ultracentrifuged at 100,000*g* for 60 min at 4 °C. The resulting supernatant (cytosolic fraction) was quickly frozen in liquid nitrogen and stored at −80 °C. The pellet was taken up in S-TKM buffer and re-centrifuged at 100,000*g* for 60 min. The final pellet (microsomal fraction) was resuspended in 150 mM Tris–HCl (pH 7.2) buffer, and was used freshly or was 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 [23]. Purity of the microsomes was checked by measuring ER, mitochondrial, and cytoplasmic markers in the samples. Contaminants accounted for less than 5% of the total amount of purified microsomal samples [24]. 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 leakiness by monitoring the reduced shrinking of vesicles [25]. Intactness of microsomal vesicles was also detected by measuring the trypsin availability of PDI. Samples with intact vesicles higher than 95% were used in the experiments.

Assessment of the levels of microsomal stress and redox proteins. Western blot was used to estimate stress and redox protein levels. Equal amounts (10–30 μg) of microsomal proteins were analyzed on SDS–PAGE using 9% (for PDI-58, Ero1-Lα, ERp57, Grp78, and Grp94) and 12% (for ERp28) gels. After semi-dry blotting to nitrocellulose membranes, blots were blocked by 2% (w/v) bovine serum albumin and visualized by antibodies indicated above, using the ECL chemiluminescence kit (Bio-Rad, Richmond, CA, USA). Loading efficiency was controlled by repeated experiments and by re-blotting the membranes for Grp94. Photographic images were quantified by densitometry using an LKB Ultroscan XL laser densitometer (Bromma, Sweden).

Determination of the GSH/GSSG ratio. Reduced/oxidized glutathione (GSH/GSSG) ratio was measured according to the method described by Tietze [26]. For the determination of total glutathione

levels, 0.25 ml of microsomal samples was incubated together with 0.24 ml of 50 mM Tris–HCl (pH 7.2), 5 μl NADPH solution (416.7 μg in 5 μl Tris–HCl), and 1 U 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 the addition of 0.75 ml of 5% (w/v) trichloroacetic acid, then the mixture was centrifuged at 3000*g* for 3 min. One milliliter of the supernatant was transferred into a 1 cm cuvette and mixed with 2 ml of 400 mM Tris–HCl (pH 7.2) and 0.1 ml Ellmann's reagent solution (99 mg DTNB in 25 ml methanol). After 5 min, 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, 0.1 ml microsome samples were mixed with 0.4 ml of 5% (w/v) trichloroacetic acid, and were centrifuged at 3000*g* for 3 min. A 0.5 ml aliquot of the supernatant was transferred into a 1 cm cuvette and 2 ml of 400 mM Tris–HCl (pH 7.2) as well as 0.1 ml Ellmann's reagent solution were added. After 5 min, GSH content was measured at 412 nm with an UV–vis spectrophotometer.

Determination of total and protein thiol contents. Total microsomal thiol content was measured from alamethicin- or detergent-permeabilized vesicles by the Ellman's method [27] using an alamethicin or sodium-deoxycholate concentration of 0.1 mg/mg protein or 0.5% (v/v), respectively. The two methods of microsome permeabilization gave identical results. Microsomal proteins were isolated by 10% (w/v) trichloroacetic acid precipitation followed by a centrifugation at 1200*g* for 10 min at 4 °C. Samples were washed twice with 70% (v/v) acetone and were resuspended in a buffer of 100 mM Tris–HCl (pH 7.2), 2% (w/v) SDS, and 8 M urea using an Eppendorf tube homogenizer. The thiol content of the protein fraction was determined using the Ellman's method [27].

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 [9]. Microsomes were suspended in 50 mM Tris–HCl (pH 7.0), their protein fraction was precipitated by 10% (w/v) trichloroacetic acid, washed twice with 70% (v/v) acetone, and was resuspended in 100 mM Tris–HCl buffer (pH 7.2) containing 8 M urea and 2% (w/v) 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 discontinuous 9% SDS–polyacrylamide gel, and analyzed by the standard Western blot procedure as described above.

Protein-disulfide oxidoreductase activity. Microsomal protein-disulfide oxidoreductase (PDOR) activity was measured by using a highly sensitive fluorescent assay based on the method of Heuck and Wolosiuk [18]. This assay is suitable to measure PDOR activity in crude systems as microsomes with high reproducibility [17]. Briefly, 150 μg of microsomal protein was added to 0.2 ml of 100 mM sodium phosphate buffer (pH 7.4) containing 2 mM EDTA, a GSH:GSSG redox buffer (5:1, final concentration: 2 mM), and 1–2 μM of the fluorescent substrate difluorescein-thiocarbamyl-insulin (di-FTC-insulin). Fluorescence was followed at 37 °C using a Hitachi F-4500 spectrofluorimeter, the excitation and emission wavelengths were 495 and 520 nm, respectively.

Re-oxidation of diabetic microsomes. Intact diabetic microsomes were suspended in 50 mM Tris–HCl (pH 7.2) in the absence or presence of oxidizing agents (2 mM oxidized glutathione, dehydroascorbic acid or ascorbic acid and 0.2 mM FAD) and incubated for 15 min at room temperature. Incubation was stopped by addition of 10% (w/v) trichloroacetic acid. Precipitated protein fraction was resuspended, and the protein thiol content and protein redox state were measured as written above.

Immunoprecipitation. For immunoprecipitation of Ero1-Lα/PDI-58 complexes, 2 mg of microsomal protein from 8 control and 8 diabetic animals was dissolved in IP buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 0.1% (w/v) SDS, and 1% (v/v) NP-40). After preclearing the samples by the addition of protein A beads in IP buffer, 5 μl of the anti-Ero1-Lα antibody, D5 serum was added. Samples were incubated overnight at 4 °C. Immunocomplexes were collected by protein A beads, washed extensively with IP buffer three times, and resuspended in sample buffer for SDS–PAGE containing 50 mM DTT. Samples were analyzed by SDS–PAGE and Western blot with anti-PDI-58 antibody to estimate the PDI-58 content of the immunoprecipitates using isolated bovine liver PDI (Sigma Chemicals, St. Louis, MO, USA) as a molecular weight control. The amount of precipitated Ero1-Lα protein was also checked by Western blots with the A29 anti-Ero1-Lα antibody. Photographic images were quantified by densitometry using an LKB Ultroscan XL laser densitometer (Bromma, Sweden).

Statistical analysis. The statistical analysis of the samples was performed using the unpaired Student's *t* test. A value of $p < 0.05$ was accepted as indicating statistically significant difference.

Results

Characterization of the diabetes model

In our experiments, we have used the streptozotocin (STZ)-induced diabetes model, where the changes in redox status have been partially characterized [17,28]. We used livers of animals as an insulin-sensitive organ highly active in protein secretion and protein folding in the lumen of the ER. Diabetes was ascertained by monitoring the blood glucose level and the weight loss of the animals. A significant increase in lipid peroxidation (characterized by the level of the thiobarbituric acid reactive substances) and the more than twofold elevated serum fructosamine level (Table 1) indicated a significant diabetic oxidative stress and protein damage.

Level of stress proteins of the endoplasmic reticulum in diabetes

First, we analyzed the overall level and changes of various proteins of the oxidative folding machinery present in the lumen of diabetic ER. Data of Fig. 1A

Table 1
Parameters of streptozotocin-treated diabetic rats

	Increase of body weight (g)	Serum glucose (mM)	Serum fructosamine (μM)	Serum TBARS (pmol/mg protein)
Control	180 ± 25	6.7 ± 1.2	142 ± 18	35 ± 3
Diabetic	70 ± 45*	36.5 ± 4.1*	319 ± 23*	46 ± 9*

Animals were sacrificed and analyzed on the 28th day of hyperglycemia. Body weights and blood glucose levels were measured in all the 20 control and 33 diabetic rats. Serum fructosamine and thiobarbituric acid (TBARS) levels were determined only from 7 control and 8 diabetic animals as described in Materials and methods. Data represent means ± SD.

* Significant change compared to control ($p < 0.05$).

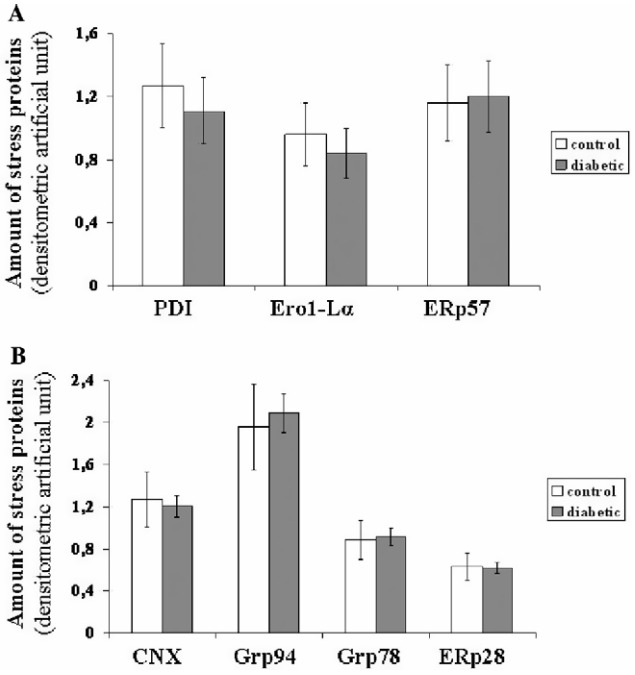

Fig. 1. Expression of members of the hepatic microsomal protein folding machinery in STZ-diabetes. Microsomes were isolated from control and diabetic rats and levels of PDIs (PDI-58 and ERp57), the re-oxidizing membrane protein (Ero1-Lα) (A) as well as other ER chaperones Grp94, Grp78, calnexin and the inactive PDI homolog, and ERp28 (B) were estimated by Western blot as described in Materials and methods. Blots were quantified by laser densitometry. Data are means ± SD of three independent experiments measuring microsomes from 20 control and 33 diabetic animals.

show that both the major PDI (PDI-58) and its re-oxidizing protein, Ero1-Lα had a marginal (but not significant) decrease of expression in diabetes. Levels of another PDI, ERp57 as well as those of other ER stress proteins, like calnexin, the inactive PDI-homolog ERp28, Grp78, and Grp94 were unchanged (Figs. 1A and B).

Microsomal redox changes in STZ diabetes

The negligible changes in stress protein levels in the diabetic ER made us curious about the possible changes in the oxidative status of the ER. Extending our earlier observations on an increase in protein sulfhydryl levels as well as total –SH content of microsomes in short-term (3-day) hyperglycemia [17], we found a significant increase in both parameters after long-term (4-week) diabetes as well (Table 2). The ratio of reduced and oxidized glutathione was also shifted to a more reduced state. However, this change was not significant.

Changes of the redox status of luminal stress proteins in diabetes

Next, we wanted to know if the reduced environment of the luminal content of the diabetic ER is reflected in the redox status of PDIs, which have a key role in oxidative protein folding. To monitor the redox status of PDIs, we used AMS, which has been successfully applied to assess the amount of free sulfhydryl groups on specific proteins [9]. Data of Figs. 2A and B show that in diabetic samples both major PDIs, PDI-58 and ERp57 had a large fraction of the reduced enzyme form compared to control, i.e., healthy animals. Interestingly, while PDI-58 showed an almost complete shift to its most reduced form in diabetes, only about two third of ERp57 became reduced in the same samples. The effect was selective on PDI-58 and ERp57, which are directly involved in disulfide bond formation: the redox state of other proteins, such as the major molecular chaperones of the ER, calnexin, Grp78, and Grp94 as well as that of another protein, the inactive PDI-homolog ERp28 was unchanged (Fig. 2C).

Interestingly, the luminal protein responsible for the binding and reoxidization of PDI-58, Ero1-Lα, showed an opposite change becoming more oxidized in diabetic microsomes than in control samples (Fig. 2D). Despite these changes, the amount of immunoprecipitable PDI-58/Ero1-Lα complexes from diabetic microsomes was similar to control (data not shown).

Table 2
Changes of the microsomal thiol-disulfide redox system in diabetes

	Control	Diabetic
Total –SH content (nmol –SH/mg protein)	139 ± 18	187 ± 24*
Protein –SH content (nmol –SH/mg protein)	56 ± 5	79 ± 8*
GSH/GSSG ratio	1.92 ± 0.06	2.25 ± 0.3

Microsomes were isolated from all control and diabetic rat liver samples, and the thiol content was measured by the Ellman's method. The GSH/GSSG ratio was determined from 12 control and 14 diabetic samples as described in Materials and methods. Data represent means ± SD.

* Significant change compared to control ($p < 0.05$).

Protein-disulfide oxidoreductase activity of control and diabetic microsomes

The possible concomitant or parallel changes in the catalytic activity of PDIs were also tested. Using a redox buffer to neutralize the differences of the small molecular redox systems in the samples, we determined PDOR activity in 6 control and 6 diabetic microsomes. A significant decrease of the enzyme activity in diabetic samples compared to control microsomes was detected in the first 15 min of the measurement, which is not caused by a decrease of the protein level (Fig. 3).

In vitro reoxidation of luminal environment in diabetic microsomes

After characterizing the significant changes in the redox state of ER proteins in diabetes, we wanted to assess

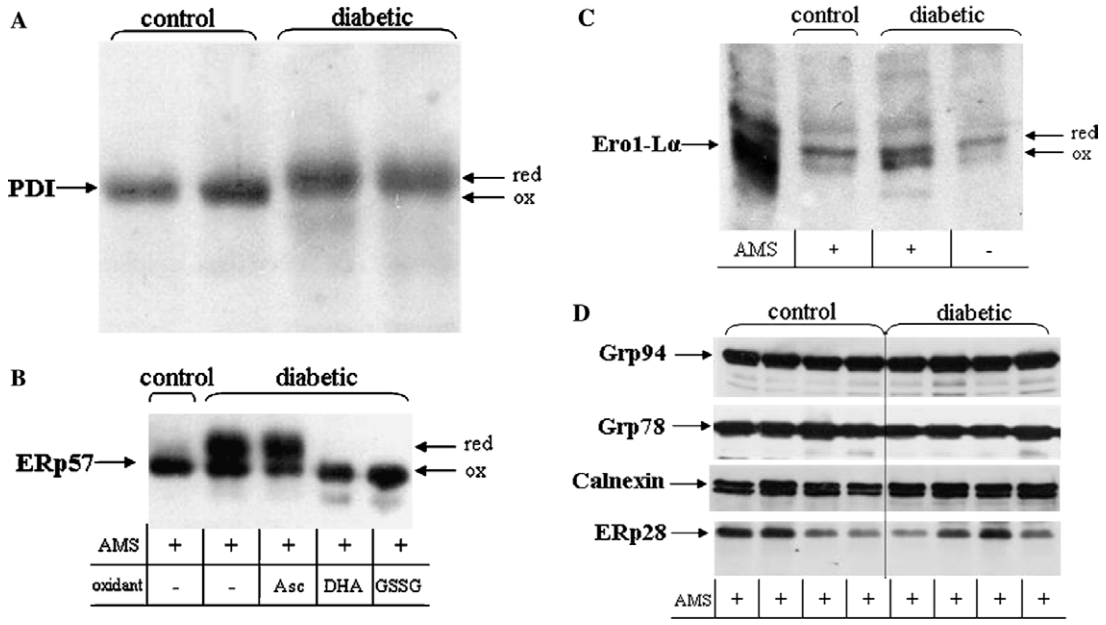

Fig. 2. Changes in the redox status of PDIs and Ero1-Lα in liver microsomes of STZ-diabetic rats. Proteins were purified from 20 control and 33 diabetic rat liver microsomes, and were labeled AMS to visualize their redox state. AMS-labeled proteins were separated by nonreducing SDS–PAGE and the different redox forms of PDIs (PDI-58, A; ERp57, B), as well as of the re-oxidizing protein (Ero1-Lα; C) and other ER chaperones Grp94, Grp78, calnexin and the inactive PDI homolog, and ERp28 (D) were detected by Western blot as described in Materials and methods. In ERp57 samples (B), AMS labeling was also performed after the incubation of microsomes from 14 diabetic rats with different oxidative agents to induce the re-oxidation of ERp57. Asc, ascorbic acid; DHA, dehydroascorbic acid; and GSSG, oxidized glutathione. Blots are representatives of at least three independent experiments showing images of microsomal proteins from control and diabetic animals.

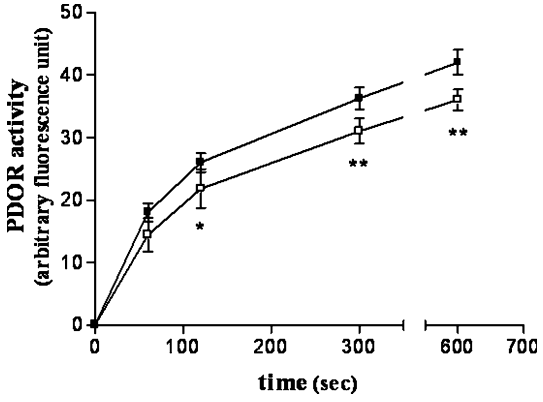

Fig. 3. PDOR activity in control and diabetic microsomes. Microsomal samples were measured by a fluorescent insulin based substrate (di-FTC-insulin) in a GSH/GSSG redox buffer on 37 °C as described in Materials and methods. Six control (filled squares) and six diabetic samples (open squares) were tested and data are means ± SD of three independent experiments. * and ** denote a significant change compared to control, with $p < 0.05$ and $p < 0.01$, respectively.

which redox systems play a role in the redox changes detected. For this, we incubated intact microsomes with different compounds in millimolar range (similar to their intracellular concentration), such as oxidized glutathione, dehydroascorbic acid, and ascorbic acid, which were previously identified (glutathione [29]) or suspected (ascorbate/dehydroascorbate [17]) to play an important role in setting the redox status of the ER in vivo. We also tested the oxidative effect of FAD, the redox prosthetic group of Ero1-Lα, to check the role of a possible FAD shortage or decreased FAD-dependent activity of Ero1-Lα in the reductive shift detected. FAD (0.2 mM) was used, as this concentration was proven to be effective in microsomal oxidation in recent experiments (Papp et al., submitted for publication). Data of Table 3 show that both oxidized glutathione and dehydroascorbic acid were able to restore the abnormally increased protein

Table 3
Re-oxidation of protein thiols in diabetic microsomes

	Total –SH content (nmol –SH/mg protein)
Control	56 ± 5
Diabetic	79 ± 8
Diabetic + GSSG	48 ± 11*
Diabetic + DHA	59 ± 4*
Diabetic + Asc	68 ± 4
Diabetic + FAD	70 ± 7

Intact microsomes isolated from diabetic animals were treated with different redox agents and protein thiol content was determined by Ellman's method as described in Materials and methods. GSSG, oxidized glutathione; DHA, dehydroascorbic acid; Asc, ascorbic acid; FAD, flavine adenine dinucleotide. The total –SH content was determined from 20 control and 33 diabetic samples. Data represent means ± SD.

* Significant change compared to control ($p < 0.05$).

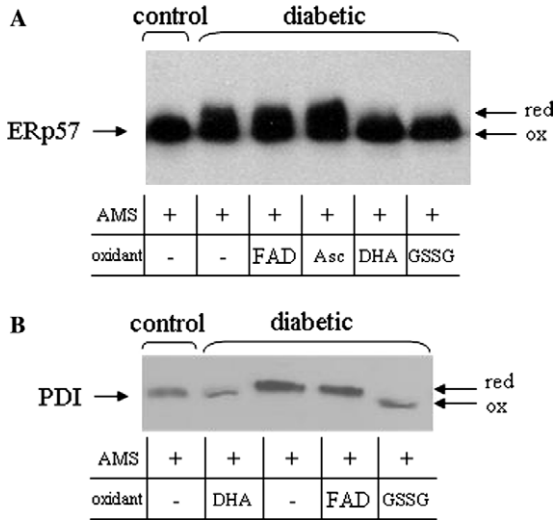

Fig. 4. Re-oxidation of PDIs by small molecular oxidants. In microsomal samples of control or diabetic rat livers, AMS labeling was performed after the incubation of microsomes with different oxidative agents for 15 min at 25 °C to induce the re-oxidation of ERp57 (A) or PDI (B). Microsomal proteins were analyzed on Western blots as described in Materials and methods. Asc, ascorbic acid (used in 2 mM final concentration); DHA, dehydroascorbic acid (used in 2 mM final concentration); FAD, flavine adenine dinucleotide (used in 0.2 mM final concentration); GSSG, oxidized glutathione (used in 2 mM final concentration). Blots are representatives of at least three independent experiments showing images of microsomal proteins from control and diabetic animals.

sulfhydryl levels of diabetic microsomes to those of the control samples, while FAD failed to induce any significant change. Similarly, the reduction of ERp57 and PDI-58 was reversed by oxidized glutathione and dehydroascorbic acid, while ascorbic acid and FAD were ineffective again (Figs. 4A and B).

Discussion

As the most important finding of our work, we identified a significant reduction of the sulfhydryl groups of both the PDI-58 and another major PDI, ERp57, and a parallel decrease of the protein-disulfide oxidoreductase activity in livers of STZ-diabetic rats. This finding explains the reduction of microsomal protein thiol groups and gives the first report on possible functional defects of molecular chaperones in diabetes.

In recent results, mammalian oxidoreductases were shown as they are in a reduced in vivo state [29]. In our experiments, we found oxidoreductases in a partially oxidized form in control samples. A possible explanation of the differences can be: (a) the other experiments were made on yeast and nonhepatic cell cultures, where the balance between the oxidase and reductase/isomerase activity of these proteins can be essentially different; (b) in the other studies authors used slightly different labeling procedure and different labeling agents.

In our recent results, we found that a short term (3 days) of hyperglycemic state in BB/Wor rats results in a reduction of the ER lumen environment, but does not cause any change in PDI expression and PDOR activity ([17], and Table 4). These findings show that the reductive defects develop quite early and are sustained later. The decrease in PDOR activity after 4-week diabetes can be explained by the assumption that protein damaging processes typical to diabetes, such as glycation and carbonylation require a longer time to incapacitate a significant amount of redox folding enzymes. These slowly developing, long-term damages are characteristic to diabetes mellitus causing functional impairment in of many enzymes [31], particularly ER chaperones [32].

The functional defect of PDIs was not accompanied by a significant change either in PDI content or in the level of any other chaperones tested (Fig. 1). These findings seem to be rather surprising, since the presumable increase in unfolded or misfolded proteins in the ER of diabetic animals usually provoke an unfolded protein response [33] leading to a compensatory increase in chaperone levels. The current findings, however, are in agreement with our previous results [12] and other reports showing no change in the protein level of two major chaperones, Grp78 [13] and Grp94 [14] in livers of STZ-diabetic rats.

Similarly, we could not detect an increase in ER chaperones in an in vivo animal model, the AAT-Z mice having a bona fide liver ER protein folding defect, overexpressing a mutant, and aggregating form of α1-antitrypsin in their liver [34]. The corroborating data may indicate that in whole animal models, where the ER experiences a long-term stress of unfolded proteins, the unfolded protein response gets exhausted, the ER chaperones cannot be induced and their levels become normalized. Under these conditions the damaged proteins

Table 4
Effects of 3-day versus 28-day diabetes on ER redox state and PDI

	Protein-SH content	PDOR-activity	PDI level	PDI redox state
3-day diabetes	Increased	Not changed	Not changed	More reduced
28-day diabetes	Increased	Decreased	Not changed	More reduced

Comparison of recent results from 3-day hyperglycemic BB/Wor rats [17] and current data from 28-day hyperglycemic STZ-diabetic Wistar rats. PDOR, protein-disulfide oxidoreductase activity; PDI, protein disulfide isomerase.

are probably transported back to the cytoplasm [35], and degraded by the proteasome. In connection with this assumption, an increase of proteasomal degradation has been detected in diabetes [36].

The reductive shift of the ER seems to be a rather surprising finding taking into account the well-established diabetic oxidative damage. Endoplasmic reticulum is also affected in some aspects [32], however, the oxidative changes occur mainly extracellularly or in the cytoplasm, the latter being related to mitochondrial malfunction [43]. Our data are in agreement with some earlier reports showing a reductive shift of the ER in some extrahepatic tissues in diabetes [44].

The reason for higher ER –SH levels in diabetes is unknown. We hypothesize that protein thiol/disulfide ratio is not affected directly by processes responsible for the oxidative stress in diabetes (e.g., NAD/NADH imbalance, polyol pathway, AGE formation, and alteration of protein kinase C) but changes are triggered by early disturbances of the intermediary metabolism in diabetes. Our results showing the restoration of the redox status after the in vitro addition of oxidized glutathione or dehydroascorbic acid (Table 3) and the oxidative shift of Ero1-Lα raise the possibility that a defective step in the transfer of electrons from the ER proteins to the cytoplasm or a diabetic disturbance of the small molecular redox systems is responsible for the effects observed (Fig. 5). We should note that the final electron acceptor of the oxidative folding (cytoplasmic partner of Ero1-Lα) is not yet exactly identified in mammalian systems. To uncover this missing link could help us to explain surprising results. Among small molecular redox systems both ascorbate/dehydroascorbate and GSH/GSSG are in millimolar concentration in the ER. A severe imbalance of both redox pairs is well characterized in diabetes [45,46]. Although the relative amount of dehydroascorbate increases transiently due to the oxidative change of the cytoplasm, the absolute amount and later on the ratio of both ascorbate and dehydroascorbate decrease. Dehydroascorbate is transported to the ER via a GLUT-like transporter [47], which may cause a shortage of dehydroascorbate in the ER due to a competitive inhibition by the increased hepatic glucose levels [17]. Our results raise the possibility that the decreased level of luminal dehydroascorbate and altered metabolism of glutathione in diabetes can induce or accelerate the reduction of the luminal proteins, especially of PDIs. However, only a few pieces of data support the interaction of dehydroascorbate and PDI so far [48,49]. We should also note that the significant differences in ascorbate metabolism between rats and humans can influence the evaluation of these data in respect of human diabetes.

In addition to dehydroascorbic acid, FAD also participates in the redox changes of the ER [50,51]. Earlier data showed that the level of FAD and the activity of FAD-enzymes are both decreased in diabetes [52]. Here, we showed FAD was unable to sufficiently re-oxidize protein thiols in diabetic microsomes despite of the potent oxidation of ER proteins by extramicrosomal FAD [51]. This finding together with the other observation that Ero1-Lα was more oxidized in diabetic samples (Fig. 2C) suggests that the disulfide bond formation process is disrupted somewhere after Ero1-Lα, and the

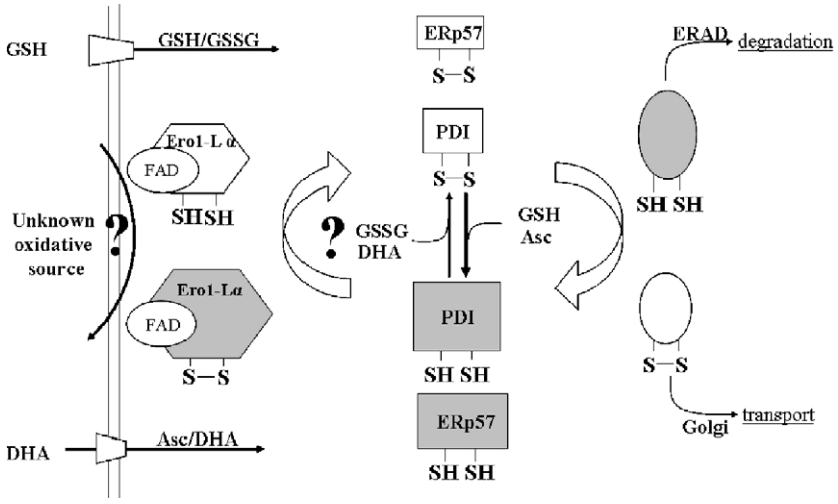

Fig. 5. Summary of changes in ER redox protein folding in STZ-diabetes. PDIs become more reduced in the lumen of the ER in diabetes and are unable to assist in the correct oxidative folding of secreted proteins. Both oxidized glutathione and dehydroascorbic acid are able to reverse this effect. The protein helping in the reoxidation of PDI-58, Ero1-Lα, becomes more oxidized either as a compensation for the defect in PDI function or reflecting a block in the oxidative chain after the protein. Proteins remain partially unfolded and most probably leave the ER via retrograde transport and proteasomal degradation causing a defect in hepatic protein secretion. Asc, ascorbic acid; DHA, dehydroascorbic acid; ERAD, endoplasmic reticulum-associated protein degradation; Ero1-Lα, human endoplasmic reticulum oxidoreductin1-Lα; ERp57, 57 kDa PDI; FAD, flavin adenine dinucleotide; GSH, reduced glutathione; GSSG, oxidized glutathione; PDI, 58 kDa protein disulfide isomerase; –SH, sulfhydryl.

oxidizing power gradually accumulates on Ero1-Lα-despite of an overall reductive change of the ER environment in diabetes.

Recently, a number of impressive studies uncovered multiple links between redox imbalance, ER-stress, protein misfolding, and diabetes mellitus [37–40]. Redox stress can cause ER stress directly or by the concomitant protein misfolding. These provoke the ER-specific unfolded protein response (UPR), might lead to apoptosis in some cells, such as in pancreatic β-cells and worsen diabetes [38,39]. There are some data about a parallel ER stress-induced insulin action inhibition on other tissues, which can deteriorate metabolic disturbances caused by diabetes mellitus [37,40]. These results suggest that ER redox stress could have a central role in the pathophysiology of diabetes mellitus. However, further studies are needed to give a full account on the fate of misfolded proteins in the diabetic ER. These studies will be interesting all the more, since protein degradation in the ER is regulated by the redox changes [41] and PDI-58 itself is participating in the retrograde transport of misfolded proteins to the cytoplasmic protein degradation machinery, the proteasome [42].

As a summary of our results, parallel with a gross reductive change in the redox status of the ER, a significant reductive shift of two important PDIs and a significant decrease of protein-disulfide oxidoreductase activity were reported (Fig. 3). These effects may contribute to the post-translational defects [30] of hepatic protein secretion in diabetes. Our data indicate the possible involvement of the ascorbate/dehydroascorbate redox pair in the disulfide bond formation, but more experiments are needed to uncover its exact contribution to the effects observed.

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