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Biochemical and Biophysical Research Communications 338 (2005) 938–945
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FAD oxidizes the ERO1-PDI electron transfer chain: The role of membrane integrity ☆,☆☆

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Received 17 September 2005
Available online 17 October 2005

Abstract

The molecular steps of the electron transfer in the endoplasmic reticulum from the secreted proteins during their oxidation are relatively unknown. We present here that flavine adenine dinucleotide (FAD) is a powerful oxidizer of the oxidoreductase system, Ero1 and PDI, besides the proteins of rat liver microsomes and HepG2 hepatoma cells. Inhibition of FAD transport hindered the action of FAD. Microsomal membrane integrity was mandatory for all FAD-related oxidation steps downstream of Ero1. The PDI inhibitor bacitracin could inhibit FAD-mediated oxidation of microsomal proteins and PDI, but did not hinder the FAD-driven oxidation of Ero1. Our data demonstrated that Ero1 can utilize FAD as an electron acceptor and that FAD-driven protein oxidation goes through the Ero1-PDI pathway and requires the integrity of the endoplasmic reticulum membrane. Our findings prompt further studies to elucidate the membrane-dependent steps of PDI oxidation and the role of FAD in redox folding.

Keywords: Redox folding; Membrane integrity; Nutritional regulation; Electron transfer chain; FAD

Oxidative protein folding is necessary for the maturation of most secreted and plasma membrane proteins. The correct formation of disulfide bonds needs a sensitively regulated redox environment. In eukaryotes, this environment is provided by the endoplasmic reticulum (ER) [1,2].

Protein disulfide isomerases help the formation of disulfide bonds in the ER [3,4]. The major, 58 kDa protein disulfide isomerase of the ER (PDI) constitutes ~2% of ER proteins [5]. PDI has disulfide isomerase and chaperone activity also [6]. Later, several other protein disulfide isomerases have been identified, which participate in specialized protein folding pathways in the ER [7,8].

Ero1, a conserved, ER-resident enzyme was identified as an electron acceptor of PDI [9–11]. Ero1 interacts with PDI in eukaryotes initiating the transfer of the oxidizing equivalents to folding proteins [12,13]. In yeast, Ero1 association to the membranes required to this action [14]. Human Ero1s also behave as peripheral membrane proteins [15,16].

Molecular oxygen was found to be an electron acceptor for yeast Ero1 [17]. Since yeast Ero1 can enhance disulfide bridge formation under anaerobic conditions [18], the existence of an alternative electron acceptor molecule is possible.

In yeast FAD accelerated the disulfide bridge formation by Ero1 [17] and riboflavin depletion resulted in a defective oxidative folding [19]. Furthermore, over-expression of the

☆ This work was supported by research grants from the EU (FP6-506850, FP6-016003), Hungarian Science Foundation (OTKA T37357, T48939, TS40865, and F47281), Hungarian Ministry of Social Welfare (ETT 32/03, 613/03), and by the Hungarian National Research Initiative (NKFP-1A/056/2004 and KKK-0015/3.0).
☆☆ *Abbreviations:* AMS, 4-acetamido-4′maleimidylstilbene 2,2′-disulfonic acid; DIDS, 4,4′(diisothiocyanato)stilbene-2-2′-disulfonic acid; DOC, deoxycholic acid; ER, endoplasmic reticulum; Ero1, ER-oxidizing protein 1; ERp44 and ERp72, members of the ER protein disulfide isomerase family; FAD, flavine adenine dinucleotide; Grp94, 94 kDa glucose regulated protein, a major ER chaperone; GSH, glutathione-reduced form; GSSG, oxidized glutathione; PDI, the 58 kDa major protein disulfide isomerase of the endoplasmic reticulum.
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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.10.027

enzyme FAD1, which converts flavine mononucleotide (FMN) to FAD, can compensate Ero1 deficiency in yeast [19]. In Jurkat cells, flavine deficiency resulted in decreased secretion and intracellular accumulation of IL-2 [20]. A recent publication demonstrates that riboflavin depletion results in impaired oxidative folding, triggering stress response in human hepatoma HepG2 cell line [21]. In mammals, Varsanyi et al. [22] showed that FAD enters the ER lumen and can promote the oxidation of protein thiols as well as the oxidation of glucose-6-phosphate. These data support the idea that in mammalian cells, similar to yeast, FAD may contribute to oxidative protein folding.

We demonstrated that mammalian Ero1 utilizes FAD as an electron acceptor and that FAD-driven protein oxidation goes through the Ero1-PDI pathway. We also demonstrated that FAD transport is a prerequisite for its action on the ER lumenal proteins, since DIDS, an anion transporter inhibitor, which blocks FAD transport [22] could hinder the action of FAD in the ER on the oxidation of protein thiols, PDI and Ero1.

Experimental

Materials. Bacitracin was purchased from Calbiochem (La Jolla CA, USA) and AMS was Molecular Probes (Eugene OR, USA) product. The polyvinylidene difluoride (PVDF) membranes and electrophoresis reagents were from Bio-Rad (Hercules, Canada). The PDI monoclonal mouse antibody was purchased from StressGen (SPA 891 Lot# B205409, Victoria BC, Canada) and the Ero1-L polyclonal rabbit antibody was a kind gift of Ineke Braakman and Roberto Sitia [23]. The enhanced chemiluminescence (ECL) kit was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). ERp72, Grp94, and calnexin antibodies were purchased from StressGen (SPA-720 Lot# 708430, SPA 850 Lot# 709074, and SPA860 Lot# 708408, respectively, Victoria BC, Canada). Alamethicin, FAD, DIDS, and all other chemicals were purchased from Sigma (St. Louis MO, USA).

Microsome preparation. Liver microsomes were prepared from male Sprague–Dawley rats (fed ad libitum, 180–230 g of body weight; Charles River, Hungary) using the method of Varsanyi et al. [22]. Intactness of microsomal vesicles was routinely checked by measuring the change in light scattering after the addition of the non-permeable compound, sucrose [24]. The intactness of the microsomes was also checked by measuring enzyme latencies (Table 1). Purity of microsomal preparations was determined by measuring the activities of glucose-6-phosphatase, UDP-glucuronosyltransferase, cytochrome c oxidase, and 5′-nucleotidase as markers [24,25]. To avoid uncontrolled oxidation, we used fresh samples and de-gassed, relatively 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 using the Bradford method [26] using bovine serum albumin as standard.

Cell culture. Human HepG2 hepatoma cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C and treated with bacitracin at a final concentration of 3 mM for 18 h. Cells were harvested, pelleted with low-speed centrifugation, and microsomes were prepared following the method of Ref. [27].

Measurement of the thiol content of ER proteins. Microsomes (containing 2.5 mg protein per ml) were diluted in 50 mM Tris–HCl, pH 7.2, and were incubated in the presence or absence of FAD at 37 °C at the concentrations indicated for 20 min. After the incubation, the protein fraction was precipitated by 10% trichloroacetic acid, washed three times by 70% acetone, and resuspended in a buffer containing 50 mM Tris–HCl, pH 6.8, 8 M urea, and 2% sodium dodecyl sulfate. Thiol content of the resuspended protein fraction was measured by the Ellman method [28] applying a molar extinction coefficient of 14,150 for 2-nitro-5-thiobenzoic acid and using a Hitachi U-1500 spectrophotometer.

Measurement of the redox state of Ero1 and PDI. Liver microsomes were dissolved in 0.1 M Tris–HCl, pH 7.2, and were pre-treated with various agents as specified in the actual experiments. Proteins were precipitated by 5% trichloroacetic acid, and after centrifugation the pellet was washed three times with 70% acetone and with the 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, and were treated with 20 mM 4-acetamido-4′maleimidylstilbene 2,2′-disulfonic acid (AMS) for 30 min at 37 °C using the method of Frand and Kaiser [29]. AMS binds to the free thiol groups of cysteine residues resulting in a decreased mobility of the proteins. In each experiment, we used the thiol reducing agents glutathione (GSH) or DTT (50 mM each) and oxidized glutathione (GSSG) or the thiol-specific oxidant, diamide (at 50 mM or 10 mM final concentrations, respectively), to check the efficiency of AMS labeling and detect the position of maximally reduced and oxidized state of proteins. AMS-labeled proteins (15 μg) were separated by electrophoresis on non-reducing 9% SDS–PAGE gels and were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h with washing buffer containing 5% non-fat milk powder and then probed with primary antibodies against Ero1 or PDI 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 washes, labeled protein bands were detected using the enhanced chemiluminescence (ECL) technique according to the manufacturer's instructions.

Statistical analysis. Data are presented as means ± SD of minimum three independent experiments unless otherwise indicated and were analyzed with unpaired Student's t test. A value of $p < 0.05$ was accepted as indicating a statistically significant difference compared with controls.

Results

FAD is a potent oxidizer of the ER lumenal proteins

To characterize the effect of FAD-induced oxidation of ER proteins, rat liver microsomes were incubated in the presence of FAD ranging from 5 to 500 μM. Fig. 1 shows that FAD was able to oxidize ER proteins in a concentra-

Table 1
Enzyme latencies of the microsomal preparations

Transported substrate	Enzyme activity measured	Reference (for the method)	Latency (%)
Mannose-6-phosphate	Mannose-6-phosphatase	[31]	90.9 ± 1.9
UDP-glucuronic acid	UDP-glucuronosyltransferase	[45]	90.7 ± 1.1
$NADP^+$	Hexose-6-phosphate dehydrogenase	[46]	92.0 ± 4.1

Measurement of various enzyme activities was performed as described earlier (see reference numbers of column 3) in the absence or presence of the pore-forming alamethicin (0.1 mg/mg protein) ensuring full permeabilization of the microsomes. Enzyme latency data refer to the percent of the enzyme activities, which become exposed after the permeabilization of microsomes taking the enzyme activity of permeabilized microsomes as 100%. Data represent means ± SD of 4–6 measurements on independent preparations.

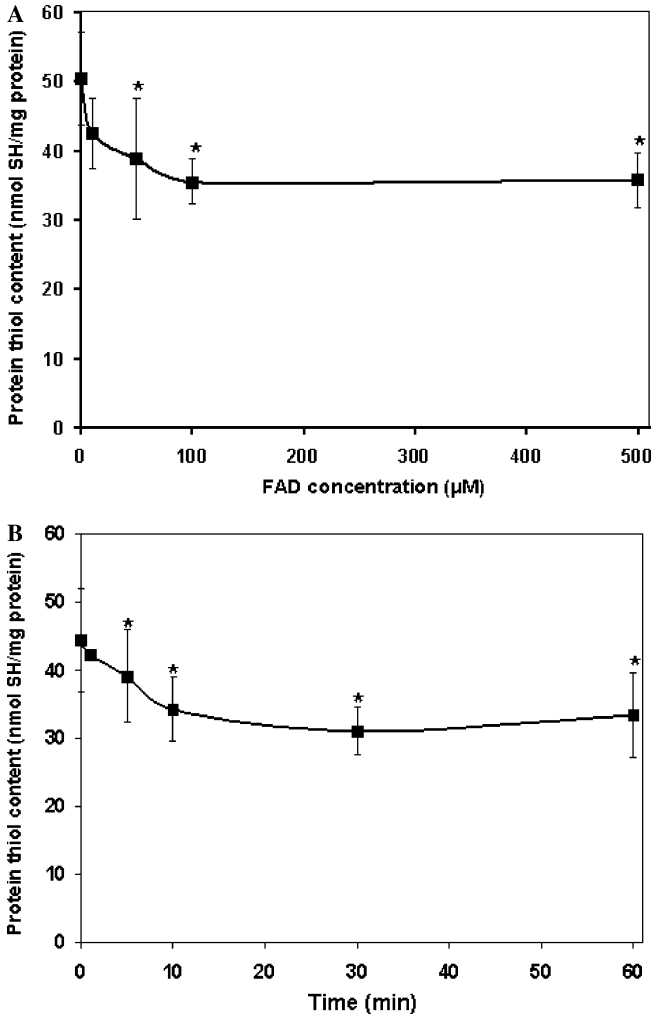

Fig. 1. Concentration and time dependence of FAD-induced oxidation of ER proteins. Rat liver microsomes containing 300 μg proteins in 100 μl of incubation buffer were incubated at 37 °C with the amounts of FAD and for times indicated. Microsomal proteins were precipitated with 5% trichloroacetic acid, and their free thiol content was measured using Ellman's method as described in Experimental. The decrease in the amount of free thiol groups indicates the extent of FAD-induced protein oxidation. (A) Concentration dependence of FAD-induced protein oxidation. The incubation time with FAD was 30 min. (B) Time dependence of FAD-induced protein oxidation. The FAD concentration was 100 μM. Data are means ± SD of three independent experiments. Asterisks indicate significant differences of FAD-treated samples from control, non-treated samples ($p < 0.05$).

tion-dependent manner. The maximal effect was reached at 100 μM FAD, which is in agreement with earlier data [22]. A similar extent of protein oxidization was caused by 50 mM GSSG or 10 mM diamide (data not shown).

The time course of FAD-induced protein oxidation was also measured. In this set of experiments, 100 μM FAD was used at all points for different times from 0 to 60 min. Protein oxidation reached a plateau after 30 min. Neither GSSG, nor dehydroascorbate, which are all potent oxidizers of ER proteins in the millimolar concentration range [22], was effective, if we used them in the same, 100 μM concentration as we had for the maximal effect of FAD (data not shown). Besides FAD, other potential electron acceptors, coenzyme Q10, coenzyme Q1, menadione, as well as pyridine nucleotides were all used in 100 μM concentration, but none of them had any effect on the microsomal thiols (data not shown), in agreement with earlier data [30].

Membrane integrity is needed for FAD-induced oxidation of ER proteins

To ensure a free access of FAD to the ER lumen, we measured the action of FAD and other oxidizing agents in the presence of deoxycholic acid (DOC), a strong detergent, and alamethicin, a pore-forming agent. To our surprise, FAD-generated oxidation of ER lumenal proteins was suspended by both forms of membrane disruption (Fig. 2A). In contrast, GSSG-mediated oxidation was promoted by adding detergent to the microsomes (Fig. 2A). The action of GSSG at a 10-fold higher concentration and of other oxidizers, like high concentration (50 mM) of dehydroascorbate, did not depend on membrane integrity (Fig. 2A). These data showed that entrance of free FAD to the ER lumen is not enough to promote the formation of protein disulfide bonds.

Next we checked, whether inhibition of FAD transport influences its oxidizing potential. We used DIDS, an anion transporter inhibitor, which was shown to inhibit FAD-uptake to the ER lumen [22]. DIDS treatment resulted in a suspended FAD action showing that uptake of FAD is a prerequisite for its oxidative effect (Fig. 2B). Mechanical disruption of ER membranes was also examined by sonicating microsomes prior to FAD treatment. Sonication also hindered FAD-related protein oxidation (Fig. 2B).

Membrane integrity is also needed for FAD-induced oxidation of PDI and Ero1

To extend our finding that the membrane integrity is important for the FAD-dependent oxidation of ER proteins, and to characterize the molecular mechanism of FAD action, we checked how the redox state of the key ER redox proteins, Ero1 and PDI, changes after the addition of FAD in the absence and presence of membrane disrupting agents. The redox state of Ero1 and PDI was monitored by adding the thiol-reagent, 4-acetamido-4′maleimidylstilbene 2,2′-disulfonic acid (AMS), and analyzing the characteristic shift in the mobility of either reduced Ero1 or reduced PDI after this treatment as described earlier [29] and successfully applied in our laboratory [32,33]. In Fig. 3, the results of a typical experiment are shown. Ero1 is in a moderately reduced state in the microsomes: FAD could oxidize Ero1 to the same extent as 50 mM GSSG (cf. lanes 2 and 8 in the upper panel of Fig. 3). Similarly, PDI was in a partially oxidized state in the microsomes, and FAD was able to oxidize PDI comparable to that after GSSG treatment (middle panel of Fig. 3).

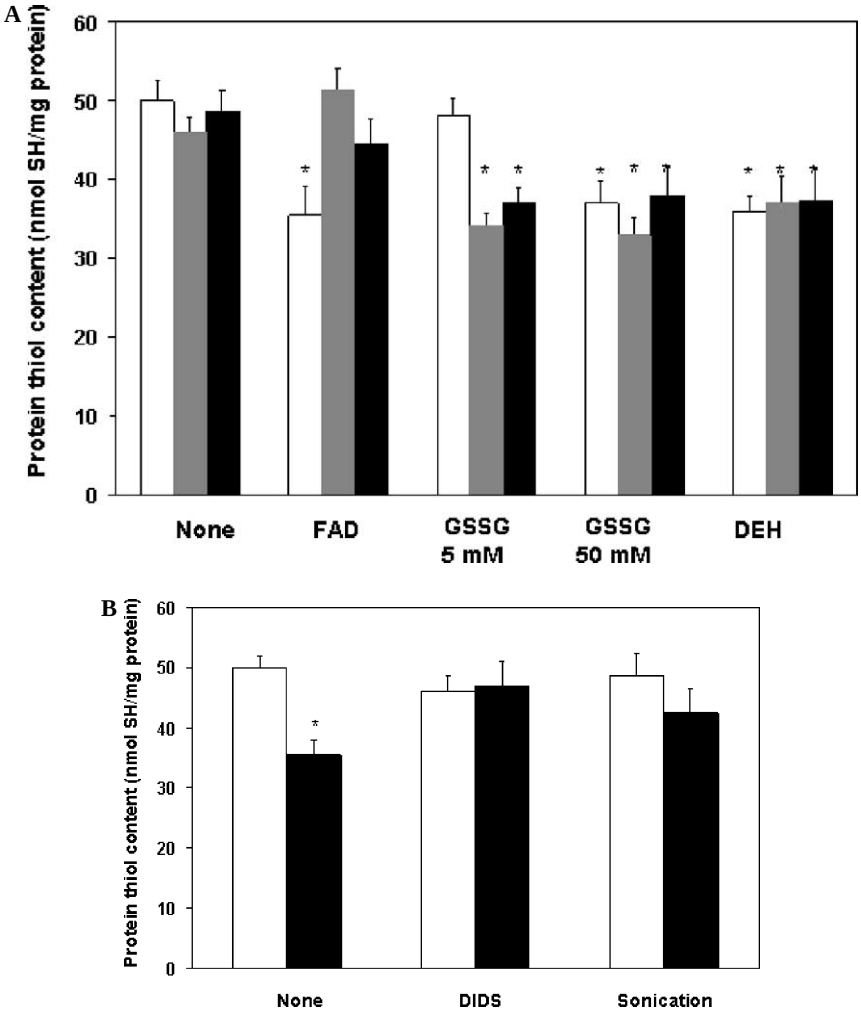

Fig. 2. Effect of various treatments on the oxidation of ER proteins. (A) A 100 μl aliquot of rat liver microsomes (3 mg protein/ml) was pre-incubated for 15 min in the absence (white bars) or in the presence of the pore-forming agent, alamethicin (10 mM) (gray bars), or the detergent deoxycholic acid (DOC, 50 mM) (black bars) at 37 °C. After these treatments, samples were incubated in the absence or presence of 100 μM FAD or 5 or 50 mM GSSG or 50 mM dehydroascorbate for an additional 30 min at 37 °C as indicated. Microsomal proteins were precipitated, washed, and their thiol content was measured as described in Experimental. (B) Microsomes were incubated for 15 min at 37 °C in the presence or absence of the anion transport inhibitor, DIDS (100 μM), or were sonicated for 3 s (at 35 W setting of a Sonic 300 dismembrator—Farmingdale, NY, USA) on ice, as indicated. After these treatments, samples were incubated in the absence (white bars) or in the presence of 100 μM FAD (black bars) for an additional 30 min. Microsomal proteins were precipitated, washed, and their thiol content was measured as described in Experimental. Data are means ± SD of minimum three, maximum six independent experiments. In panel A, asterisks denote significant differences between control (columns marked with "None") and the matching column of treated samples ($p < 0.05$). In panel B, the asterisk denotes a significant difference between control (white) and FAD-treated (black) samples of the columns marked with "None" ($p < 0.05$).

We checked the effect of FAD on other ER chaperones, such as ERp72 (lower panel on Fig. 3), calnexin, and Grp94, but no change in the redox state was seen (data not shown).

DIDS and alamethicin preincubations did not alter the redox state of Ero1 (cf. lane 1 with lanes 3 and 5 on the upper panel of Fig. 3), but were able to suspend FAD action (cf. lane 2 with lanes 4 and 6 on the upper panel of Fig. 3). A similar picture was seen in the case of PDI, where DIDS and alamethicin did not influence the redox state of PDI (cf. lane 1 with 3 and 5 in the lower panel of Fig. 3), but were able to diminish the effect of FAD (cf. lane 2 with lanes 4 and 6 in the lower panel of Fig. 3).

PDI inhibition blocks the FAD-induced oxidation of ER proteins

In agreement with our findings shown in Figs. 1 and 2, data of Fig. 3 demonstrated that both FAD transport and membrane integrity are needed for the FAD-induced oxidation of Ero1 and PDI, but did not tell whether these key ER oxidoreductases are directly involved in the FAD-induced oxidation of ER proteins. Since no selective inhibitor for Ero1 has been established so far, we went one step forward, and monitored the redox state of ER proteins after PDI inhibition using bacitracin, a metalloprotein known to inhibit PDI [34]. Bacitracin is regularly used to

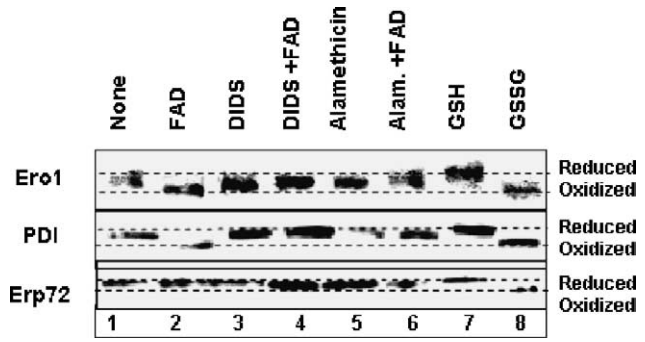

Fig. 3. FAD-induced oxidation of major ER redox proteins. Microsomes containing 40 μg ER proteins were pre-incubated for 15 min in 100 μl of 15 mM Tris buffer (pH 7.2) at 37 °C in the presence or absence of 100 μM of the anion transporter inhibitor, DIDS, or 10 mM of the pore-forming agent, alamethicin, respectively. After pre-incubation, 100 μM FAD was added and samples were incubated further for 30 min at 37 °C. Sample proteins were then precipitated and washed as described in Experimental. To selected samples 50 mM GSH or GSSG was added to reach the maximally reduced or oxidized state of the proteins, respectively. AMS labeling, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and Western blots were performed as described in Experimental. Upper panel: oxidation state of Ero1. Middle panel: oxidation state of PDI. Lower panel: oxidation state of ERp72. The gels shown are representative of 5 experiments with similar results.

examine the role of PDI in the processing of secretory proteins [35,36]. For these experiments we used the human hepatoma cell line, HepG2. Fig. 4 shows that the redox state of ER proteins is slightly more reduced in bacitracin-treated hepatoma cells than in non-treated cells, however, this change is not significant. FAD could oxidize the non-treated microsomal proteins up to 30%, which is in agreement with the previous data shown in Figs. 1 and 2. Bacitracin inhibited the effect of FAD. In contrast, the diamide-induced direct, chemical oxidation did not differ in the absence or presence of bacitracin, which excludes non-specific effects of bacitracin on ER proteins.

These results demonstrate that FAD utilizes the Ero1-PDI pathway, and inhibition of PDI is sufficient to block the FAD effect.

PDI inhibition blocks the FAD-induced oxidation of PDI but not of Ero1

Finally, we checked the redox state of the key redox proteins in bacitracin-treated cells. This time we used DTT and diamide as a control for maximally reduced and oxidized state of proteins, since glutathione acts on the Ero1-PDI pathway [37,38] and bacitracin treatment may diminish its effect. Data of Fig. 5 show that both Ero1 and PDI are more reduced in the bacitracin-treated cells than in control cells, correlating results shown in Fig. 4. FAD could oxidize Ero1 in both control and bacitracin-treated cells (cf. lanes 1 and 4 with 2 and 5 in the upper panel of Fig. 5). In both cases DIDS, the inhibitor of FAD transport, blocked FAD action (cf. lanes 3 and 6 with 2 and 5 in the upper panel of Fig. 5). In the lower panel of Fig. 5, changes of PDI mobility are shown. In control cells, the picture is similar to that of Fig. 3, i.e., PDI can be oxidized by FAD and this effect is reversed by DIDS (cf. lanes

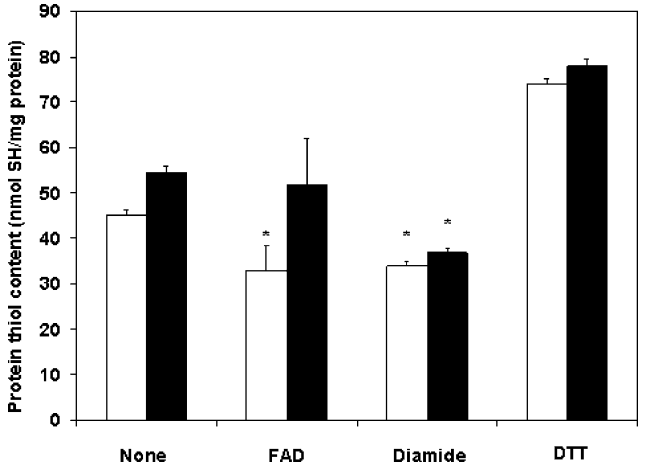

Fig. 4. Effect of PDI inhibition on the FAD-induced oxidation of ER proteins. HepG2 hepatoma cells (~80% confluent monolayer of cells on a 12 cm tissue culture dish) were incubated for 18 h in the absence (open bars) or presence (filled bars) of 3 mM of the PDI inhibitor, bacitracin, at 37 °C. Cells were harvested and 100 μl of freshly isolated microsomes (300 mg protein/ml) was incubated in the absence or presence of 100 μM FAD or 5 mM diamide or 50 mM DTT as indicated. Microsomal proteins were precipitated, washed, and their thiol content was measured as described in Experimental. Data are means ± SD of three independent experiments. The asterisks denote significant differences between control (columns marked with "None") and the matching column of treated samples ($p < 0.05$).

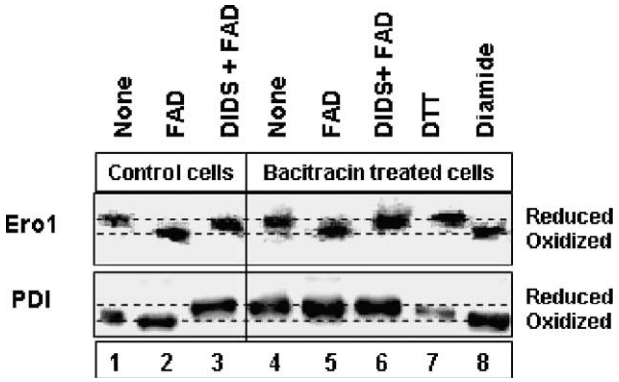

Fig. 5. Effect of PDI inhibition on the FAD-induced oxidation of major ER redox proteins. HepG2 hepatoma cells (~80% confluent monolayer of cells on a 12 cm tissue culture dish) were incubated in the absence or presence of 3 mM of the PDI inhibitor, bacitracin, for 18 h at 37 °C. Cells were harvested and their microsomes were isolated as described in Experimental. Hundred microliters of both control and bacitracin-treated microsomal samples (4 mg protein/ml) was incubated with 100 μM FAD or 100 μM DIDS where indicated as described in the legend of Fig. 3. We used reduced DTT (50 mM) and the thiol-specific oxidant, diamide (10 mM), to check the efficiency of AMS labeling and detect the position of maximally reduced and oxidized state of proteins. AMS labeling, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blots were performed as described in Experimental. Upper panel: oxidation state of Ero1. Lower panel: oxidation state of PDI. The gels shown are representatives of three experiments with similar results.

1 through 3 in the lower panel of Fig. 5). Bacitracin treatment resulted in a complete block of the FAD-driven oxidization of PDI (cf. lanes 1 and 2 with 4 and 5 in the lower panel of Fig. 5). This was not due to the incapacitation of PDI for oxidation, since the thiol-specific oxidant, diamide, oxidized PDI effectively (cf. lanes 4 and 8 in the lower panel of Fig. 5) An additional band is appearing above PDI in this figure. This protein is calreticulin, which is also recognized by the PDI-specific antibody according to the producer's technical specification sheet. This protein did not show redox sensitivity in these experiments.

Similar experiments were done on the Jurkat human lymphoma cell line. In Jurkat cells, the proteins examined here were already in a totally oxidized state and FAD supplementation could not alter their redox state (data not shown).

Discussion

Formation of disulfide bonds is a key event in the maturation of secreted or plasma membrane proteins. The role of small molecules in this oxidation process is relatively unknown. Our experiments showed that FAD was a powerful oxidizer of Ero1 and PDI besides other microsomal proteins. Approximately 30% of the total thiol content of lumenal proteins can be oxidized with different agents, such as FAD, GSSG [22], or dehydroascorbate [39]. FAD is an efficient oxidizer, since 100 μM FAD could reach maximal oxidation of the microsomal proteins, while all the other oxidants were effective only in the millimolar range.

In yeast, Tu et al. [17] found that the redox status of Ero1 is sensitive to the free FAD content of the cell. The maximal oxidoreductase activity of yeast Ero1 was reached by adding FAD in 50 μM concentration. The profound differences between yeast and mammalian cells might explain such a twofold difference in the required optimal FAD concentration. Camporeale and Zempleni [20] found an impaired folding of IL-2 in a flavine-deficient cell line, which demonstrated the unique role of FAD in the redox folding process of mammalian cells. Since Ero1 is a membrane associated, lumenal protein [14–16], it was likely that FAD entering the lumen is sufficient to fulfill its oxidative role. Indeed, the anion transporter, DIDS, which was proven to block FAD intake into the microsomal space [22], hindered the FAD-driven oxidation of ER proteins as well as those of Ero1 and PDI.

FAD-induced protein oxidation is specific, since neither ERp72, a member of the PDI family, nor other ER proteins, such as calnexin or Grp94, were oxidized by FAD.

These results are in agreement with the suggestion that the membrane association of Ero1 is necessary for its FAD binding ability [40]. Inhibition of PDI (by bacitracin) stops the electron flow at an earlier step, since the redox balance of Ero1 can be changed in the presence of the electron acceptor FAD, but the electron flow between PDI and Ero1 (and probably between substrate proteins and PDI) is blocked.

We demonstrated that membrane integrity per se is needed for FAD-induced oxidization of ER proteins. Many oxidative agents, such as 50 mM GSSG or dehydroascorbate, can oxidize ER proteins directly. In contrast, disruption of the membrane by alamethicin, a pore-forming agent, as well as the strong detergent, DOC, or mechanical membrane disruption by sonication, blocked the effect of FAD on protein thiol oxidation.

Multi-component electron transfer systems are often membrane associated to ensure the segregation and proper transfer of electrons between their components. The permeabilizing agents used in our study may disturb the lipid–protein interactions in the membrane with a subsequent impairment of the electron transfer. This can be the case after the addition of detergents, such as DOC, but the pore-forming alamethicin seems not to influence the conformation of membrane proteins [41].

Alamethicin permeabilization selectively releases low molecular compounds [42]. The release of the components of glutathione redox buffer may influence the redox potential in the lumen. However, glutathione is not essential in the process of oxidative protein folding in yeast [20]. Alternatively, an escape of other small molecular weight compounds could be responsible for the effect. Since permeabilization stops the electron flow between Ero1 and FAD, these putative compounds could transfer electrons from Ero1 to FAD or, more likely, could have a permissive role as a positive regulator of Ero1.

Treatment with both DIDS and alamethicin blocked the effect of FAD on the redox state of Ero1 and PDI. FAD-driven oxidation was also hindered by bacitracin treatment. During these experiments, we found that bacitracin, the inhibitor of PDI oxidoreductase activity [34–36], completely inhibited the FAD-driven oxidation of ER lumenal proteins. These data suggest that FAD uses the Ero1-PDI pathway for the oxidation of ER proteins. Many members of the PDI family have disulfide-isomerizing potential. Since no data are available with regard to the interaction of bacitracin with other members of the PDI family, we cannot exclude that bacitracin inhibited more than one type of PDIs in our experiments. On the other hand, Ero1 was found to form mixed disulfide only with PDI [12], which supports the idea that protein is performed by the Ero1-PDI pathway. Thus, FAD emerges as a specific oxidative agent for the PDI-mediated oxidation of ER proteins.

In HepG2 cells, the inhibition of PDI did not lead to accumulation of oxidized Ero1. When an electron acceptor was added (i.e., FAD), the oxidized form of Ero1 appeared. This is in good correlation with the fact that in yeast Ero1 could not promote the oxidation of ER proteins in a FAD-free environment [17].

In a recent publication, bacitracin was found to inhibit IL-12 αβ and ββ dimers, which are substrates of PDI, but did not influence the mRNA levels or the expression of the β monomer, which is folded independently from PDI [36], supporting the idea that in spite of its multiple

targets within the cell, its major influence is due its ability to inhibit PDI.

Stress is often coupled by oxidative damage in the mitochondria, nucleus, cytoplasm, and plasma membrane. Marciniak et al. [43] found that cells lacking the stress-inducer CHOP gene have an impaired induction of Ero1, a relatively hypo-oxidizing cellular environment and a better survival. Thus, hypo-oxidizing environment may help to protect cells from lethal consequences of ER stress. In chronic stress redox protection of the ER can be even more important to avoid a massive cell death. This idea is in good correlation with earlier observations that the endoplasmic reticulum became more reducing in case of streptozotocin-induced diabetes, and also in a transgenic mice model accumulating a misfolded, mutant human alpha-1 antitrypsin in the ER of liver cells [32,33]. The redox imbalance in the case of diabetic samples could be reversed by GSSG or dehydroascorbate, but was insensitive for FAD treatment [44]. This may reflect a functional switch of PDI from redox enzyme to a chaperone as a response for changing redox environment [45].

Our findings give a starting framework to assess the molecular steps of FAD action in the oxidation of ER lumenal proteins in healthy organisms and under chronic stress induced by aging or various diseases. Our data raise the possibility to use FAD as a PDI-specific oxidative agent, and prompt further studies to elucidate the membrane-dependent steps of Ero1-oxidation.

Acknowledgments

The authors thank Katalin Mihály (Department of Medical Chemistry, Semmelweis University, Budapest, Hungary) for her valuable help in the cell culture work. The kind gift of Ero1 antibodies from Ineke Braakman (Bio-Organic Chemistry Utrecht University, Utrecht, The Netherlands) and from Roberto Sitia (San Raffaele Scientific Institute, Milano, Italy) is gratefully acknowledged.

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