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Accumulation of overoxidized Peroxiredoxin III in aged rat liver mitochondria

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ABSTRACT

Overoxidation and subsequent inactivation of Peroxiredoxin III (PrxIII), a mitochondrial H_2O_2 scavenging enzyme, have been reported in oxidative stress conditions. No data are available in the literature about the presence of overoxidized forms of PrxIII in aged tissues. Liver mitochondria from 12-month-old rats and 28-month-old rats were here analyzed by two-dimensional gel electrophoresis. A spot corresponding to the native form of PrxIII was present in adult and old rats with the same volume, whereas an additional, more acidic spot, of the same molecular weight of the native form, accumulated only in old rats. The acidic spot was identified, by MALDI-MS analysis, as a form of PrxIII bearing the cysteine of the catalytic site overoxidized to sulphonic acid. This modified PrxIII form corresponds to the irreversibly inactivated enzyme, here reported, for the first time, in aging. Three groups of 28-month-old rats treated with acetyl-L-carnitine were also examined. Reduced accumulation of the overoxidized PrxIII form was found in all ALCAR-treated groups.

1. Introduction

Mitochondria play a key role in aging since they are the major source of intracellular reactive oxygen species (ROS) and, at the same time, the primary target of their action. Mitochondria, which utilize 90% of cellular oxygen, produce ROS as by-products of the respiration: in physiological conditions 0.2% of the utilized oxygen is converted into ROS [1]. ROS are highly reactive molecules harmful to the cell macromolecules: mitochondrial macromolecules, which are nearby the major source of ROS, are primarily damaged. ROS production increases during aging and seems to be responsible for the oxidative damage to mitochondrial macromolecules verified in different aging tissues [2]. To cope with ROS the cell expresses several antioxidant enzymes which are localized in different cellular compartments [3].

Peroxiredoxins (Prxs) are a family of peroxidases, with ubiquitous distribution [4]. Mammalian Prxs include six members (Prx I–VI). Peroxiredoxin III (PrxIII) is exclusively located into mitochondria and it is involved in the regulation of mitochondrial H_2O_2 concentration. It is about 30-fold more abundant than glutathione peroxidase I in mitochondria of HeLa cells [5]. PrxIII belongs to 2-Cys Prxs subgroup, which contains two conserved Cys residues, both required for the catalytic function. In the catalytic cycle of 2-Cys Prx proteins, the conserved "peroxidatic" cysteine residue of each subunit of the Prx homodimer is selectively oxidized by H_2O_2 to sulphenic acid (Cys-SOH), which then reacts with the conserved "resolving" Cys of the other subunit to form an intermolecular disulfide. In order to complete the enzymatic catalytic cycle, the peroxiredoxin is reduced back to its active thiol form by the thioredoxin/thioredoxin reductase system [6]. However, the formation of the disulfide is a slow process, thus under oxidative stress conditions, the sulphenic intermediate can be easily overoxidized to cysteine sulphinic (Cys-SO_2H) or sulphonic acid (Cys-SO_3H), resulting in the inactivation of peroxidase activity [7]. Nevertheless, the cysteine sulphinic acid form of 2-Cys Prxs, in vitro, can be reduced to the catalytically active thiol form by sulfiredoxin [8], whereas the cysteine sulphonic acid form cannot be reduced to the active form [7].

The presence of overoxidized forms for Prx I, II, III and IV has been reported in cell cultures treated with H_2O_2 [9,10] or with other

Abbreviations: 2-DE, two-dimensional polyacrylamide gel electrophoresis; ALCAR, acetyl-L-carnitine; CAM, carboxyamidomethyl; CHAPS, 3-((3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate; Cys-SO_2H, sulphinic acid; Cys-SO_3H, sulphonic acid; Cys-SOH, sulphenic acid; DTT, dithiothreitol; ESI–MS, electrospray ionization–mass spectrometry; IPG, immobilized pH gradient; MALDI-MS, matrix assisted laser desorption/ionization-mass spectrometry; Mn SOD, Manganese Superoxide Dismutase; mtNOS, mitochondrial Nitric Oxide Synthase; NRF-1, nuclear respiratory factor-1; PGC-1alpha, peroxisome proliferator-activated receptor gamma coactivator 1alpha; Prx, peroxiredoxin; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFAM, mitochondrial transcription factor A

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hydroperoxides [11]. In oxidative stress conditions, overoxidized forms of Prxs were identified after ischemia/reperfusion occurring during human liver transplantation [10]. At the present, nothing is known about the status of the mitochondrial PrxIII in the oxidative stress conditions associated with aging.

The purpose of the current study was to investigate about the presence of oxidized forms of PrxIII in aged rat liver where oxidative stress and loss of Complex I, Complex IV, Mn SOD and mtNOS activities have been described [12,13].

Acetyl-L-carnitine (ALCAR) is a naturally occurring substance that has been reported to limit age-linked mitochondrial macromolecular damages and mitochondrial function alterations in heart, brain and skeletal muscle [14–24]. In perfused old rat livers, 1 month of ALCAR treatment slows down the age-associated decline of mitochondrial respiration and biosynthetic functions [25]. Furthermore, in hepatocytes of old rats, ALCAR supplementation in drinking water for 1 month reversed the age-associated decline of mitochondrial membrane potential; additionally it restored the cardiolipin content, which declined significantly with age, and raised cellular oxygen consumption to the level of young rats [26]. In several oxidative stress conditions a protective role of ALCAR was reported, mainly in the brain, on generalized proteins oxidation [27–32]. By using a redox proteomic approach, the protective effect of ALCAR treatment on the increased carbonylation of specific proteins in the brain was reported by Poon et al. [33].

Results reported here show that, as revealed by two-dimensional gel electrophoresis, an acidic form of PrxIII was present and accumulated in old rats whereas the native form of PrxIII was present, at the same level, both in adult and in old animals. Mass spectrometry analysis showed that the acidic form of PrxIII was the form with the cysteine of the catalytic site overoxidized to cysteine sulphonic acid. ALCAR treatment of old rats reduced the content of this overoxidized form of PrxIII in liver mitochondria.

2. Materials and methods

2.1. Animals

Male Fisher-344 Charles–River rats were maintained on a 12:12 h light–dark cycle at 25 °C and had access to standard laboratory chow and water ad libitum. All procedures were in accordance with the Italian Ministry of Health Guidelines (no. 86/609/EEC). Experiments were performed on five groups of rats belonged to the following groups: 12-month-old rats (adult group, $n=4$), 28-month-old rats (old group, $n=4$), 28-month-old rats treated with ALCAR for 1 month ($n=4$), 28-month-old rats treated with ALCAR for 2 months ($n=4$), 28-month-old rats treated with ALCAR for 2 months and analyzed after 1 month of treatment suspension ($n=4$). Treated rats were allowed to drink ad libitum a 1.5% (w/v, pH adjusted to 6.5) solution of ALCAR. No difference in food and water assumption was noticed among the five experimental groups. Rats typically drank ~20 ml of ALCAR solution per day, which would provide a daily dose of about 0.5 g/kg of body weight. Old animals were all sacrificed at 28 months of age.

2.2. Mitoplast preparation

Rats were sacrificed, livers were quickly excised and immediately used for mitoplast preparation according to Greenawalt [34]. Briefly, after centrifugation at 660 ×*g* of homogenized liver, mitochondria were collected from the supernatant at 15,000 ×*g* and resuspended in an isotonic buffer containing 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, pH 7.4, and 0.5 mg/ml BSA. After treating the mitochondrial suspension with 6 mg/ml digitonin, mitoplasts were collected from the supernatant at 10,000 ×*g*, resuspended in the above-mentioned buffer, with 15% glycerol and 0.05 mM EDTA and immediately frozen. Protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.3. Two-dimensional polyacrylamide gel electrophoresis (2-DE)

Mitoplast proteins were solubilized in rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) pH 4–7 IPG buffer (GE Healthcare Life Sciences, Piscataway, NJ, USA) and 100 mM DTT. The sample was loaded on 18 cm 4–7 pH gradient IPG strips (GE Healthcare Life Sciences, Piscataway, NJ, USA) by in-gel sample rehydration; 100 μg of proteins were loaded on analytical gels, and 800 μg of proteins were loaded on preparative gels. The isoelectric focusing was performed using the Ettan IPGphor 3 system (GE Healthcare Life Sciences, Piscataway, NJ, USA), for 29 h for a total of 50,000 VhT. The gel strips were then equilibrated twice for 15 min with gentle shaking in equilibration solution containing 0.12 M Tris pH 7.5, 6 M urea, 30% (v/v) glycerol, 2.5% (w/v) SDS; 65 mM DTT was added in the first step and 240 mM iodoacetamide was added in the second step. The second dimension was carried out using 10% polyacrylamide gels. Analytical gels were stained with silver [35] and preparative gels with colloidal Coomassie [36]. Silver-stained gels were acquired from the Image Scanner (GE Healthcare Life Sciences, Piscataway, NJ, USA) and analyzed by Image Master 2D v 6.0 software (GE Healthcare Life Sciences, Piscataway, NJ, USA). Three gels for each animal were analyzed. The spots were detected and matched between different samples. Individual spot volume values were obtained, by the software, as percentage spot volume calculated by considering the total volume over all the spots in the gel. This relative measure takes into account variations due to protein loading and staining.

2.4. Western blotting analysis

Fifty micrograms of mitoplast proteins were separated by 2-DE, as described above, 10 μg of mitoplast proteins were separated on 12% SDS-polyacrylamide slab minigels. Proteins were electroblotted onto PVDF membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). After transfer, the membranes containing blotted proteins were treated with a blocking buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100 and 5% (w/v) non-fat dry milk for 1 h, then were incubated with rabbit anti-Peroxiredoxin III polyclonal antibody (LabFrontiers, Seoul, South Korea), diluted 1:5000 in blocking buffer or with anti-peroxiredoxin-SO_3 polyclonal antibody (LabFrontiers, Seoul, South Korea) diluted 1:10,000. After four washes with blocking buffer, membranes were incubated for 1 h with peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:5000 in blocking buffer, and washed again. Blots were visualized by chemiluminescence using the ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Piscataway, NJ, USA), according to the supplier's instructions, and exposed to ECL films (GE Healthcare Life Sciences, Piscataway, NJ, USA). Autoradiographs were acquired from the Chemi Doc System and analyzed by Quantity One software (Bio-Rad Laboratories Inc, Hercules, CA, USA). After the exposure the membranes were stained with Coomassie Blue and acquired from the Chemi Doc System to estimate for each lane the total protein content electroblotted on the membrane. The densitometric value of O.D. units of each band was then related to the total amount of proteins in the corresponding lane.

2.5. Mass spectrometry analysis

2.5.1. Nano electrospray ionization (ESI) tandem MS

The protein spots were excised from stained gels and subjected to in-gel trypsin digestion according to Shevchenko et al. [37]. The recovered peptide mixtures were then separated by using a nanoflow high performance liquid chromatography system (Ultimate/

Switchos/Famos; LC Packings, Amsterdam, The Netherlands) coupled with a high-capacity ion trap, HCTplus (Bruker-Daltonik, Germany). Protein identification was performed by searching in the National Center for Biotechnology Information nonredundant database (NCBInr) by using the Mascot program (http://www.matrixscience.com). For positive identification, the score of the result had to be over the significance threshold ($p<0.05$).

2.5.2. MALDI-MS

For MALDI-MS, mass measurements were carried out on a Bruker BIFLEX III™ MALDI-time-of-flight mass spectrometer equipped with SCOUT™ High Resolution Optics with X-Y multisample probe and gridless reflector. Samples preparation was carried out according to Wagner et al. [7]. Monoisotopic peptide masses were assigned and used for database searches.

2.6. Statistical analysis

In two-dimensional gel analysis the results are shown as the mean ± SEM of spot volumes. Statistical analysis was carried out using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test using SPSS Base 11.5 software (SPSS Inc., Chicago, IL); $p<0.05$ was considered statistically significant.

For MALDI-MS all statistical analyses were performed using Microsoft excel data analysis program for Student's *t*-test analysis and using statistical analysis program for ANOVA with the Scheffe multiple comparison test; $p<0.05$ was considered statistically significant.

3. Results

3.1. Analysis of different PrxIII forms in adult and old rat liver mitochondria by two-dimensional gel electrophoresis, western blot and mass spectrometry

The presence of different forms of PrxIII in liver mitochondria of adult (12-month-old) and old (28-month-old) rats was analyzed by two-dimensional gel electrophoresis since the native and the different oxidized forms of Prxs have different p*I* [9]. Mitoplasts were prepared to reduce extra mitochondrial protein contamination. Mitoplasts were freshly isolated from the livers of four rats for each group and immediately frozen. Mitoplast proteins (50 μg) were separated on 18 cm IPG strips pH 4–7, in the first dimension, and on 10% polyacrylamide gels in the second dimension, electro-transferred on PVDF membrane and probed against PrxIII antibodies. As representatively reported in Fig. 1 (panels A, B), two spots of PrxIII, an acidic and a basic form with the same molecular weight, were revealed in adult and old rat liver samples. However, the acidic spot was very weak in adult rats, but as intense as the basic spot in old rats. The basic spot should correspond to the native form of PrxIII, whereas the acidic spot to one of the oxidized forms of the enzyme [9].

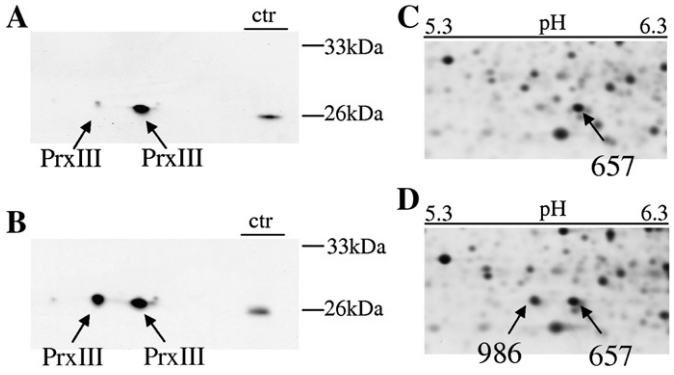

Fig. 1. Two-dimensional analysis of PrxIII spots in adult and old rat liver. Only the regions of the gels containing PrxIII spots are shown. Left panels: representative western blots of adult (A) and old (B) rat liver mitoplast proteins separated on 2-DE gels and probed against PrxIII antibodies. Fifty micrograms of mitoplast proteins were loaded into the first dimension IPG strips. Ten micrograms of mitoplast proteins were loaded, as a control (ctr), onto the second dimension gels; molecular weight markers (kDa) were included on the right. Arrows indicate two PrxIII spots. Right panels: representative silver-stained gels of proteins from adult (C) and old (D) rat liver mitoplasts. One hundred micrograms of mitoplast proteins were loaded into the IPG strips; the gels were stained with silver; arrows indicate the basic (657) and the acidic (986) PrxIII spots.

Table 1
Spot volume values (%) of PrxIII in the liver mitochondria of adult and old rats treated or not with ALCAR.

Rats	Spot 657	Spot 986	Percentage of spot 986 volume with respect to old rats
Adult	2.53 ± 0.25	–	–
Old	2.32 ± 0.27	2.03 ± 0.11	–
Old, 1m ALCAR	2.80 ± 0.28	1.41 ± 0.13*	−30%
Old, 2m ALCAR	2.89 ± 0.24	1.22 ± 0.04*	−40%
Old, 2m ALCAR, 1m treatment suspension	2.67 ± 0.26	1.35 ± 0.12*	−34%

Spot volume values were obtained as percentage spot volume calculated by considering the total volume over all the spots in the gel. Volume values are means ± SEM derived from three 2-DE gels for each of the four animals belonging to the same experimental group.
Old rats were all sacrificed at 28 months of age; m = month.
* $p<0.05$ versus old rat group.

To localize the two spots of PrxIII on the analytical 2-DE gels, we separated 100 μg of mitoplast proteins of adult and old rats. By comparison of the images of western blots and silver-stained gels, we localized, in a region corresponding to MW around 26 kDa, at a p*I* value around 5.9 a basic spot indicated as spot 657 and at a p*I* value around 5.7 an acidic spot indicated as spot 986 (Fig. 1, panels C, D). The spot 657 was present both in adult and old rats, whereas the spot 986 was absent in adult and present in the old rat mitochondrial proteome. The gels of adult and old rats were compared with Image Master 2D Platinum v 6.0 software, three gels for each animal were analyzed. In Table 1 the spot volumes of the two PrxIII forms in adult and old rat are reported. The volume of spot 657 did not change with age.

To confirm, by mass spectrometry, that the spots 657 and 986, on the two-dimensional silver-stained gels, corresponded to PrxIII we separated 800 μg of mitoplast proteins on 2-DE gels stained with Colloidal Coomassie. The excised spots were trypsin-digested and analyzed by ESI–MS/MS mass spectrometry. We identified the basic spot (spot 657) and the acidic spot (spot 986) as PrxIII; the Mascot score was 152 and 357 and the coverage 11% and 28%, respectively.

3.2. Identification of PrxIII cysteine modification

In rat PrxIII the cysteine of the catalytic site is Cys109 included in the tryptic peptide 95–119. In ESI–MS/MS analysis here used for the identification of the acidic and basic spots this peptide was not observed. This could be expected since the negative charge carried by the modified cysteine is known to create problems in ionization and/or fragmentation, at least in the positive mode of ion acquisition [7]. This was indeed the case and the Cys-containing peptides could not be observed in positive mode, neither by MALDI-MS nor by ESI–MS. When in MALDI-MS analysis the spectra were acquired in negative mode, the peptide containing the Cys109, as shown in Fig. 2, could be observed. During sample preparation for the second dimension analysis, iodoacetamide alkylation of cysteines occurs so that all cysteines present in the sample should be in the form of CAM (carboxyamidomethyl) Cys except the oxidized Cys. In MALDI spectra of the basic spot (Fig. 2A), signals at m/z 3031.22 were observed and assigned to peptide 95–119 containing the CAM Cys109 (2974.50 +

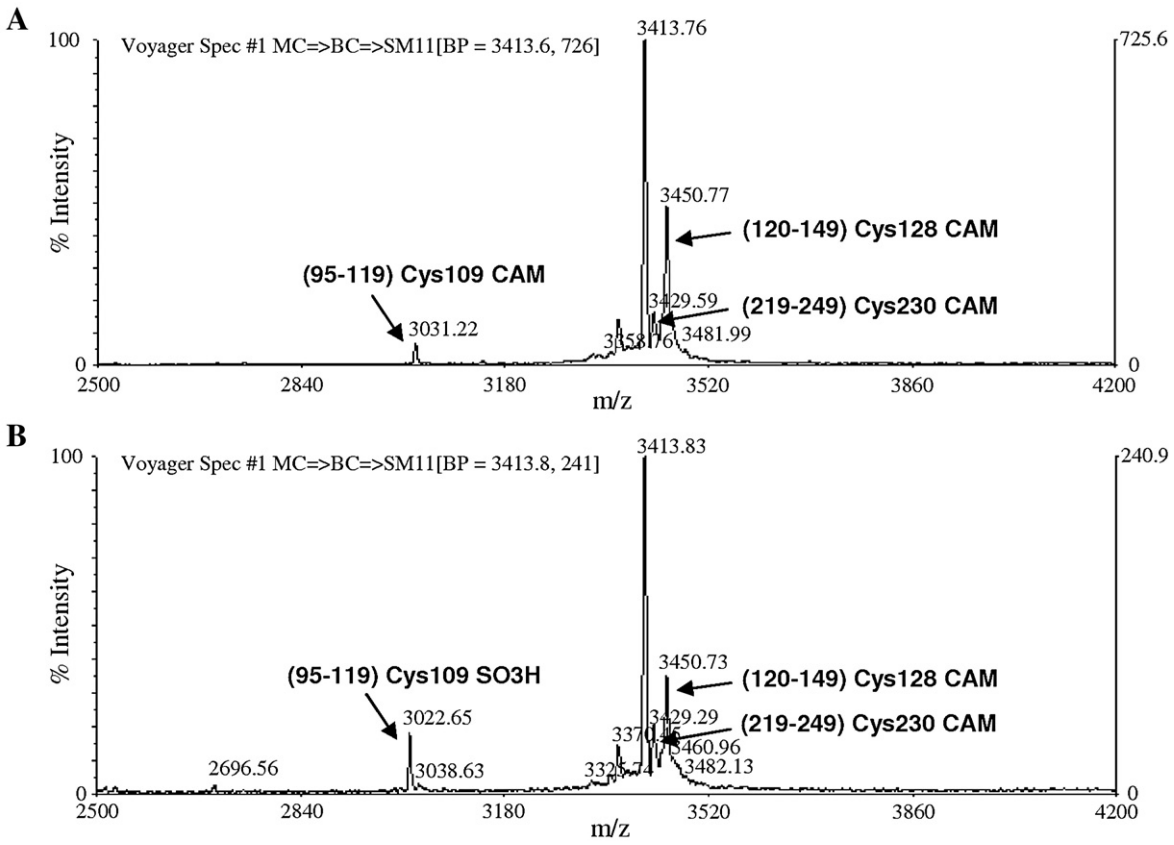

Fig. 2. Identification of cysteine-containing peptides of PrxIII by MALDI-MS. The spectra were acquired in negative mode. Peaks corresponding to Cys-containing peptides are indicated with arrows. (A) Spectrum of the basic spot 657. (B) Spectrum of the acidic spot 986.

57 = 3031.50), whereas in MALDI spectra of the acidic spot (Fig. 2B), signals at m/z 3022.65 were assigned to the same peptide 95–119, containing the Cys109 with the thiol group oxidized to sulphonic acid (2974.50 + 48 = 3022.50). The mature form of PrxIII contains other two cysteines in the peptide 120–149 and in the peptide 219–249, respectively. As expected, the two peptides showed the same m/z in the acidic and in the basic spots since these CAM Cys were not modified in old rats (Figs. 2A, B). MALDI-MS analysis of the basic and acidic spots, identified as PrxIII, allows to conclude that the p*I* shift of PrxIII spot in old rat liver mitochondria is due to the overoxidation of the cysteine of the catalytic site to cysteine sulphonic acid.

3.3. Analysis of PrxIII forms in liver mitochondria of ALCAR-treated rats

To estimate whether ALCAR treatment affected the content of the overoxidized form of PrxIII in old rats, mitoplast proteins from liver of 28-month-old rats treated with ALCAR for 1 month, for 2 months and for 2 months followed by 1 month of treatment suspension were analyzed by two-dimensional gel electrophoresis experiments. Proteins (100 μg) were separated on 18 cm IPG strips pH 4–7, in the first dimension, and on 10% polyacrylamide silver-stained gels in the second dimension. In Fig. 3 representative two-dimensional gels of mitoplast proteins from old control rats and ALCAR-treated old rats are reported. The results of treated rats were compared with those of the control group by Image Master 2D Platinum v 6.0 software, three gels for each animal were analyzed. In Table 1 the spot volume values of the two forms of PrxIII in 28-month-old rats treated with ALCAR for 1 month, for 2 months and for 2 months followed by 1 month of treatment suspension and untreated rats are reported. The spot volume of the native form of PrxIII (spot 657) did not change in a significant way in the three groups of treated animals with respect to the untreated old ones, whereas the spot volume of the overoxidized form of PrxIII (spot 986) decreased significantly with the treatment as reported in Table 1. No significant differences were found between the three ALCAR-treated groups of old rats.

Western blotting experiments on mitoplast proteins probed with peroxiredoxin-SO_3 antibodies were then performed to confirm the quantitative data obtained by two-dimensional and mass spectrometry analysis. Ten micrograms of mitoplast proteins from each animal of the five experimental groups were separated on 12% polyacrylamide gels, electro-transferred on PVDF membrane and probed with Prx-SO_3 antibodies. In Fig. 4A a representative western blotting of mitoplast proteins from adult rats (lane 1), old control rats (lane 2) and ALCAR-treated old rats (lanes 3–5) is reported. The densitometric value of O.D. units of each band was then related to the total amount of proteins transferred on the membrane in the corresponding lane. In the histogram of Fig. 4B bars show the average values obtained for each group of animals referred to the mean value of the old rat group. As it is shown, the overoxidized form of PrxIII in adult rats represents about 10% with respect to the overoxidized PrxIII in the old group. As far as the ALCAR-treated groups of old rats, the overoxidized PrxIII is significantly decreased of about 35% in all groups and no significant differences were found among them.

4. Discussion

The data here reported reveal, for the first time, the presence and the accumulation in the liver of four groups of 28-month-old rats of an acidic form of PrxIII in which the cysteine of the catalytic site has been oxidized to sulphonic acid. This acidic form, present in adult rat liver in a very low amount, corresponds to the irreversibly inactivated antioxidant enzyme [7]. The overoxidation of Cys109 of PrxIII is selective, since Cys128 and Cys230 remain in the reduced form. ALCAR

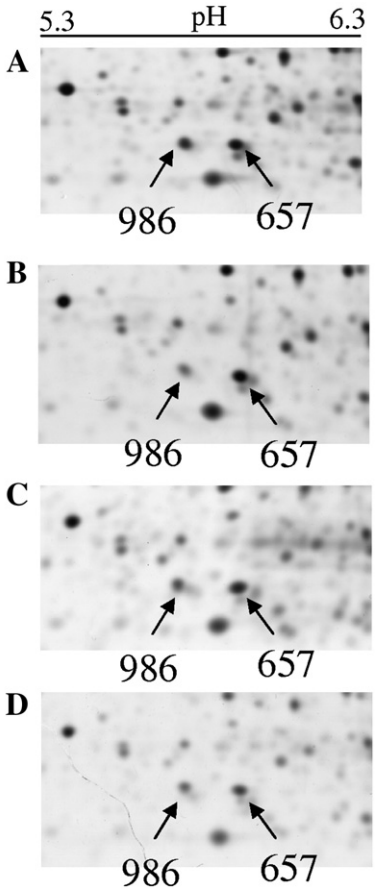

Fig. 3. Two-dimensional gel electrophoresis of PrxIII spots in 28-month-old rats treated with ALCAR. Only the regions of the gels containing PrxIII spots are shown. Representative silver-stained two-dimensional gels of liver mitochondrial proteins from: old rats (A), rats treated with ALCAR for 1 month (B), rats treated with ALCAR for 2 months (C), rats treated with ALCAR for 2 months and analyzed 1 month after treatment suspension (D). All rats were sacrificed at 28 months of age. In the panels, arrows indicate the 657 basic spots and the 986 acidic spots.

feeding to old rats reduces the content of this overoxidized form of PrxIII.

The presence of the sulphonic form of PrxIII in liver mitochondria of old rats is probably due to the age-related oxidative stress conditions already reported in this tissue [12,13,38]. Contrary to the sulphonic form (Cys-SO_3H), the sulphinic PrxIII form (Cys-SO_2H) was not found probably because it can be reduced to cysteine by cytosolic sulfiredoxin (Srx) which translocates into mitochondria [39]. Furthermore, since the reduction of the sulphinic form by Srx is a very slow process [40], the sulphinic PrxIII form might spontaneously overoxidize to the sulphonic acid form due to the oxidative environment. However, it has been reported that in yeast the sulphonic form of the peroxiredoxin Tsa1p (Tsa1p-SO_3H) is not a spontaneous oxidation product of the sulphinic form, suggesting the existence of an enzymatic system that facilitates the formation of the sulphonic form [41].

The accumulation of the acidic inactive form of PrxIII in old rat liver might be due to the decrease of protease activities involved in the degradation of oxidized proteins. Aggregates of modified proteins are typically observed in aging tissues and the age-dependent dysfunction of the protein quality control system has been reported [42–44]. Among the mitochondrial proteases [45] the ATP-stimulated Lon protease plays an important role in the removal of oxidized proteins in the mitochondrial matrix [44]. A decrease of the activity of Lon protease has been reported in liver of old rats [46]. A reduction of expression and activity of this enzyme was associated with an increased level of oxidized proteins in mice skeletal muscle [47].

The post-translational changes of the redox state of a key thiol protein such as PrxIII might be critical for the mitochondrial as well as for the cellular functions since the global antioxidant defense system of the cell and, in particular, of mitochondria should be affected [3]. However, as revealed by this study, the native PrxIII enzyme is still present in old rat and in the same amount as in the adult counterpart. This could mean that PrxIII is constitutively expressed and that its expression is not influenced by the accumulation of the acidic form of PrxIII. Otherwise, we have to hypothesize that aged rat liver is able to feel the gap and keep constant its antioxidant defense level in a situation of chronic ROS production as that of rat liver during aging [12,13,38]. This last hypothesis seems to be supported by an increased amount of transcript for the PrxIII gene found in bovine aortic endothelial cells exposed to oxidative stress as well as by an increased protein content found in the rat ischemic myocardium [48].

However, the irreversibly overoxidized inactive PrxIII might not be an impaired dead-end product destined to be removed as usually believed. In fact, in mammalian cells, oxidation of mitochondrial PrxIII, followed only later on by oxidation of PrxI and PrxII, has been reported during the initiation of the receptor-mediated apoptosis [5,49]. Furthermore, in the yeast, the irreversibly hyperoxidized peroxiredoxin (Tsa1p-SO_3H), in oxidative stress conditions, functions as an effective and stable molecular chaperone [41].

The ability of the ALCAR treatment to maintain mitochondrial homeostasis and/or energy levels in different aged tissues and in various age-related degenerative conditions [15–26,29] might be responsible for the partial prevention of overoxidation and/or the accumulation of oxidized protein here reported. ALCAR, in fact, might buffer the oxidative stress and prevent the depletion of ATP [17,29]. ALCAR might pursue such goals with the same mechanism suggested by Guarente for caloric restricted diet, that is by inducing mitochondrial biogenesis [50]. In fact, ALCAR feeding during unloading, a condition that accelerates the negative effect of age on skeletal muscle [51], triggers in rat soleus muscle the coordinated expression of genes involved in mitochondrial biogenesis like PCG-1alpha, NRF1 and TFAM

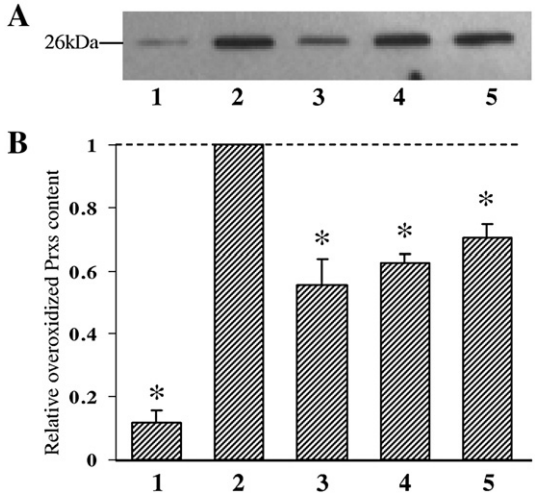

Fig. 4. Quantitative estimate of the overoxidized PrxIII content by western blotting using Prx-SO_3 antibodies. (A) Representative western blotting carried out on liver mitochondrial proteins. Samples were from: adult rat (lane 1); old rat (lane 2); rat treated with ALCAR for 1 month (lane 3); rat treated with ALCAR for 2 months (lane 4); rat treated with ALCAR for 2 months and analyzed 1 month after treatment suspension (lane 5). The densitometric value of O.D. units of each band was related to the total amount of proteins transferred on the membrane. (B) The histogram shows the amount of the overoxidized PrxIII related to the old rat content. Bars, corresponding to the gel lanes, represent the average ± standard error of values obtained from 3 experiments for each animal belonging to the same experimental group ($n=4$). *$p<0.05$ versus old rat group.

[23] and increases the mitochondrial mass [23,24]. Two months of ALCAR treatment prevented in the soleus muscle of 28-month-old rats the age-related decrease of TFAM [22] as well as of PGC-1alpha protein content (Pesce, V., personal communication). PGC-1alpha, a master regulator of mitochondrial biogenesis [52], controls also the expression of the enzymes of the mitochondrial antioxidant defense systems like PrxIII [53] and mitochondrial Lon protease [54]. Therefore ALCAR, by stimulating the expression of PGC-1alpha might favor mitochondrial biogenesis and, in the same time, reduce the overoxidation of PrxIII and/or induce the degradation of overoxidized PrxIII.

In conclusion, this is the first report demonstrating, in a tissue of old rat, the presence as well as the accumulation of the PrxIII containing the Cys sulphonic acid in the catalytic site. Furthermore, for the first time, ALCAR involvement in a partial prevention of overoxidation and/or accumulation of the overoxidized form of a specific mitochondrial enzyme is reported, although with a yet unknown mechanism.

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