FEBS Letters 584 (2010) 2837–2844

FEBS Letters
journal homepage: www.FEBSLetters.org

Swapping of the N-terminus of VDAC1 with VDAC3 restores full activity of the channel and confers anti-aging features to the cell

Simona Reina [a], Vanessa Palermo [b], Andrea Guarnera [a], Francesca Guarino [a], Angela Messina [a], Cristina Mazzoni [b,*], Vito De Pinto [a,**]

[a] Department of Chemical Sciences, University of Catania, and Istituto Nazionale di Biomembrane e Biosistemi, Sezione di Catania, Viale A. Doria 6, 95125 Catania, Italy
[b] Department of Cell and Developmental Biology, Pasteur Institute–Cenci Bolognetti Foundation, University of Rome 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome, Italy

ARTICLE INFO

Article history:
Received 11 March 2010
Revised 22 April 2010
Accepted 23 April 2010
Available online 29 April 2010

Edited by Peter Brzezinski

Keywords:
Voltage-dependent anion-selective channel
Reactive oxygen species
Mitochondria
N-terminal chimaera
Saccharomyces cerevisiae

ABSTRACT

Voltage-dependent anion-selective channels (VDACs) are pore-forming proteins allowing the permeability of the mitochondrial outer membrane. The VDAC3 isoform is the least abundant and least active in a complementation assay performed in a yeast strain devoid of porin-1. We swapped the VDAC3 N-terminal 20 amino acids with homologous sequences from the other isoforms. The substitution of the VDAC3 N-terminus with the VDAC1 N-terminus caused the chimaera to become more active than VDAC1. The VDAC2 N-terminus improved VDAC3 activity, though to a lesser extent. The VDAC3 carrying the VDAC1 N-terminus was able to complement the lack of the yeast porin in mitochondrial respiration and in modulation of reactive oxygen species (ROS). This chimaera increased life span, indicating a more efficient bioenergetic metabolism and/or a better protection from ROS.

1. Introduction

The mitochondrial porin voltage-dependent anion-selective channel (VDAC) forms the proteinaceous aqueous passage between the cytosol and the inner of mitochondria [1,2]. Its privileged position and special function makes it a preferred site for the regulation of the whole mitochondrial metabolism. An increasing body of evidence indicates that VDAC plays a major role in the metabolite flow in and out of mitochondria, resulting in the regulation of mitochondrial functions [3]. This means that VDAC, as a consequence of closing and opening, may act as a "governor" of mitochondrial bioenergetics [4]. VDACs have also been described in association with apoptotic processes [5] and as interacting with cytoskeletal proteins [6,7].

In higher eukaryotes three different VDAC genes encode three proteins whose sequences are homologous [8]. VDAC isoforms, despite the substantial sequence conservation, do not show the same activity [9,10]. Functional experiments demonstrated that recombinant VDAC1 and VDAC2 isoforms are able to form pores in lipid bilayers [11], different from VDAC3 that shows a reduced pore-forming ability [12]. Human and mouse recombinant VDAC1 and VDAC2 isoforms can complement the yeast VDAC1 deficiency, while VDAC3 was unable to completely rescue the VDAC-deficient yeast phenotype [12,13]. VDAC K.O. mice were generated. VDAC1 deficient mice are viable and show that mitochondrial functions are slightly affected; VDAC2 gene deletion is lethal [14]; the spermatozoa are rich in VDAC3 [15]: mice lacking VDAC3 are healthy, but males are infertile [16].

The structure of VDAC1 has been recently solved by NMR and crystallization experiments [17–19]. It shows a rather compact transmembrane channel, formed by 19 amphipathic beta strands connected by short turns and loops, with the striking addition of the N-terminal moiety structured with α-helix segments. The N-terminal sequence is located inside the channel, forming a partial obstruction of the wide pore, possibly exerting a structural reinforcement to the channel walls. In agreement with this finding, CD and NMR spectroscopy of the isolated N-terminal peptide showed that there is the need of a special environment to force the peptide in an ordered α-helix secondary structure [20]. In water, for example, it is usually disordered. The conditions for the peptide to assume in vitro the structured α-helix were the presence of negative charges together with a hydrophobic

Abbreviations: CLS, chronological life span; VDAC, voltage-dependent anion-selective channel; N1-VDAC3, human VDAC3 swapped with the N-terminal of human VDAC1; N2(1–31)-VDAC3 and N2(12–31)-VDAC3, human VDAC3 swapped with the N-terminal sequence 1–31 or 12–31 of human VDAC2; ROS, reactive oxygen species

* Co-corresponding author.
** Corresponding author. Fax: +39 095580138.
E-mail addresses: cristina.mazzoni@uniroma1.it (C. Mazzoni), vdpbiofa@unict.it (V. De Pinto).
URL: http://www.unictbiolmol-lab.it (V. De Pinto).

0014-5793/$36.00
doi:10.1016/j.febslet.2010.04.066

environment [20]. These data thus suggest that the structural constraints observed in the crystal might be a necessary prerequisite for the peptide to assume the α-helix conformation.

The VDAC1 N-terminal sequence has always been the subject of considerable interest. In previous papers it was reported that during voltage-gating or interaction specifically with other molecules like cytochrome *c*, some motion of the protein occurred [21]. Its deletion did not affect the correct targeting of the protein to mitochondria [20] but had serious effects on the pore-forming activity and the voltage-dependence of the protein [22,23]. More recently, the N-terminal domain of VDAC1 was indicated to be a main effector in the apoptotic events depending on VDAC1. Its deletion decreases the pro-apoptotic effect of VDAC1 over-expression [24]. It is considered one of the docking sites where HK1 binds VDAC1 [25]. It has been proposed that the mechanism responsible of the gating of the pore was due to conformational changes or movements of the N-terminal sequence [26].

In this work we studied the effect of the substitution of the N-terminal sequence of the human VDAC3 by the homologous sequences of VDAC1 and VDAC2. The activity of these chimaeras was monitored in a *Saccharomyces cerevisiae* strain in which the endogenous gene por1 was deleted. Our experiments clearly show that this substitution is sufficient to complement the lack of the endogenous porin and to give to the strain a clear advantage in several living features of the cell. This result underlines the decisive role of the VDAC1 N-terminus in the protein activity.

2. Materials and methods

2.1. Yeast strains and growth conditions

Wild type *S. cerevisiae* strain BY4742 [MAT α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) and porin-depleted mutant (Δpor1) derived from the above parent (MATα, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*, *por1*::kanMX4) were obtained from EUROSCARF (Frankfurt, Germany). Yeast strains were grown on rich medium YP (1% yeast extract, 2% peptone) supplemented with 2% glucose (YPD) or 3% glycerol (YPY) and minimal medium (0.67% yeast nitrogen base) containing glucose (SD) or glycerol (SY) supplemented with 10 μg/ml of the appropriate nutritional requirements according to the genotype of the strains. Agar (2%) was added for solid plates. Δpor1 strain was used for heterologous expression of native or mutant VDACs; BY4742 was used for reference.

2.2. PCR amplifications and construction of the chimaeras

Each isoform was designed by PCR amplification. The details of the amplification conditions (including the primers' sequences) and the construction of chimaeras is described in the Supplementary data.

2.3. Expression of HVDACs in yeast

Native- or mutant-VDACs coding sequences were restriction digested with CpoI and cloned into the 2μ multi-copy shuttle vector pYX212 (Novagen) carrying the URA3 gene under the control of the constitutive promoter TPI1. Each construct was introduced into Δpor1 strain by the lithium-acetate transformation. Transformants were selected on agar plates containing 2% glucose in yeast nitrogen base supplemented with essential nutrients except uracil. The transformation of the human VDACs was verified by Western blot of yeast extracts assayed with antibodies anti-VDAC 3 isoform (provided by K.D. Hinsch, Giessen, Germany) or anti-VDAC1 N-terminus (provided by F. Thinnes, Göttingen, Germany) (Supplementary data and Supplementary Fig. S1).

2.4. Chronogical aging in yeast

Cell suspensions (5 μl) containing approximately 6×10^6 cells/ml were poured onto a thin layer of YPD agar on a microscope slide. A cover slip was placed over the samples and after 24 h viable and non-viable cells were scored on the basis of their ability to form microcolonies (% CFU).

2.5. H_2O_2 resistance

Hydrogen peroxide stress was performed by incubating yeast cultures in the presence of 0.8, 1.2 and 3 mM H_2O_2 for 4 h. Following hydrogen peroxide stress, cell survival and proliferation were assessed by spotting in triplicate 0.15 OD_{600nm} of cultures onto YPD agar microscope slides and incubated at 28 °C for 48 h. Survival was determined by their ability to form microcolonies (% CFU).

2.6. Fluorescence microscopy

Dihydro-rhodamine 123 (Sigma) was added at a concentration of 5 μg/ml of cell culture from a 2.5 mg/ml stock solution in ethanol and cells were viewed without further processing through a rhodamine optical filter after 3 h incubation [27].

Active mitochondrial membranes in vivo were detected by mitochondrial staining with dimethyl-aminostyryl-methylpyridinium iodine (DASPMI, Sigma). Cells were resuspended in 10 μl of DASPMI 1 μg/μl.

An Axioskop2 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (micro-CCD) was used for image acquisition.

3. Results

3.1. Swapping of the N-terminal domain of VDAC3 with the same domain from the other isoforms confers an increased life span to the host cell

To study the influence of the N-terminal moieties upon the barrel activity, we designed a set of VDAC3 mutants whose first 20 amino acids were swapped with the VDAC1 corresponding sequence and with the amino acids 1–31 or 12–31 of VDAC2. VDAC2 has indeed an 11 amino acids extension at the N-end with a second methionine in frame. This corresponds to the typical starting position of the other isoforms. We were interested to determine whether the two N-terminal extensions of VDAC2 are both active. Fig. 1A shows the alignment of the three human VDAC isoforms N-terminal sequences and the yeast porin-1, showing the conserved residues. A scheme of the swapped proteins used in this work is shown in Fig. 1B.

The microtiter assay exploits the growth deficiency of a yeast devoid of the endogenous porin-1 (Δpor1) upon a non-fermentable carbon source at 37 °C. The yeast growth could be recovered after transformation with vectors expressing VDAC1 and VDAC2 [13]. VDAC3 typically showed a growth defective phenotype [12]. In this work we compared the yeast growth phenotype after transformation of the defective yeast with the chimaeras described above.

Microtiter assay indicates that the growth efficiency of Δpor1 yeast cells containing the VDAC3 chimaeras at 37 °C becomes comparable, but much lower, than with the same strain containing whole, native VDAC1 or 2 (data not shown and [28]).

To get quantitative information about the changes in the yeast phenotype, we determined the chronological life span (CLS) either upon fermentable or non-fermentable carbon sources. The growth of cells on non-fermentable carbon sources, such as glycerol and

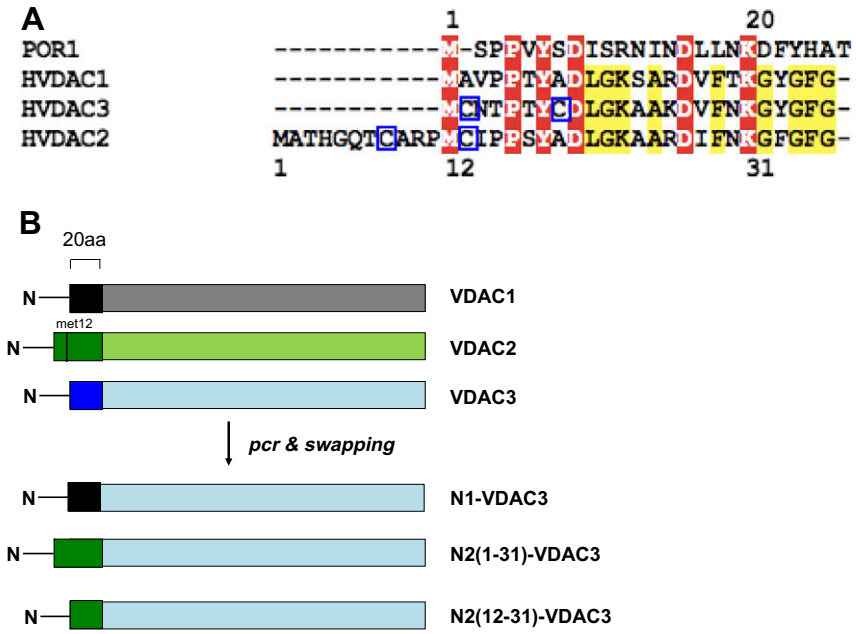

Fig. 1. (A) Alignment of the N-terminal sequences of *S. cerevisiae* porin-1 with human isoforms VDAC1, VDAC2 and VDAC3. In red are the residues identical among the four sequences. In yellow are outlined the residues identical among the three human isoforms. Blue boxes indicate the location of cysteines in VDAC2 and VDAC3. (B) Scheme of the swapped proteins used in this work.

ethanol, provides a direct way to study the effect of respiratory condition on CLS. Caloric restriction in yeast cells provokes a metabolic shift from fermentation to aerobic respiration and, in turn, this causes a longer life span [29]. In Fig. 2, we show yeast ageing curves in both media at 28 °C. We have already shown that the Δpor1 yeast transformed with VDAC1 and VDAC2 live longer than the w.t. [28]. Even upon transformation with VDAC3 the cell growth is similar to the yeast wild type, at least on glucose. The same curves were determined in the presence of the VDAC3 chimaeras (Fig. 2A and B). The VDAC3 mutant containing the VDAC1 N-terminal is the most active in complementing the absence of the wild type porin-1 protein. The addition of the VDAC1 N-terminal to the VDAC3 barrel structure extends the cellular life span of 50%. The VDAC3 modified by the amino acids 1–31 of the VDAC2 N-terminus lived longer than the homologous mutant containing the amino acids 12–31 of the VDAC2 N-terminus in glycerol but not in glucose, suggesting a specific influence of the fermentative metabolism upon these chimaeras.

These results support the notion that the N-terminal moiety of VDAC is a major determinant in the activity modulation of the channel.

3.2. Swapping of the N-terminal domain of VDAC3 with the same domain from the other isoforms confers resistance to the yeast against incubation with reactive oxygen species (ROS) producing chemicals

It has been reported that the inactivation of porin in yeast induces susceptibility to apoptosis and high sensitivity to ROS-producing agents [30]. In yeast also [31], new lines of evidence connect VDAC with the ROS metabolism. To investigate this functional feature, we exposed the Δpor1 yeast cells transformed with the VDAC3 chimaeras to pro-apoptotic agents. Their survival was quantified. In Fig. 3A, the yeast strains were exposed to growing concentrations of H_2O_2 for 4 h. The yeast cell deleted of the endogenous porin-1 was found to be highly sensitive to the exposure to this ROS generating chemical. In this strain, in general, the complementation with various VDAC isoforms and chimaeras produces an increase of the viability depending on the particular isoform or chimaeras used for the complementation assay. VDAC3 is able to support cell viability but a lesser extent than the other isoforms and the chimaeras. The swapping mutant human VDAC3 swapped with the N-terminal of human VDAC1 (N1-VDAC3) showed the best results and conferred to the cells a resistance comparable to that of the wild type (Fig. 3A). Similar results were obtained upon exposure of yeast strains to 50 mM acetic acid, another known apoptosis inducing agent in yeast [32]: VDAC3 transformed cells showed the same poor viability comparable to that of Δpor1 yeast. The cells expressing the chimaeric proteins exposed to acetic acid showed a cell viability similar to that of the wild type or to the VDAC1 expressing cells (Fig. 3B).

The action of ROS species on the various VDAC chimaeras was quantified by DHR123 fluorescence. Wild type yeast cultures assayed in the exponential growth phase showed very low levels of DHR123 fluorescence. In Δpor1 the ROS level went out of control, a condition very dangerous for the cell (Fig. 4A). The complementation of Δpor1 with VDAC1 and VDAC2 re-established a low level of ROS in the cell [28], while VDAC3 could not (Fig. 4). The substitution in VDAC3 of the natural N-terminal sequence with either the VDAC1 or VDAC2 N-terminal sequences (in particular the N2(1–31) sequence), either in exponential (Fig. 4A) or in the early stationary phase (Fig. 4B), conferred to the yeast features very similar to those of the wild type strain. There is no special difference in the ROS amount experimentally determined in these assays in comparison with the whole VDAC1 or 2 or the wild type, different from the striking results found with the native VDAC3 [28].

The same strains were subjected to the quantification of the cells with mitochondria carrying the canonical membrane potential by DASPMI staining. Yeast cells in exponential phase showed a high level of active mitochondria, with the exception of the Δpor1 mutant strain (Fig. 5A). Interestingly, the VDAC3 strain also showed high levels of mitochondrial potential. Actually, similar to mammalian cells, yeast apoptosis is sometimes accompanied by a sharp transient increase of mitochondrial membrane potential that can directly cause accumulation of ROS [33]. A decrease of mitochondrial potential is considered a general marker for in vivo ageing both in mammals and yeast cells [34]. In fact, DASPMI staining

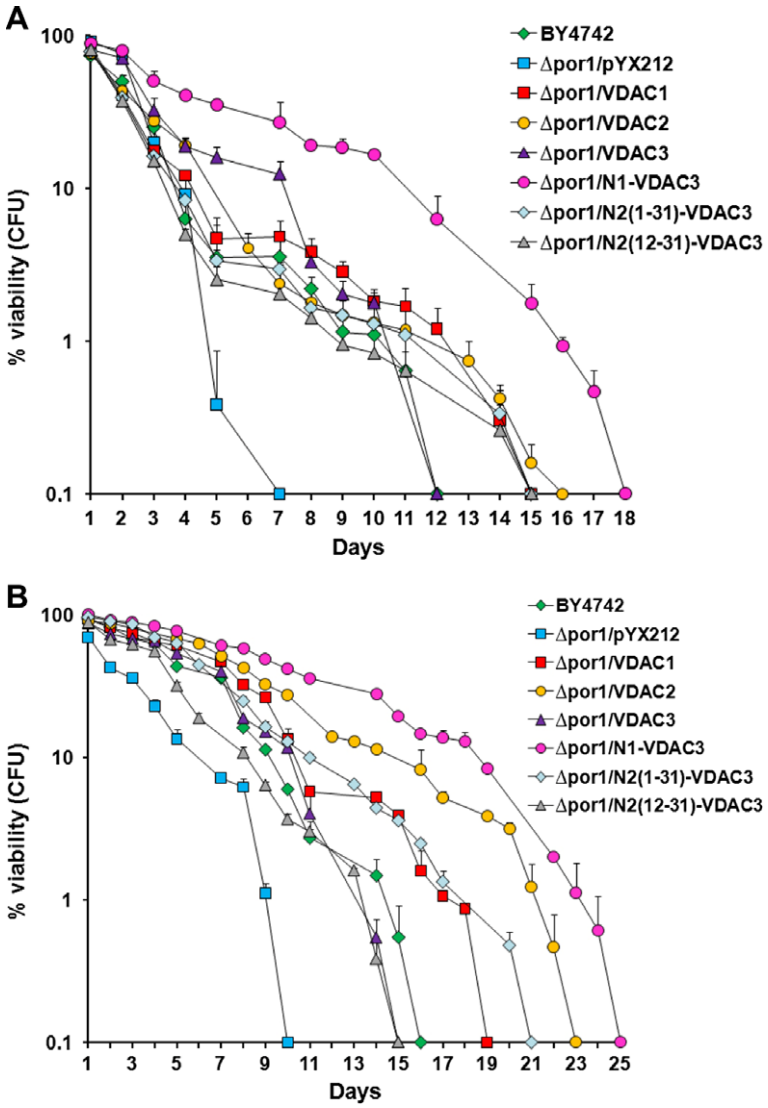

Fig. 2. The substitution of the VDAC3 N-end sequence with the homologous sequences from VDAC1 and 2 restores the growth phenotype of Δpor1 yeast. (A) Chronological ageing of yeast strains in glucose. Wild type (BY4742) and Δpor1 strains transformed with the empty plasmid pYX212 or with the same plasmid carrying coding sequences for the following proteins: human VDAC1, VDAC2 and VDAC3, N1-VDAC3, N2(1–31)-VDAC3, N2(12–31)-VDAC3 were grown in glucose medium at 28 °C and their viability, expressed as the percentage of the micro-colony forming units (CFU), was determined. The average and standard deviation of three independent experiments is shown. (B) Chronological ageing of yeast strains in glycerol at 28 °C of the same strains as in (A).

performed upon human VDAC-expressing strains in cells aged 3 days showed a dramatic decrease of mitochondrial potential, with an especially negative decrease for VDAC3 carrying cells (Fig. 5B). Once again, the simple substitution of the N-terminal changed the properties of VDAC3, transforming it in a replica protein of the other VDACs (Fig. 5).

4. Discussion

4.1. Role of the VDAC N-terminus

The N-terminal end of VDAC contains amphipathic α-helix elements [19]. This structure represents an addition to the β-barrel pore structure and was proposed to have functionally relevant properties [20–26,35]. It has been recently proposed that the final N-terminal sequence (i.e. amino acids 1–20 in VDAC1) might swing from side to side of the wall realizing a structural gate. Such swinging would be dependent on the short sequence 21–25 that is a gly-containing motif [26].

We produced a set of VDAC3 mutants where the 1–20 N-terminal of the protein was swapped with the homologous sequences derived from VDAC1 and VDAC2. The results obtained are very clear: the change of the N-terminus of VDAC3 with the same from VDAC1 transforms the apparently inactive isoform into a very active protein. This property confirms that this moiety of the protein is really decisive for the functional features of the channel.

We also designed two VDAC3 mutants whose first 20 amino acids were swapped with the amino acids 1–31 or 12–31 of VDAC2. We wanted to know whether the two N-terminal extensions of VDAC2 are both active. Our experiments showed that a difference exists between these chimaeras. The VDAC3 modified by the longer VDAC2 N-terminus usually performs better than the shorter, either in terms of activity recover, life span upon glycerol and after ROS treatment, indicating that the shorter N-terminus confers functional properties half way between VDAC1 and VDAC3. This observation could be interpreted on the basis of protein structural predictions (see below) and of the cysteine content of these sequences.

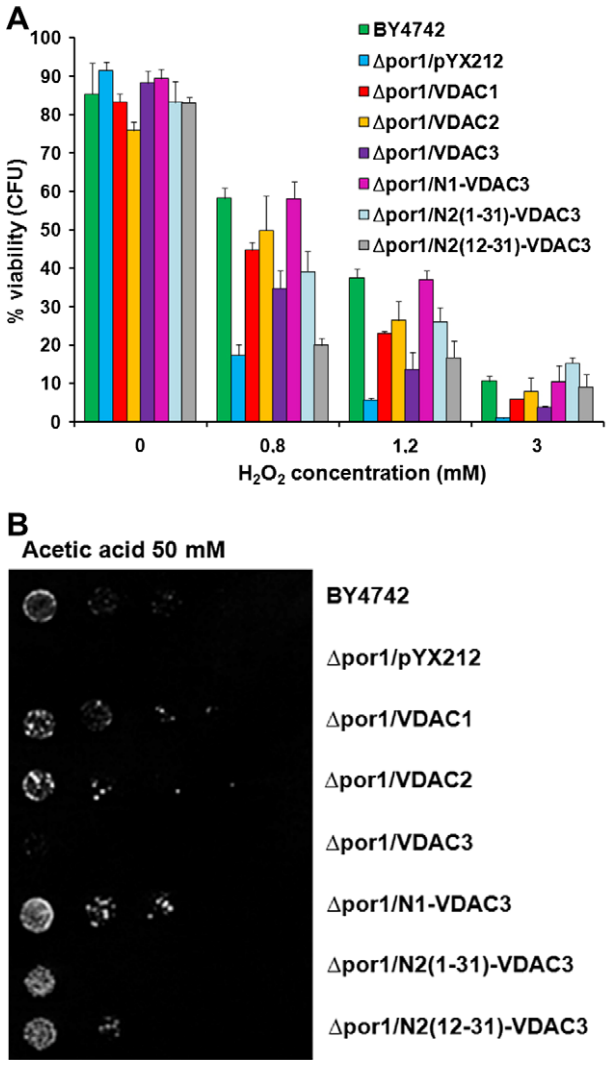

Fig. 3. Swapping of the VDAC3 N-terminal with the homologous sequences from VDAC1 and 2 confers resistance to the yeast against incubation with ROS producing drugs. (A) Wild type (BY4742) and Δpor1 strains were transformed with the empty plasmid pYX212 or with the same plasmid carrying coding sequences for the following proteins: VDAC3, N1-VDAC3, N2(1–31)-VDAC3, N2(12–31)-VDAC3. These transformed yeast were incubated with the indicated concentrations of H_2O_2. After 4 h the percentage of viability was determined as described in Section 2. (B) Serial dilutions of the yeast cells described were spotted on YPD medium containing 50 mM acetic acid. Plates were recorded after 5 days of incubation at 28 °C.

The VDAC 1–20 N-terminal sequence with its typical α-helix content is one of the most conserved structure in the protein and the only part where an α-helix is present (76 upon 80 animal VDACs show the same motif [8]). Its primary sequence shows moderate difference between the isoforms. There are seven out of 20 different amino acids in the N-end terminal between VDAC3 and VDAC1. In the N-end exchange VDAC3 loses alanine 13 against a serine. In VDAC1 serine 13 has been found to be a phosphorylation site in proteomic analysis [36], thus its presence may condition the function of this segment. In addition VDAC1 and 2 N-termini have, respectively, 1 or 2 additional proline and 1 or 2 additional arginine residues: these residues are the target of carbonylation reactions, while VDAC3 does not have them. This could be one explanation for the lack of any effect in VDAC3 against ROS. VDAC1 and the yeast porin-1 do not show any cysteine in the N-terminal sequence. VDAC3 has two cysteines in the 20 aa sequence under study in position 2 and 8. VDAC2 has also two Cys, but only one of them corresponding to the VDAC3 Cys2, while the other is in the disorganized extension. Leucine 10, considered a crucial pivot for the hydrophobic interaction with the barrel walls [26], is conserved in the mammalian isoforms, and it is conservatively replaced by a Ile in the yeast Por1. The counterpart of the N-terminus, i.e. the barrel structure facing the N-terminus, is indeed important because it is considered to interact with the N-terminus [19,26]. The residues considered critical for this interaction, i.e. Val 153 and Leu 150, are conserved in the mammalian isoforms and also in the yeast sequence.

3D models of VDAC2 and VDAC3 have been proposed [28]. These models are useful because they allow us to speculate about the amino acid localization in the structure. In particular the cysteine disposition in these models is interesting, since one of the most relevant difference among the human isoforms are cysteines (for example human VDAC1 has 2 Cys, VDAC2 9 Cys and VDAC3 6 Cys). From the reconstruction shown in Supplementary Fig. S2 it is clearly visible that cysteines in VDAC2 and VDAC3 are apparently clustered on the same side of the membrane, standing either in the N-terminal sequence and in the tight junctions connecting β-strands. This is a main difference with VDAC1. This isoform contains only two cysteines and they are located far from one another, protruding into the water-exposed interior (Cys232) or into the phospholipid hydrophobic moiety (Cys127) [37,38]. Cysteines are residues usually involved in the disulfide bridge formation or in redox modifications. Thus it is tempting to speculate that the different functionalities of the VDAC isoforms might depend upon this sequence composition and structural asymmetry. VDAC2 also has two cysteines in the N-terminus but they are placed differently than in VDAC3 N-terminus. In addition VDAC2 has a longer, unstructured 11 amino acid long extension containing one N-terminal cysteine. The presence of this moiety could hinder the protein to reach the same conformation as VDAC3. The experimental behavior of N2(12–31)-VDAC3 and N2(1–31)-VDAC3 might be explained by this difference.

The scenario appearing from these considerations is that the few amino acid exchanges in the 1–20 N-terminus VDAC sequences could represent a sufficient reason for the presence of alternative isoforms with different functional activities.

4.2. VDACs and ROS

One of the discriminating features among the VDAC isoforms is their behavior or susceptibility towards ROS [28]. This point has already been dealt with but the multiplicity of VDAC isoforms has not yet been taken into consideration. It is generally accepted that VDAC is a way mitochondria escape of an aliquot of the $O_2^{-\cdot}$ produced by the complex III [39]. Han et al. demonstrated that the closure of VDAC with the specific inhibitor dextran sulfate decreases the amount of ROS available in the cytosol [40]. They suggested that the opening and closing of VDAC modulates the flux of mitochondrially generated $O_2^{-\cdot}$ into cytosol. Unfortunately these authors did not consider that in the outer mitochondrial membrane there are three different VDACs. Since dextran sulfate acts as a kind of clog upon the pore and since the VDAC isoforms are structurally very similar, it is very probable that the overall effect measured for example in [41] was not specific of VDAC1 but possibly the sum of the effect of the isoforms. In the literature there are systemic surveys of ROS-dependent chemical modifications of the yeast proteome and it was shown that VDAC was usually involved. In yeast mutants lacking MnSOD (SOD2) or CuZnSOD (SOD1) superoxide dismutase, it was found that few, specific mitochondrial proteins are modified (carbonylated) to a greater extent than in the wild type after exposure to paraquat, an oxidant-generating molecule [42]. CuZnSOD has been found in the cytosol and in the mitochondrial intermembrane space. A relatively small number of proteins are affected by the absence of either MnSOD or CuZnSOD, as demonstrated by their carbonylation upon generation

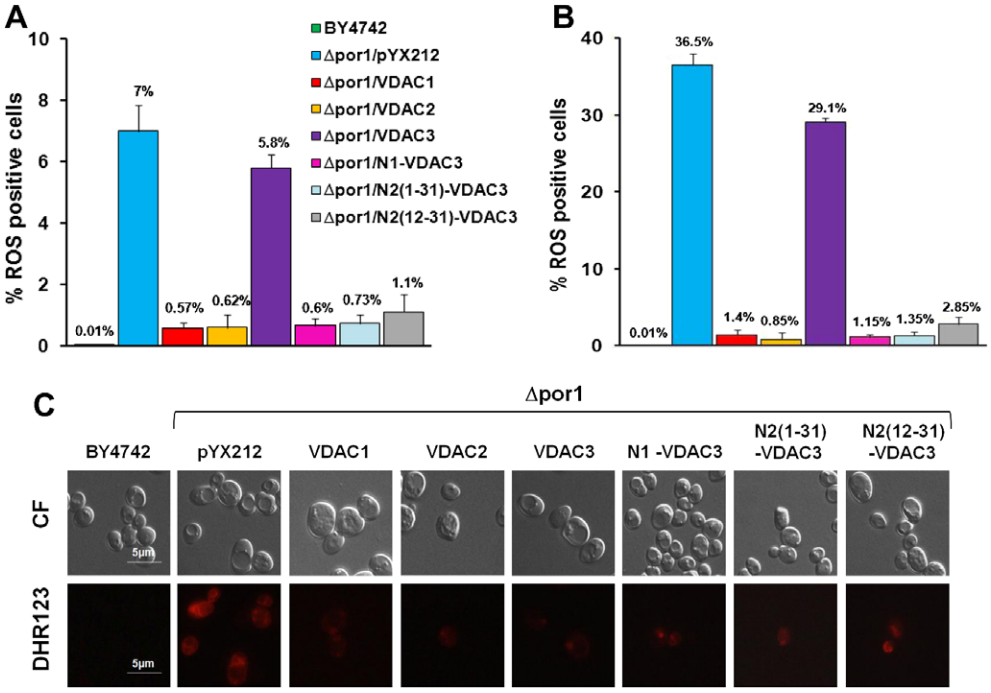

Fig. 4. ROS detection by DHR123 shows that the VDAC3 N-terminal chimaeras are able to lower the ROS level in opposition to Δpor1 yeast and the same transformed with VDAC3. Wild type (BY4742) and Δpor1 strains were transformed with the empty plasmid pYX212 or with the same plasmid carrying coding sequences as in Fig. 3. These transformed yeast were incubated with DHR123 and after 3 h observed at the fluorescence microscope. The ROS positive cells were counted with respect to the negative cells. Panels (A) and (B) show the percentage of ROS positive cells of the wild type (BY4742) and Δ*por1* strains transformed with the VDACs and chimaeras in exponential and stationary phase, respectively. Average and standard deviations, obtained from three independent experiments, are also indicated. (C) Phase contrast and fluorescent microscopy of ROS positive cells.

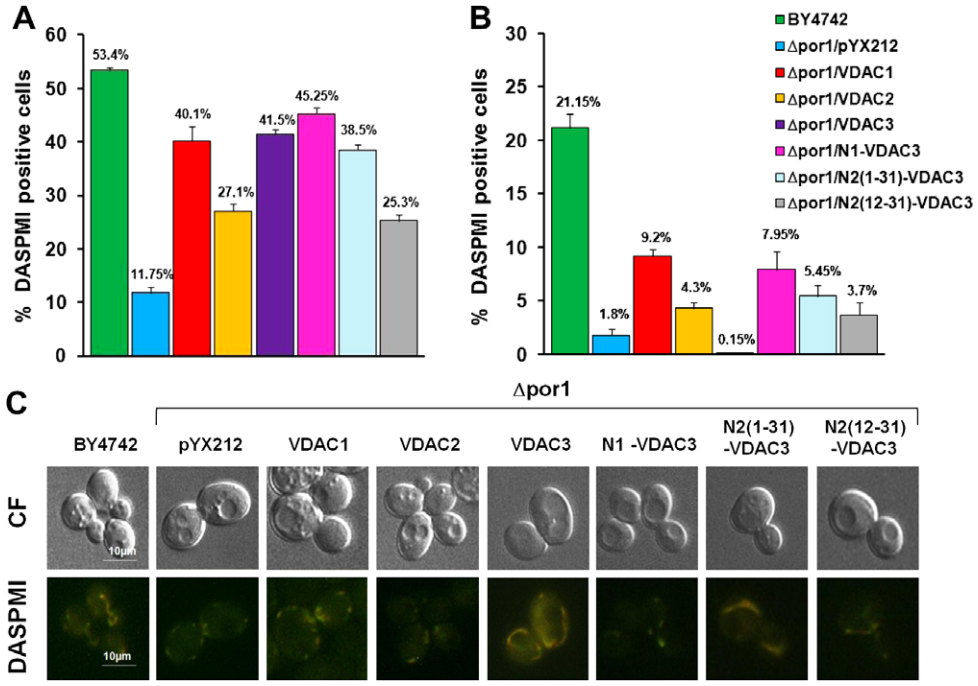

Fig. 5. Evaluation of the membrane potential of Δpor1 yeast transformed with VDAC3 N-terminal chimaeras by DASPMI. Wild type (BY4742) and Δpor1 strains were transformed with the empty plasmid pYX212 or with the same plasmid carrying coding sequences as in Fig. 3. These transformed yeast were incubated with DASPMI and observed at the fluorescence microscope. The DASPMI positive cells were counted with respect to the negative cells. Panels (A) and (B) show the percentage of DASPMI positive cells of BY4742 (wild type) and Δ*por1* strains transformed with VDAC3 and its chimaeras during exponential and stationary phase, respectively. Average and standard deviations, obtained from three independent experiments, are indicated. (C) Phase contrast and fluorescent microscopy of DASPMI positive cells.

of oxidants by paraquat [42]. This suggests that these superoxide dismutases protect specific proteins. One of the proteins specifically carbonylated either in the *sod1*- or in *sod2*-mutant was the endogenous yeast porin-1 [42]. Also in mammalian synaptosomes the exposure to acrolein, a chemical suspected to cause Alzheimer's disease, enhances the ratio of carbonylation of VDAC1 13-fold; this is a very

specific effect [43]. We showed that in the absence of porin-1 (yeast strain Δpor1) the ROS-derived fluorescence of DHR123 is enhanced. The same result was found in VDAC3 transformed yeast: this strain was not able to replace the function of the other VDAC isoforms and this was also confirmed by the reduced mitochondrial membrane potential in aged VDAC3-expressing cells [28].

In another systematic study focused onto the oxidation of protein thiols in yeast, the oxidized proteome of unstressed cells was identified [44]. In this survey, among 200 mostly cytoplasmatic proteins, cysteines were reported to be oxidized at a various extent. It was also found that porin-1 is predominantly present with its cysteine oxidized. The in vivo redox state of cysteine residues is another indication of the susceptibility of VDAC to be modified by ROS. Lefièvre et al. [45] demonstrated the S-nitrosylation of VDAC3 in spermatozoa. Saaed et al. [46] downregulated Glutaredoxin1 (Grx1) in cell and found that it was associated with the oxidation of thiol groups of VDAC leading finally to mitochondrial dysfunction. They did not distinguish among the different VDAC isoforms, that have a strikingly different cysteine content.

It is thus tempting to speculate that porin-1 in yeast and VDAC1 and VDAC2 in higher eukaryotes might bear modification by ROS species and these modifications could be important for the protein activity. Oxidation of specific residues might change the conformation of the pore, possibly switching to the closed pro-apoptotic state. Reduction of the same residues by enzymatic activities could re-open the pore.

4.3. VDACs and cell ageing

The CLS experiments clearly show that the presence of porin-1 is essential for the yeast's life. Little is known about the role of the VDAC isoforms during ageing. Proteomic analysis of the human normal colon epithelial tissue, revealed that VDAC1 and VDAC2 protein levels increase about 5- to 6-fold during ageing, indicating a protecting role for these proteins [47]. In the absence of porin, yeast cell loose viability in stationary phase, suggesting that this protein is required for the maintenance of cell viability during ageing. Most important, the substitution of the endogenous porin-1 with higher eukaryotic VDAC1, VDAC2 and VDAC3 restores this feature [28]. The chronological aging assay gave surprising results when the Δpor1 strain was transformed with the VDAC3 chimaeras and, in particular, the N1-VDAC3. The transformation of the yeast with N1-VDAC3 was able to almost double the yeast's life span. Our results are thus a direct indication that VDAC, at a meeting point between energy metabolism and the ROS pathways, is also implicated in the mechanisms determining the life span of the cell.

Acknowledgements

V.D.P. and S.R. thank Simone Ottonello (Parma, IT), who generously provided the yeast strains used in this work and hosted SR for training in yeast technologies. The financial support of FIRB RBRN07BMCT and PRIN MIUR 2008SW44CS_004 to V.D.P. is acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.066.

References

[1] Schein, S.J., Colombini, M. and Finkelstein, A. (1976) Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from paramecium mitochondria. J. Membr. Biol. 30, 99–120.
[2] Benz, R. (1994) Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. Biochim. Biophys. Acta 1197, 167–196.
[3] Shoshan-Barmatz, V., Israelson, A., Brdiczka, D. and Sheu, S.S. (2006) The voltage-dependent anion channel (VDAC): function in intracellular signalling, cell life and cell death. Curr. Pharm. Des. 12, 2249–2270.
[4] Lemasters, J.J. (2007) Modulation of mitochondrial membrane permeability in pathogenesis, autophagy and control of metabolism. J. Gastr. Hepat. 22 (Suppl. 1), S31–S37.
[5] Zaid, H., Abu-Hamad, S., Israelson, A., Nathan, I. and Shoshan-Barmatz, V. (2005) The voltage-dependent anion channel-1 modulates apoptotic cell death. Cell Death Differ. 12, 751–760.
[6] Xu, X., Forbes, J.G. and Colombini, M. (2001) Actin modulates the gating of *Neurospora crassa* VDAC. J. Membr. Biol. 180, 73–81.
[7] Schwarzer, C., Barnikol-Watanabe, S., Thinnes, F.P. and Hilschmann, N. (2002) Voltage-dependent anion-selective channel (VDAC) interacts with the dynein light chain Tctex1 and the heat-shock protein PBP74. Int. J. Biochem. Cell Biol. 34, 1059–1070.
[8] Young, M.J., Bay, D.C., Hausner, G. and Court, D.A. (2007) The evolutionary history of mitochondrial porins. BMC Evol. Biol. 7, 31.
[9] Xu, X., Decker, W., Sampson, M.J., Craigen, W.J. and Colombini, M. (1999) Mouse VDAC isoforms expressed in yeast: channel properties and their roles in mitochondrial outer membrane permeability. J. Membr. Biol. 170, 89–102.
[10] Aiello, R., Messina, A., Schiffler, B., Benz, R., Tasco, G., Casadio, R. and De Pinto, V. (2004) Functional characterization of a second porin isoform in *D. melanogaster*. DmPorin2 forms voltage-independent cation-selective pores. J. Biol. Chem. 279, 25364–25373.
[11] Menzel, V.A., Cassarà, M.C., Benz, R., De Pinto, V., Messina, A., Cunsolo, V., Saletti, R., Hinsch, K.D. and Hinsch, E. (2009) Molecular and functional characterization of VDAC2 purified from mammal spermatozoa. Biosci. Rep. 29, 351–362.
[12] Sampson, M.J., Lovell, R.S. and Craigen, W.J. (1997) The murine voltage-dependent anion channel gene family. Conserved structure and function. J. Biol. Chem. 272, 18966–18973.
[13] Blachly-Dyson, E., Zambronicz, E.B., Yu, W.H., Adams, V., McCabe, E.R., Adelman, J., Colombini, M. and Forte, M. (1993) Cloning and functional expression in yeast of two human isoforms of the outer mitochondrial membrane channel, the voltage-dependent anion channel. J. Biol. Chem. 268, 1835–1841.
[14] Cheng, E.H., Sheiko, T.V., Fisher, J.K., Craigen, W.J. and Korsmeyer, S.J. (2003) VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science 301, 513–517.
[15] Hinsch, K.D., De Pinto, V., Aires, V.A., Schneider, X., Messina, A. and Hinsch, E. (2004) Voltage-dependent anion selective channels VDAC2 and VDAC3 are abundant proteins in bovine outer dense fibers, a cytoskeletal component of the sperm flagellum. J. Biol. Chem. 279, 15281–15288.
[16] Sampson, M.J., Decker, W.K., Beaudet, A.L., Ruitenbeek, W., Armstrong, D., Hicks, M.J. and Craigen, W.J. (2001) Immotile sperm and infertility in mice lacking mitochondrial voltage-dependent anion channel type 3. J. Biol. Chem. 276, 39206–39212.
[17] Hiller, S., Garces, R.G., Malia, T.J., Orekhov, V.Y., Colombini, M. and Wagner, G. (2008) Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. Science 321, 1206–1210.
[18] Bayrhuber, M., Meins, T., Habeck, M., Becker, S., Giller, K., Villinger, S., Vonrhein, C., Griesinger, C., Zweckstetter, M. and Zeth, K. (2008) Structure of the human voltage-dependent anion channel. Proc. Natl. Acad. Sci. USA 105, 15370–15375.
[19] Ujwal, R., Cascio, D., Colletier, J.P., Faham, S., Zhang, J., Toro, L., Ping, P. and Abramson, J. (2008) The crystal structure of mouse VDAC1 at 2.3 Å resolution reveals mechanistic insights into metabolite gating. Proc. Natl. Acad. Sci. USA 105, 17742–17747.
[20] De Pinto, V., Tomasello, F., Messina, A., Guarino, F., Benz, R., La Mendola, D., Magri, A., Milardi, D. and Pappalardo, G. (2007) Determination of the conformation of the human VDAC1 N-terminal peptide, a protein moiety essential for the functional properties of the pore. Chembiochem 8, 744–756.
[21] Stanley, S., Dias, J.A., D'Arcangelis, D. and Mannella, C. (1995) Peptide-specific antibodies as probes of the topography of the voltage-gated channel in the mitochondrial outer membrane of *Neurospora crassa*. J. Biol. Chem., 16694–16700.
[22] Popp, B., Court, D.A., Benz, R., Neupert, W. and Lill, R. (1996) The role of the N and C termini of recombinant Neurospora mitochondrial porin in channel formation and voltage-dependent gating. J. Biol. Chem. 271, 13593–13599.
[23] Koppel, D.A., Kinnally, K.W., Masters, P., Forte, M., Blachly-Dyson, E. and Mannella, C.A. (1998) Bacterial expression and characterization of the mitochondrial outer membrane channel. Effects of N-terminal modifications. J. Biol. Chem. 273, 13794–13800.
[24] Abu-Hamad, S., Arbel, N., Calo, D., Arzoine, L., Israelson, A., Keinan, N., Ben-Romano, R., Friedman, O. and Shoshan-Barmatz, V. (2009) The VDAC1 N-terminus is essential both for apoptosis and the protective effect of anti-apoptotic proteins. J. Cell Sci. 122, 1906–1916.
[25] Arzoine, L., Zilberberg, N., Ben-Romano, R. and Shoshan-Barmatz, V. (2009) Voltage-dependent anion channel 1-based peptides interact with hexokinase to prevent its anti-apoptotic activity. J. Biol. Chem. 284, 3946–3955.
[26] Hiller, S. and Wagner, G. (2009) The role of solution NMR in the structure determinations of VDAC-1 and other membrane proteins. Curr. Opin. Struct. Biol. 19, 396–401.

[27] Bereiter-Hahn, J. (1976) Dimethylaminostyrylmethylpyridiniumiodine (daspmi) as a fluorescent probe for mitochondria in situ. Biochim. Biophys. Acta 423, 1–14.
[28] De Pinto, V., Guarino, F., Guarnera, A., Messina, A., Reina, S., Tomasello, F.M., Palermo, V. and Mazzoni, C. (2010) Characterization of human VDAC isoforms: a peculiar function for VDAC3? BBA – Bioenergetics (February 6, epub ahead of print).
[29] Lin, S.J., Kaeberlein, M., Andalis, A.A., Sturt, L.A., Defossez, P.A., Culotta, V.C., Fink, G.R. and Guarente, L. (2002) Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. Nature 418, 344–348.
[30] Pereira, C., Camougrand, N., Manon, S., Sousa, M.J. and Corte-Real, M. (2007) ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome *c* release in yeast apoptosis. Mol. Microbiol. 66, 571–582.
[31] Jazwinski, S.M. (2005) Yeast longevity and aging: the mitochondrial connection. Mech. Ageing Dev. 126, 243–248.
[32] Ludovico, P., Sousa, M.J., Silva, M.T., Leao, C. and Corte-Real, M. (2001) *S. cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology 147, 2409–2415.
[33] Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S.J., Wolf, D.H. and Frohlich, K.U. (1999) Oxygen stress: a regulator of apoptosis in yeast. J. Cell Biol. 145, 757–767.
[34] Pozniakovsky, A.I., Knorre, D.A., Markova, O.V., Hyman, A.A., Skulachev, V.P. and Severin, F.F. (2005) Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. J. Cell Biol. 168, 257–269.
[35] De Pinto, V., Reina, S., Guarino, F. and Messina, A. (2008) Structure of the voltage dependent anion channel: state of the art. J. Bioenerg. Biomembr. 40, 139–147.
[36] Distler, A.M., Kerner, J. and Hoppel, C.L. (2007) Post-translational modifications of rat liver mitochondrial outer membrane proteins identified by mass spectrometry. Biochim. Biophys. Acta 1774, 628–636.
[37] De Pinto, V., Al Jamal, J.A., Benz, R., Genchi, G. and Palmieri, F. (1991) Characterization of SH groups in porin of bovine heart mitochondria. Porin cysteines are localized in the channel walls. Eur. J. Biochem. 202, 903–911.
[38] Aram, L., Geula, S., Arbel, N. and Shoshan-Barmatz, V. (2010) VDAC1 cysteine residues: topology and function in channel activity and apoptosis. Biochem. J. 427, 445–454.
[39] Orrenius, S., Gogvadze, V. and Zhivotovsky, B. (2007) Mitochondrial oxidative stress: implications for cell death. Annu. Rev. Pharmacol. Toxicol. 47, 143–183.
[40] Han, D., Antunes, F., Canali, R., Rettori, D. and Cadenas, E. (2003) Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J. Biol. Chem. 278, 5557–5563.
[41] Simamura, E., Hirai, K., Shimada, H., Koyama, J., Niwa, Y. and Shimizu, S. (2006) Furanonaphthoquinones cause apoptosis of cancer cells by inducing the production of reactive oxygen species by the mitochondrial voltage dependent anion channel. Cancer Biol. Ther. 5, 1523–1529.
[42] O'Brien, K.M., Dirmeier, M., Engle, M. and Poyton, R.O. (2004) Mitochondrial protein oxidation in yeast mutants lacking MnSOD or CuZnSOD. J. Biol. Chem. 279, 51817–51827.
[43] Mello, C.F., Sultana, R., Piroddi, M., Cai, J., Pierce, W.M., Klein, J.B. and Butterfield, D.A. (2007) Acrolein induces selective protein carbonylation in synaptosomes. Neuroscience 147, 674–679.
[44] Le Moan, N., Clement, G., Le Maout, S., Tacnet, F. and Toledano, M.B. (2006) The *Saccharomyces cerevisiae* proteome of oxidized protein thiols. J. Biol. Chem. 281, 10420–10430.
[45] Lefièvre, L., Chen, Y., Conner, S.J., Scott, J.L., Publicover, S.J., Ford, W.C. and Barratt, C.L. (2007) Human spermatozoa contain multiple targets for protein S-nitrosylation: an alternative mechanism of the modulation of sperm function by nitric oxide? Proteomics 7, 3066–3084.
[46] Saeed, U., Durgadoss, L., Valli, R.K., Joshi, D.C., Joshi, P.G. and Ravindranath, V. (2006) Knockdown of cytosolic glutaredoxin 1 leads to loss of mitochondrial membrane potential: implication in neurodegenerative diseases. PLoS One 3 (6), e2459.
[47] Ming, Li, Zhi-Qiang, Xiao, Zhu-Chu, Chen, Jian-Ling, Li, Cui, Li, Peng-Fei, Zhang and Mao-Yu, Li (2007) Proteomic analysis of the aging-related proteins in human normal colon epithelial tissue. J. Biochem. Mol. Biol. 40, 72–81.