



Sprague Dawley rats: A model of successful heart aging



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ABSTRACT

Aging is a universal phenomenon involving the whole body and is characterized by metabolic and physiological decline, leading to cardiovascular defects and heart failure.

To characterize the molecular basis of physiological cardiac aging, the proteomic profiles of Sprague Dawley rat hearts of 6, 22 and 30 months were analysed by DIGE and immunoblotting.

Results indicate changes in myosin binding protein C, aldehyde dehydrogenase, serpins and sirtuin-3 which protects from the opening of the mitochondrial permeability transition pore induced by cyclophilin D increment.

Conversely, an increase of fusion, a decrease of mitochondrial fission and the activation of the non-canonical autophagy pathway were observed. These results support the hypothesis of successful aging in this rat model.

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1. Introduction

The substantial increase in life expectancy at birth combined with medical advances pose the question of how to promote healthier old age and how to age “successfully.”

Later in life, people can experience “usual aging” with normal decline in physical, social, and cognitive functioning or a “successful aging” in which functional loss is minimised [1,2]. From a molecular point of view, little is known about the successful aging of centenarians and specifically, little is known about molecular changes occurring in heart tissue that lead to a successful senescence [2,3].

Cardiovascular diseases are the major causes of death among the elderly [4] and the heart aging rate is one of the major risk factors [5]. In humans, the intrinsic aging is defined as a slow and progressive age-dependent myocardial degeneration that induces cardiovascular hypertrophy making the heart vulnerable to stress, with reduced functional reserve, contributing to morbidity and mortality. In addition, both diabetes and hypertension accelerate the process of cardiovascular aging leading to heart insufficiency

and failure, making difficult the dissociation of the aging process from other contributing factors [6].

Rodents represent a suitable model to detect markers of intrinsic cardiac aging as they are not affected by other risk factors (e.g. diabetes or hypertension) that can obscure the slowly progressive age-dependent degeneration and decline in cardiac function [5].

During aging, a swelling in size and a decrease in the number of cardiomyocytes result in a loss of function combined with myocardium interstitial fibrosis, accumulation of lipofuscin and amyloid. These features are typical of aged myocardium and are extended to the sinoatrial and atrioventricular node and to the atrioventricular bundle leading to alterations of cardiac conductivity [7].

Furthermore mitochondria, which represent ~30% of the cardiomyocyte volume [8], play a crucial role in maintaining cardiac function through the balance among mitochondrial fusion, fission and autophagy. These processes can remove and replace damaged mitochondria preventing aging [9].

In skeletal muscles, ROS overproduction and defective mitochondria lead to a progressive accumulation of lipofuscins and cytosolic protein aggregates [10] and aged muscles show a decline of autophagy [11].

The overall aim of this study is to characterize, in a Sprague Dawley rat, the molecular basis of the physiological cardiac aging

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analysing changes in heart proteome, autophagy and in mitochondrial dynamics, since these processes are strictly interrelated and their homeostasis is associated to well-being. Under moderate dietary restriction, this model does not develop heart disease within an average life of ~2 years [12]. Rats of 6 (young), 22 (old adult) and 30 (senescent) months of age were analysed. The study has been extended to senescent rats to follow changes in the heart proteome possibly occurring in centenarians.

2. Material and methods

2.1. Animals

Sprague Dawley rats were divided into three groups: young ($n = 3$, 6 months), old adult ($n = 3$, 22 months) and senescent ($n = 3$, 30 months). For sacrifice, animals were anesthetized with a hip injection of sodium thiopental (10 mg/100 g body weight) and heparin (500 units). Hearts were removed soon after the sacrifice (less than 1 min), immediately frozen in liquid nitrogen and then stored at -80°C until use. Investigation was conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Protein extraction

For two-dimensional differences in-gel electrophoresis (2-D DIGE) and immunoblots, an aliquot of each frozen heart was suspended in lysis buffer (urea 7 M, thiourea 2 M, CHAPS 4%, Tris 30 mM, and PMSF 1 mM) and solubilized by sonication on ice. Proteins were selectively precipitated using PlusOne 2D-Clean-up kit to remove non-protein impurities and re-suspended in lysis buffer. Protein extracts were adjusted to pH 8.5 by the addition of NaOH 1 M solution, and sample concentrations were determined using PlusOne 2D-Quant kit.

2.3. Proteomic analysis

Protein labelling, 2-D separation and analysis were performed as previously described [13]. Before IEF, labelled samples were diluted in an equal volume of 2x sample buffer containing 130 mM DTT and 2% v/v IPG buffer (GE Healthcare). Individual samples (40 μg) were combined with an equal amount of internal standard; rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.5% IPG buffer pH 3.5–9.5 and BBF in traces) was added to a final volume of 450 μL . Samples were separated on 24 cm, pH 3–10 nonlinear (NL) gradient IPGstrips, applying the following multistep IEF protocol: 200 V for 2 h, 500 V (2 h), 1000 V (2 h), 2000 V (1 h), 3000 V (1 h) and 8000 V until a total of 64 000 VhT was reached, using an IPGphor electrophoresis unit (GE Healthcare). After focusing IPGstrips were equilibrated in an SDS reducing-buffer (6 M urea, 2% SDS, 20% glycerol, 375 mM Tris-HCl pH 8.8, 65 mM DTT) for 15 min, then alkylated for 8 min in the same buffer containing 135 mM iodoacetamide instead of DTT. Second dimension was carried out in $20 \times 25 \text{ cm}^2$, 12%T, 2.5%C, constant concentration polyacrylamide gels at 20°C and 15 mA per gel using the Ettan Dalt II system (GE Healthcare). Each sample was analysed independently and run in triplicate.

Spot detection, background subtraction, gel normalization, spot matching and statistical analysis were performed using DeCyder 2D software version 7.0 (GE Healthcare). Statistically significant differences of 2-D DIGE data were computed by an independent one-way analysis of variance (ANOVA) coupled to Tukey's multiple-group comparison test ($p < 0.01$). False discovery rate was applied as multiple test correction in order to keep the overall

error rate as low as possible. Changed spots were further filtered on the basis of the average ratio value provided by DeCyder 2D software: only spots with an average ratio > 1.2 or < -1.2 were considered as differentially expressed.

Proteins were identified by matrix-assisted laser desorption/ionization time of flight utilizing the method previously described [13]. Spectra were processed by the FlexAnalysis software v. 3.0 (Bruker Daltonics) and search was carried out by correlation of uninterpreted spectra to Rodentia entries in NCBI nr 20140801 database (8.483.808 sequences; 2.914.572.939 residues) using in-house Mascot 2.2 software.

In cases where this approach was unsuccessful, additional searches were performed using electrospray ionization-MS/MS, as previously described [14].

2.4. Immunoblotting

Protein extracts from young, old adult and senescent hearts, were pooled and resolved by SDS-PAGE on 6–14% gradient polyacrylamide gels or 12% homogeneous polyacrylamide gels and transferred onto PVDF membranes. Each pooled sample (50 μg) was run in triplicate. Blots were incubated with rabbit, goat or mouse polyclonal primary antibodies diluted as follows: anti-beclin1 (Cell Signaling Technology, CST, #3738 1:1000), anti-Bnip3 (CST, #3769, 1:1000), anti-mTor (CST, #2972, 1:500), anti-P-mTor (CST, #2974, 1:200), anti-LC3 (CST, #2775, 1:500), anti-Bcl2 (CST, #2876 1:1000), anti-Dlp1 (BD transduction labs, 611113, 1:500), anti-Fis1 (Enzo life sciences, ALX-210-907, 1:200), anti-Mfn2 (Santa Cruz biotechnologies, sc-50331, 1:200), anti-OPA1 (BD transduction labs, 612607, 1:500), anti-CypD (Santa Cruz biotechnologies, sc-66848, 1:500), anti-Sirt3 (CST, #5490, 1:1000). Signals were visualized by chemiluminescence by ECL prime detection kit (GE Healthcare). Images were scanned using ImageQuant LAS 4000 mini digital imaging system (GE Healthcare) and each band was quantitated using ImageQuant Software (GE Healthcare). Band intensities were normalized against the total amount of proteins stained by Sypro Ruby and subjected to a Student's *t*-test by comparing old adult and senescent versus young, and senescent versus old adult rats. Differences were considered significant at $p < 0.05$.

To confirm 2D-DIGE data, the following blots were performed as described above: anti-Des (Santa Cruz biotechnologies, sc-14026, 1:500), anti-Gapdh (Santa Cruz biotechnologies, sc-25778, 1:500), anti-Hspa8 (CST, #8444, 1:1000), anti-Ldhd (Santa Cruz biotechnologies, sc-385256, 1:250), anti-Aldh2 (Santa Cruz biotechnologies, sc-48837, 1:500), anti-Eno3 (Santa Cruz biotechnologies, sc-15343, 1:500).

3. Results

3.1. Proteome profile

The proteome profiles of 6-month-old (young), 22-month-old (old adult) and 30-month-old (senescent) rat heart tissues were obtained through 2-D DIGE analysis and MALDI-ToF or ESI MS/MS.

Overall, 700 spots were matched among all gels, of which 284 spots (40.6%) were identified by mass spectrometry. Proteomic analysis revealed 112 differentially expressed spots in the three groups. Among them, 67 spots were identified and shown in a representative 2-D DIGE map (Fig. 1). Not considering proteoforms (as defined by Smith LM et al. [15]), these identified spots correspond to 40 different proteins. Identified spots are listed in supplementary Table S1 and S2 with the corresponding UniProtKB accession numbers and degree of variation expressed as average ratio.

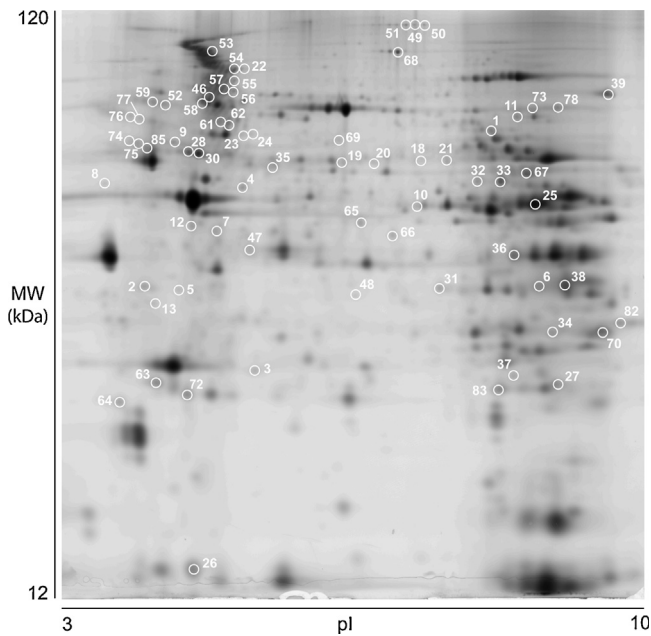


Fig. 1. Representative 2-D DIGE map of rat cardiac muscle. Spots identified as changed in old adult and senescent rats vs. young controls and in senescent vs. old adult rats were indicated and numbered accordingly to Supplementary Tables S1 and S2 listing.

The differentially expressed proteins from young vs. old adult and young vs. senescent respectively were divided into functional classes and represented with histogram bars (Figs. 2 A, 3 A and 4 A). The comparison between old adult and senescent was also divided into functional classes represented as before, in supplementary material (Fig. S1). Protein identification was validated performing a random analysis *via* immunoblotting of 13% of the identified proteins, not considering proteoforms (Figs. 2 B, 3 B and 4 B).

Proteomic results indicated that protein dysregulation took place in three major cell compartments: contractile and structural, metabolic, antioxidant/other proteins.

3.1.1. Contractile and structural proteins (Fig. 2A)

Overall, in aging we observed a decreased protein expression compared to the young. Senescent rats were characterized by a decrement of myosin-binding protein C (Mybpc3), paralleled by an increase of the intermediate filament proteins, specifically, myosins light chain (Myl4 and Myl7), troponin I (Tnni3) and actin (Actc1). Cytoskeletal proteins, including cysteine and glycine-rich protein 3 (Csrp3), also known as Muscle LIM protein (MLP), desmin (Des) and vimentin (Vim) were increased whereas beta-tubulin (Tubb4b) decreased. It should be noted that changes in old adults affected only few proteins, in particular a decrement of Myl7 and Tubb4b was observed.

Senescent rats were characterized by a decrement of 6 proteoforms of myosin-6 (Myh6) and 3 proteoforms of myosin-7 (Myh7), whereas two different proteoforms of Myh6 and Myh7 increased. Considering the overall protein expression, results showed a decrement of both proteins (average ratio -1.22 and -1.24 , respectively) in senescent vs. young rats (Fig. 2A, gray frames). In old adults, one proteoform of Myh7 was instead increased.

3.1.2. Metabolic proteins (Fig. 3A)

Among glycolytic proteins, two proteoforms of aldehyde dehydrogenase (Aldh2) were increased and one decreased in old adult vs. young, whereas only one was increased and two decreased in senescent vs. young. Given the differences in

proteoforms tendency, the overall expression of Aldh2 was considered and resulted in unchanged levels in old adult vs. young and in a significant decrement (average ratio -1.30) in senescent vs. young (Fig. 3A, gray frame). This trend was confirmed by immunoblotting (Fig. 3B). Two proteoforms of beta-enolase (Eno3), localized on the line Z of the sarcomere, were decreased both in old adult and senescent. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and phosphoglycerate mutase 2 (Pgam2) increased in senescent, whereas L-lactate dehydrogenase B chain (Ldhd) increased in old adult only. Changes in oxidative and in fatty acid metabolism were prominent in senescent rats. Isocitrate dehydrogenase [NAD] subunit alpha (Idh3a) decreased. Concerning oxidative phosphorylation, the mitochondrial ubiquinone biosynthesis protein CoQ9, also known as coenzyme Q9 homolog (Coq9), and the cytochrome c oxidase subunit 5B (Cox5b) decreased in senescent together with the electron transfer flavoprotein subunit alpha and beta (Etfb, Etfb). A proteoform of NADH dehydrogenase subunit 1 alpha subcomplex 10 (Ndufa10) decreased, whereas another proteoform increased in aging. Considering Ndufa10 as a whole, its level was significantly increased in senescent (average ratio 1.22; see Fig. 3A, gray frame). Likewise, the NADH dehydrogenase [ubiquinone] flavoprotein 1 (Ndufv1) increased. These changes were paralleled by a reduction of acyl-CoA synthetase family member 2 (Acsf2), mitochondrial delta(3,5)-delta(2,4)-dienoyl-CoA isomerase (Ech1), hydroxyacyl-coenzyme A dehydrogenase (Hadh), trifunctional enzyme subunit alpha (Hadha) and long-chain specific acyl-CoA dehydrogenase (Acadl), whereas apolipoprotein A-1 (Apoa1) increased.

3.1.3. Antioxidant defence and other proteins (Fig. 4A)

Overall, in aging six proteins involved in stress response were changed, three of them decreased in old adult (3-mercaptopyruvate sulfurtransferase, Mps1; protein disulfide-isomerase A3, Pdia3; peroxiredoxin-2, Prdx2), whereas in senescent, a reduction of the heat shock cognate 71 kDa protein (Hspa8), and increments of glutathione S-transferase P (Gstp1), and ribonuclease inhibitor (Rnh1) were observed.

The serpin proteins, were also significantly reduced in aging. In particular, the serine protease inhibitor A3K (Serpina3k), the serine protease inhibitor A3L (serpina3l) and the two proteoforms of the alpha-1-antiproteinase (Serpina1) were decreased. Notably, annexin A5 (Anxa5), a desmosome associated protein, was increased in senescent rats.

3.2. Autophagy and mitochondrial dynamics

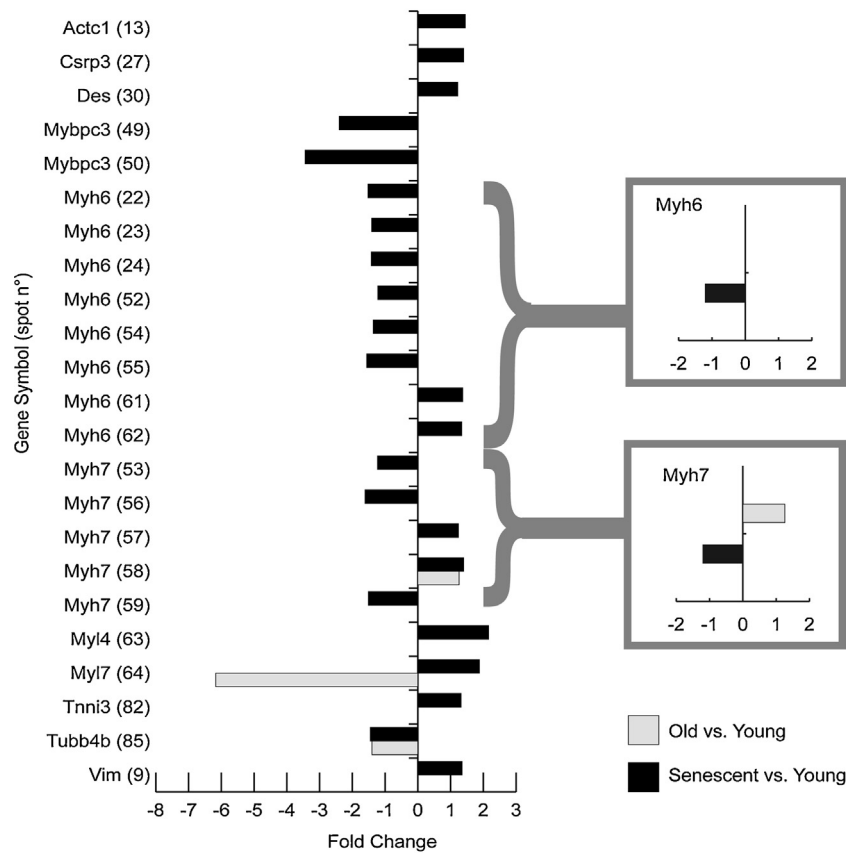
The expression of some proteins involved in autophagy and mitochondrial dynamics was determined by antigen-antibody reaction in cardiac muscle protein extracts.

Autophagic process can be activated through a canonical serine/threonine-protein kinase mTOR (mTor) and beclin1 pathway [16], or through a non-canonical beclin1 independent system [17]. Both pathways lead to the activation of an effector complex, which is part of the microtubule-associated proteins 1A/1B light chain 3B (LC3) complex.

A link between autophagy and apoptosis – programmed cell death – is given by B-cell lymphoma protein 2 (Bcl2), which blocks autophagy by blunting beclin1, whereas BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3) can induce autophagy destroying the interaction between Bcl2 and beclin1 [18].

The assessment of markers of autophagy (Fig. 5) indicated a progressive decrement of Bnip3 with aging, whereas beclin1 was not significantly changed among the experimental groups, and Bcl2 increased, progressively. Likewise, the phosphorylated form over the total amount of mTor protein (P-mTor/mTor) increased in

A Contractile and structural proteins



B Validation by immunoblotting

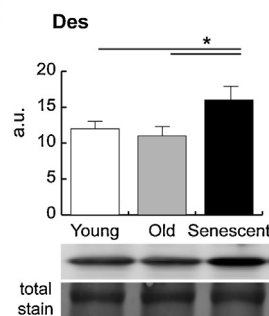


Fig. 2. A) Histograms of differential protein expression in cardiac tissue of old adult (22 months, gray bars) and senescent (30 months, black bars) rats, as detected by 2-D DIGE analysis. Proteins significantly altered (ANOVA and Tukey, $p < 0.01$) were expressed as a ratio of spot volume variations in old adult and senescent rats versus young (6 months) control, respectively. In rat hearts, 11 contractile and structural proteins were identified: Actc1, actin alpha cardiac muscle 1; Csrp3, cysteine and glycine-rich protein 3; Des, desmin (2 proteoforms); Mybpc3, myosin-binding protein C (2 proteoforms); Myh6, myosin-6 (8 proteoforms); Myh7, myosin-7 (5 proteoforms); Myl4, myosin light chain 4; Myl7, myosin regulatory light chain 2 atrial isoform; Tnni3, troponin I; Tubb4b, tubulin beta-4B chain; Vim, vimentin. For proteins having proteoforms with opposite trends in the same group, the global protein expression was reported in frames. B) Validation of Des protein by immunoblotting in young, old adult and senescent rat cardiac muscles. Statistical analysis was performed by Student's *t*-test ($n = 3$; * $p < 0.05$). Data were normalized against the total amount of loaded proteins stained with Sypro Ruby.

aged rats. For autophagosome formation the expression of the intact 18 kDa LC3 protein and of the lipidated 16 kDa LC3 form were monitored. The 18 kDa LC3 was unchanged whereas the 16 kDa increased progressively from old adult to senescent.

Mitochondrial morphology is controlled by monomeric GTPases mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), which control the external mitochondrial membrane fusion together with the multimeric protein optic atrophy 1 (OPA1) [19]. Conversely, dynamin-1 like protein (Dlp1) and fission protein 1 (Fis1), a

protein associated with the external mitochondrial membrane, have an important role in fission.

Dlp1 and Fis1 decreased in both old adult and senescent (Fig. 6). Concerning mitochondrial fusion, Mfn2 increased during aging, particularly in senescent, suggesting a progressive increase of the mitochondrial fusion. OPA1 showed an anomalous trend, as it increased in old compared to young whereas decreased in senescent. These changes observed in senescent could be associated to increased protein degradation [10].

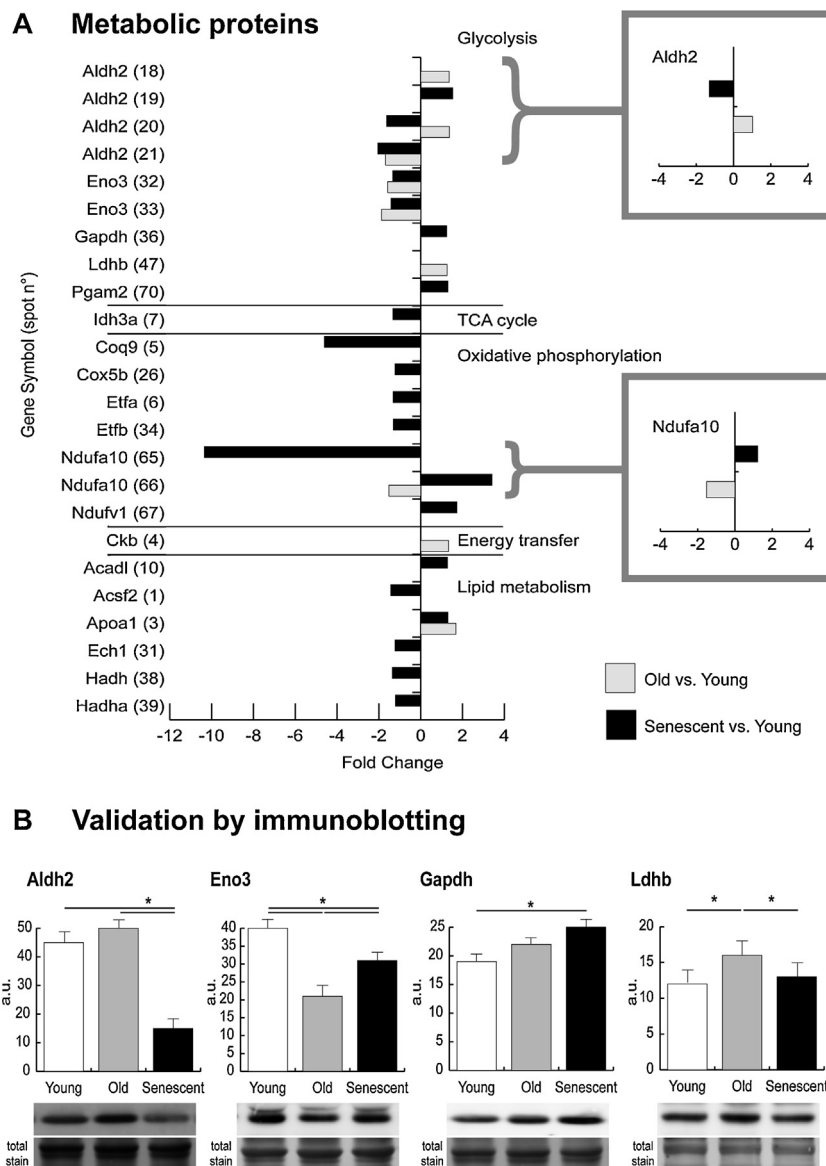


Fig. 3. A) Histograms of differential protein expression in cardiac tissue of old adult (22 months, gray bars) and senescent (30 months, black bars) rats, as detected by 2-D DIGE analysis. Proteins significantly altered (ANOVA and Tukey, $p < 0.01$) were expressed as a ratio of spot volume variations in old adult and senescent rats versus young (6 months) control, respectively. In rat hearts, 19 proteins of cellular metabolism were identified: Aldh2, aldehyde dehydrogenase (4 proteoforms); Eno3, beta-enolase (2 proteoforms); Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Ldhb, L-lactate dehydrogenase B chain; Pgam2, phosphoglycerate mutase 2; Idh3a, isocitrate dehydrogenase [NAD] subunit alpha; Coq9, ubiquinone biosynthesis protein CoQ9; Cox5b, cytochrome c oxidase subunit 5B; Etfb, electron transfer flavoprotein subunit alpha; Etfb, electron transfer flavoprotein subunit beta; Ndufa10, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 (2 proteoforms); Ndufv1, NADH dehydrogenase [ubiquinone] flavoprotein 1; Ckb, creatine kinase B-type; Acadl, long-chain specific acyl-CoA dehydrogenase; Acsf2, acyl-CoA synthetase family member 2; Apoa1, apolipoprotein A-I; Ech1, delta(3,5)-delta(2,4)-dienoyl-CoA isomerase; Hadh, mitochondrial hydroxyacyl-coenzyme A dehydrogenase; Hadha, trifunctional enzyme subunit alpha. For proteins having proteoforms with opposite trends in the same group, the global protein expression was reported in frames. B) Immunoblotting of selected proteins (identified by proteomic analysis) in young, old adult and senescent rat cardiac muscles. Statistical analysis was performed by Student's T-Test ($n = 3$; * $p < 0.05$). Data were normalized against the total amount of loaded proteins stained with Sypro Ruby.

Finally, we evaluated the expression of the protein cyclophilin D (CypD), a regulatory protein of the mitochondrial permeability transition pore (mtPTP), which increased both in old adult and senescent compared to young. Concerning sirtuin-3 (Sirt3), a deacetylase involved in metabolism and resistance to stress, it was increased in aging.

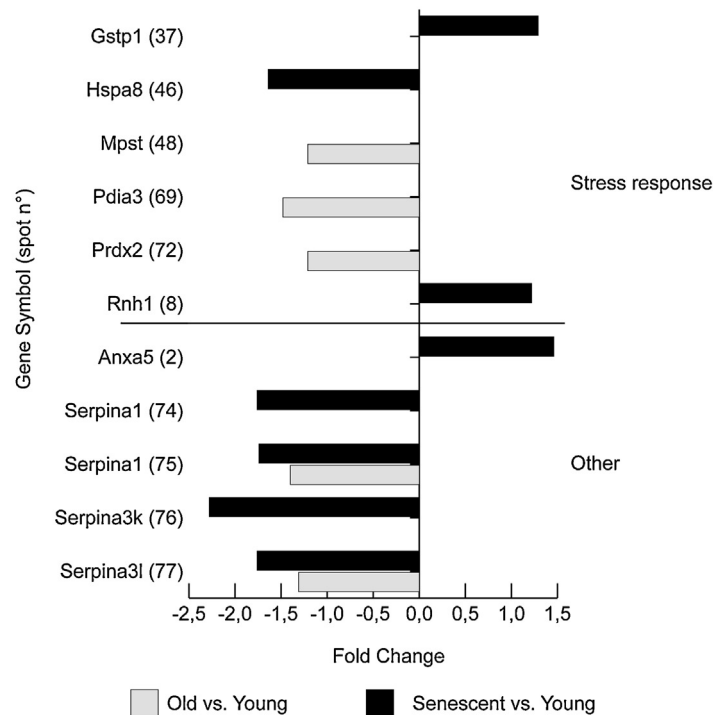
4. Discussion

The 2-D DIGE analysis highlighted changes in protein abundance in aged heart tissue that involved only some specific proteoforms of contractile proteins [20]. It should be of note that

changes followed a proportional progression with aging. The proteomic results support the hypothesis that this animal model could be associated with disease-free aging occurring in centenarians. Indeed, Sprague Dawley rats do not develop spontaneous cardiomyopathy, unlike Fisher 344 rats which are characterized by myocardial degeneration, interstitial fibrosis and chronic myocarditis (33% of males and 18% females) [21], spontaneously hypertensive rats (SHR), a model of primary (or essential) hypertension [22], or Obese Zucker rats characterized by high levels of lipids and cholesterol and insulin-resistance.

Proteomic results indicated a prevalent down regulation of proteins in senescent rats.

A Stress response and other proteins



B Validation by immunoblotting

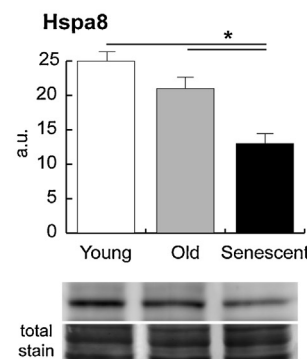


Fig. 4. A) Histograms of differential protein expression in cardiac tissue of old adult (22 months, gray bars) and senescent (30 months, black bars) rats, as detected by 2-D DIGE analysis. Proteins significantly altered (ANOVA and Tukey, $p < 0.01$) were expressed as a ratio of spot volume variations in old adult and senescent rats versus young (6 months) control, respectively. In rat hearts, 6 stress response and 4 other proteins were identified: Gstp1, glutathione S-transferase P; Hspa8, heat shock cognate 71 kDa protein; Mpst, 3-mercaptopyruvate sulfurtransferase; Pdia3, protein disulfide-isomerase A3; Prdx2, peroxiredoxin-2; Rnh1, ribonuclease inhibitor; Anxa5, annexin A5; Serpina1, alpha-1-antiproteinase (2 proteoforms); Serpina3k, serine protease inhibitor A3K; Serpina3l, serine protease inhibitor A3L. B) Validation of Hspa8 protein by immunoblotting in young, old adult and senescent rat cardiac muscles. Statistical analysis was performed by Student's T-Test ($n = 3$; * $p < 0.05$). Data were normalized against the total amount of loaded proteins stained with Sypro Ruby.

Interestingly, among contractile proteins, comparing old adult vs. young, a subunit of the myosin regulatory light chain (Myosin regulatory light chain 2) atrial isoform was significantly down-regulated (average ratio -6.17).

Comparing senescent and young heart extracts, the number of differentially expressed spots increased. In particular, two proteoforms of myosin binding protein-C (Mybpc3) were changed in advanced aging. Mybpc3 is a sarcomeric protein of thin filaments that interacts with titin, myosin and actin and regulates the assembly, the structure and function of the sarcomere and is also involved in muscle cell regeneration. This protein has three tissue-specific isoforms, exclusively expressed in skeletal (Mybpc1 and 2) and heart (Mybpc3) muscles. Knockout mice for this protein have

an abnormal structure of the sarcomere with alteration in the size of thin filaments and failure of M line in the contractile units. Knockout mice are viable but develop cardiomyopathies and contractile dysfunction at 3 months of age, indicating that the Mybpc3 is necessary for proper organization of the sarcomere and for cardiac function [23,24]. Furthermore, Mybpc3 proteoforms, which were decreased in aged heart tissue, can be released into the blood and become possible biomarkers of aging. It would therefore be interesting to investigate these protein fragments in the biological fluids of elderly subjects.

As regards metabolic proteins, acyl-CoA synthetase family member 2 (Acsf2), which catalyzes the first reaction in the metabolism of fatty acids, decreased in senescent vs. young. This

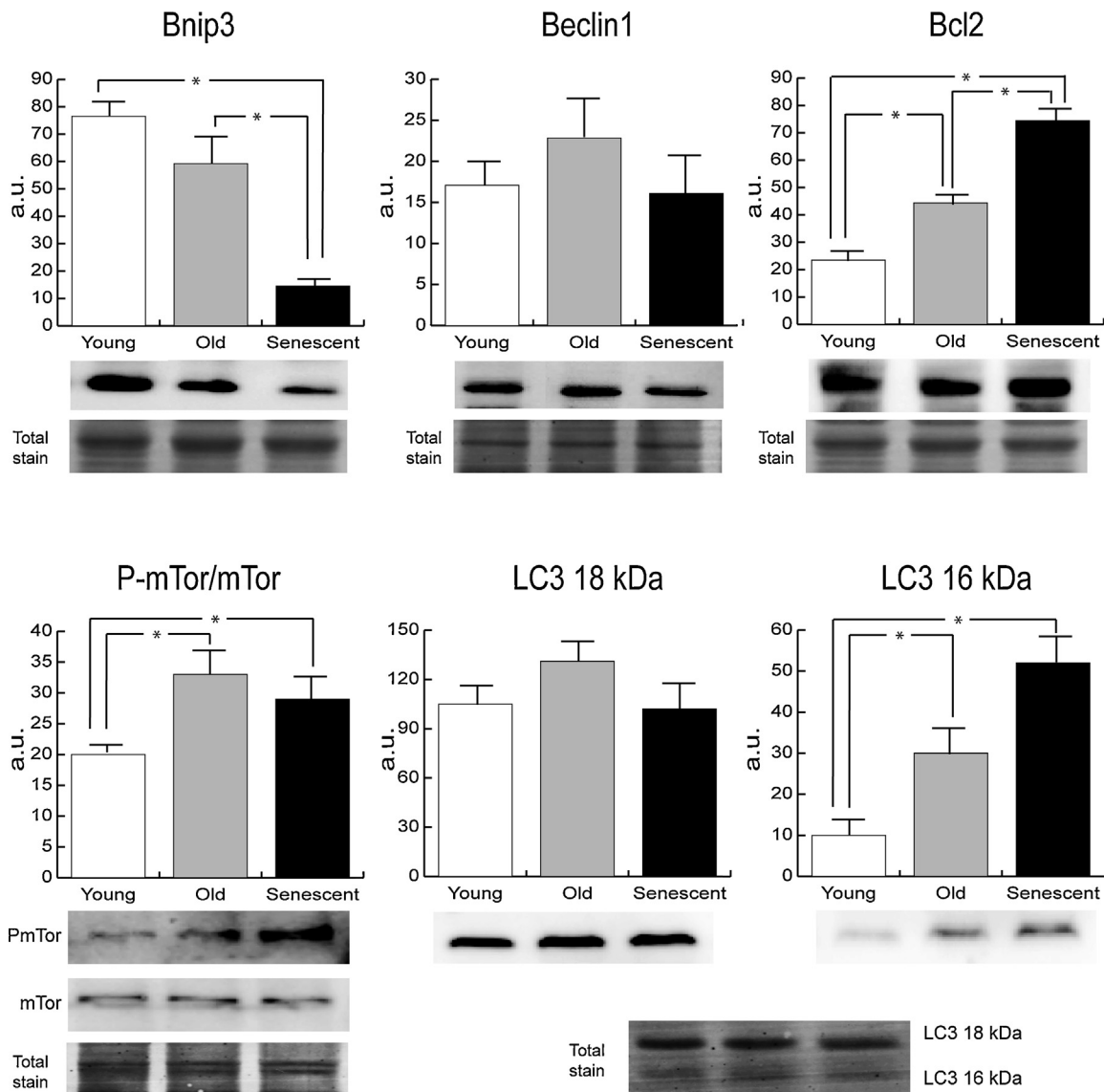


Fig. 5. Biochemical analysis of the autophagic process. Lower panel: Representative immunoblot images of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3), Beclin-1, apoptosis regulator Bcl-2 (Bcl2), the phosphorylated/total amount ratio of Serine/threonine-protein kinase mTOR (P-mTor/mTor), cytosolic (18 kDa) and lipidated (16 kDa) forms of Microtubule-associated proteins 1A/1B light chain 3B (LC3), in cardiac muscles of young (white bars), old adult (gray bars) and senescent (black bars) rats. Upper panel: Histograms of protein expression normalized to the total protein stain (mean \pm SD). *Significant differences (Student's *t*-test, $n=3$, $p < 0.05$).

result may indicate a restriction of fatty acids for the production of ATP in senescent heart, with a consequent reduction of the energy for heart contraction [25].

Among proteins involved in oxidative phosphorylation, some proteoforms displayed significant variations in aging such as NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 (Ndufa10) and NADH dehydrogenase [ubiquinone] flavoprotein 1 (Ndufv1), two subunits of the respiratory chain complex I. In addition, CoQ9 protein (Coq9), involved in the biosynthesis of ubiquinone (or coenzyme Q), decreased in senescent rats. Comparative studies of different mammalian species indicated that the amount of superoxide anion radical correlated directly to mitochondrial CoQ9 content, and inversely to the amount of CoQ10 [26]. The strong decrement of protein Coq9 suggests a decrease of free radicals production, enhancing the chances of healthy aging in this model.

The mitochondrial protein aldehyde dehydrogenase (Aldh2) is the enzyme responsible for the oxidation and detoxification of

acetaldehyde in ethanol metabolism [27,28]. The decrement of Aldh2 protein in aging (average ratio -1.30) could be considered as a putative marker for cellular aging to be specifically targeted. Recent studies indicated that an over-expression of Aldh2 could decrease the generation of ROS protecting cells from oxidative stress [28,29]. In addition, the group of Li Rui-Jian demonstrated that alpha-lipoic acid, a natural compound with antioxidant properties, reduces the oxidative stress in patients with acute coronary syndrome by increasing the antioxidant activity of Aldh2 [28].

Another class of proteins crucial in cardiac aging is represented by serpins localized in plasma and in the extracellular matrix [30]. Serpins are inhibitors of plasminogen activator and urokinase, blunting fibrinolysis and modulating both the coagulation pathway and inflammation. A general decrease of alpha-1-antitrypsin (Serpina1), serine protease inhibitor (Serpina31) and serine protease inhibitor A3 K (Serpina3k) was observed in our study, candidating them as putative biomarkers of aging.

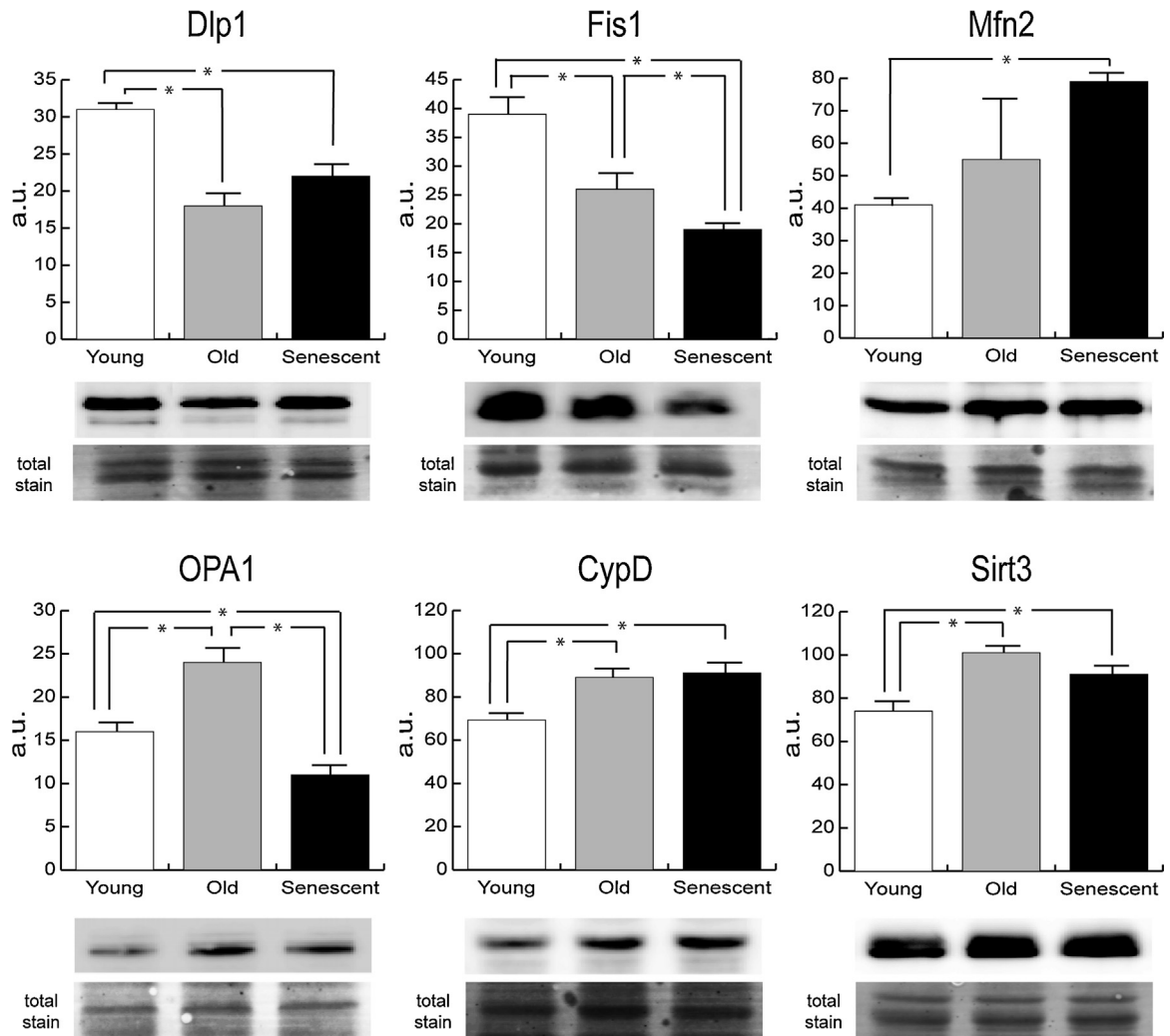


Fig. 6. Biochemical analysis of mitochondrial plasticity. Lower panel: Representative immunoblot images of Dynamin Like Protein-1 (Dlp1), Mitochondrial fission 1 protein (Fis1), Mitofusin-2 (Mfn2), Dynamin-like 120 kDa protein (OPA1), Cyclophilin D (CypD), and NAD-dependent protein deacetylase sirtuin-3 (Sirt3) in cardiac muscles of young (white bars), old adult (gray bars) and senescent (black bars) rats. Upper panel: Histograms of protein expression normalized to the total protein stain (mean \pm SD). *Significant differences (Student's *t*-test, $n=3$, $p < 0.05$).

Aside from proteomic results and in order to provide an overview of the homeostasis of cardiac tissue, in line to what has been recently observed by our group in muscle tissue [11], the levels of Sirt3 and CypD, not detectable by the present proteomic analysis, but known to be involved in the dysregulation of tissue homeostasis, mitochondrial dynamics and in the muscle capacity to clear damaged molecules, were considered.

Sirtuin-3 increment exerts a protective mechanism in the aging of this murine model counteracting the oxidative stress [31]. Likewise, CypD increased in aging. This increment can lead to mitochondrial permeability transition pore (mtPTP) activation, triggering cell death. It has been recently reported that CypD activity is controlled by acetylation [32]. In recent papers, authors hypothesized that the hypertrophic phenotype associated with aging in Sirt3-deleted mice is mediated by hyperactivation of the mtPTP via acetylated CypD [32–34]. To this point, the increment of Sirt3 deacetylase could be another important mechanism of cardioprotection activated in this animal model.

The role of mitochondrial dynamics in aging was assessed by quantitating some proteins involved in fission, fusion and in autophagy by immunoblotting. As expected, a decrease in proteins involved in fission (Fis1 and Dlp1), and an increase, albeit of borderline significance, of fusion proteins were observed [10].

Concerning autophagy, a decrement of the mTor-beclin1-induced signal, paralleled by an increment of LC3 suggest the presence of a non-canonical autophagy, which is independent from mTor. Similar results were obtained from cell cultures of centenarians, making the use of this model reasonable [35]. It is still unclear why the non-canonical autophagy was activated; we could assume that it might be an adaptive mechanism to prevent the blockage of autophagy [17].

In conclusion, aside from the typical changes of aging (*i.e.* a defective activation of the classical pathway of autophagy, a decrease in the generation of new mitochondria for fission and an increase in the mitochondrial fusion), the activation of protective mechanisms (such as limited ROS production, increased resistance to apoptosis, inhibition of mtPTP opening, and activation of the non-canonical autophagy) suggests that these elements of heart protection could be present in disease-free aging, which occurs in centenarians.

Further progress will be possible by comparing the pattern of physiological aging with other models of cardiovascular disease. The comparison will clarify if candidate biomarkers for monitoring aging can anticipate the manifestation of heart disease or if they themselves have a causal role. It would be desirable to translate the results from the animal model to humans in order to identify new

targets for the prevention or treatment of cardiovascular diseases associated with aging.

Conflict of interest

All authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.euprot.2016.03.017>.

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