Altered FoF1 ATP synthase and susceptibility to mitochondrial permeability transition pore during ischaemia and reperfusion in aging cardiomyocytes

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Summary

Aging is a major determinant of the incidence and severity of ischaemic heart disease. Preclinical information suggests the existence of intrinsic cellular alterations that contribute to ischaemic susceptibility in senescent myocardium, by mechanisms not well established. We investigated the role of altered mitochondrial function in the adverse effect of aging. Isolated perfused hearts from old mice (>20 months) displayed increased ischaemia-reperfusion injury as compared to hearts from adult mice (6 months) despite delayed onset of ischaemic rigor contracture. In cardiomyocytes from aging hearts there was a more rapid decline of mitochondrial membrane potential (Δψm) as compared to young ones, but ischaemic rigor shortening was also delayed. Transient recovery of Δψm observed during ischaemia, secondary to the reversal of mitochondrial FoF1 ATP synthase to ATPase mode, was markedly reduced in aging cardiomyocytes. Proteomic analysis demonstrated increased oxidation of different subunits of ATP synthase. Altered bionergetics in aging cells was associated with reduced mitochondrial calcium uptake and more severe cytosolic calcium overload during ischaemia-reperfusion. Despite attenuated ROS burst and mitochondrial calcium overload, mitochondrial permeability transition pore (mPTP) opening and cell death was increased in reperfused aged cells. *In vitro* studies demonstrated a significantly reduced calcium retention capacity in interfibrillar mitochondria from aging hearts. Our results identify altered FoF1 ATP synthase and increased sensitivity of mitochondria to undergo mPTP opening as important determinants of the reduced tolerance to ischaemia-reperfusion in aging hearts. Because ATP synthase has been proposed to conform mPTP, it is tempting to hypothesise that oxidation of ATP synthase underlie both phenomena.

Keywords

Mitochondria, calcium, aging, bionergetics

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Introduction

Aging of human population has substantially increased the global burden of ischaemic heart disease in the last years (1). Advanced age has been described as a major and independent risk factor of coronary syndromes (1, 2) having profound effects on their epidemiology, as the proportion of elderly persons is unprecedentedly expanding worldwide (3). Not only is there a progressive increase in the incidence of myocardial infarction with aging, but also an exacerbation of the clinical manifestations and higher mortality rates (2, 4). Although this can be partially explained by a compromised functional reserve in older patients, increased burden of comorbidities or more frequent extra-cardiac complications (5), preclinical studies in different models and current clinical data are consistent with the notion that susceptibility to ischaemia-reperfusion (IR) is increased in the old heart because of a constitutive impairment of cellular capacity to tolerate and adapt to ischaemic stress (6, 7).

A considerable number of evidences implicate mitochondria in functional and structural damage secondary to both ischaemic injury and senescence (8–10). One of the postulated mechanisms linking both phenomena is the excess of mitochondrial-derived reactive oxygen species (ROS) production and concomitant oxidative damage of some key molecules, specifically those involved in mitochondrial bioenergetic activity (11, 12). Aging is associated with electron transport chain defects in myocardial tissue, mainly

in interfibrillar mitochondria (13, 14), and it is well known that mitochondria become a source of pathological ROS production after an ischaemic insult (12, 15). To date, it is not clear whether these changes are cause or consequence of other cellular functional perturbations.

Age-dependent oxidative changes may have adverse consequences on calcium handling and bioenergetics (9, 16). In the present study we analysed cytosolic calcium handling and mitochondrial function in cardiomyocytes from aged mouse hearts in which tolerance to IR is reduced. The information on the mechanisms responsible for the reduced tolerance to ischaemia of senescent myocardium could be essential for devising interventions to limit infarct size in older patients and the deleterious consequences it has on their prognosis.

Methods

Mitochondria, cardiomyocytes and hearts were obtained from young (4–6 months) and old (>20 months) C57BL/6 mice. Animal handling was approved by the Ethical Committee of the Vall d'Hebron Research Institute and experiments were performed in accordance with the European Union legislation (EU directive 2010/63EU) and Recommendation 2007/526/EC, regarding the protection of animals used for scientific purposes.

Ischaemia-reperfusion in perfused hearts

After sodium pentobarbital overdose (150 mg/kg i.p.), mouse hearts were quickly excised and perfused through the aorta in a Langendorff apparatus with a modified Krebs–Henseleit bicarbonate buffer (in mmol/l: 140 NaCl, 24 $NaHCO_3$, 2.7 KCl, 0.4 KH_2PO_4, 1 $MgSO_4$, 1.8 $CaCl_2$ and 11 glucose, 95% O_2–5% CO_2 at 37°C) at constant flow perfusion pressure of 80 mmHg. Left ventricular (LV) pressure was monitored with a water-filled latex balloon inserted into the LV and inflated to obtain an end-diastolic pressure (LVEDP) of 6–8 mmHg. LV developed pressure (LVdevP) was calculated as the difference between LV systolic pressure and LVEDP. After 30 minutes (min) of normoxic perfusion, mouse hearts were subjected to 60 min of normothermic global ischaemia followed by 60 min of reperfusion.

Lactate dehydrogenase (LDH) activity was spectrophotometrically measured in samples collected from the coronary effluent at different times throughout the perfusion period and was considered an indirect measure of necrosis. After 60 min of reperfusion, heart slices were incubated in 1% triphenyltetrazolium chloride to outline the area of necrosis. Hearts submitted to normoxic perfusion during 120 min served as time control series.

Freshly isolated cardiomyocytes

Ischaemia-reperfusion in isolated cardiomyocytes

Freshly isolated cardiomyocytes were obtained from mouse hearts by retrograde collagenase perfusion (17). Rhod-shaped calcium tolerant cardiomyocytes were selected by differential centrifugation and albumin gradient and were plated on laminin-coated glass bottom coverslips (for confocal studies) or on 4% fetal calf serum pretreated multiwell dishes. To simulate IR in cardiomyocytes, two independent approaches were used: anoxic workstation under controlled atmosphere of 0% O_2–5% H_2 at 37°C (Invivo$_2$ Workstation Ruskinn, Bridgend, UK) and microscope-adapted microperfusion chamber (RC-43C/BS4 64–0371 Harvard Apparatus, Holliston, MA, USA). For the anoxic workstation, cardiomyocytes were plated in 96-wells dishes, placed within the anoxic atmosphere and incubated for 15 min with glucose-free acidic ischaemic buffer (previously deoxygenated in an autoclave and bubbled with N_2 for 20 min) containing (in mmol/l): 140 NaCl, 3.6 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, 20 HEPES, pH 6.4, and supplemented with 4 µmol/l resazurin, 100 µmol/l ascorbic acid, 0.5 mmol/l dithionite and 100 U/ml superoxide dismutase. For reperfusion, ischaemic buffer was washed out and oxygenated, glucose-containing control buffer (pH 7.4) was added for 10 min. To simulate ischaemia in the microperfusion chamber, laminin-attached cardiomyocytes placed on the stage of an inverted microscope were superfused, with the aid of a peristaltic pump, within a closed microperfusion chamber with ischaemic buffer (see above) at pH 6.4, continuously bubbled with N_2 for 15 min. Reoxygenation was induced by switching to oxygenated, glucose-containing control superfusion, at pH 7.4.

Rigor development, hypercontracture and cell death during ischaemia-reperfusion

To analyse the effect of IR injury on cell morphology, the rate of ischaemic rigor shortening development (defined as 25–40% reduction of cell length with preserved squared-shape morphology) and of reperfusion-induced hypercontracture (defined as >70% reduction of cell length with concomitant disruption of cytoarchitecture) was quantified. Reperfusion-induced sarcolemmal disruption was analysed by two independent methods: a) visual quantification of 0.04% trypan blue positive cells (BX41 Olympus) and b) spectrophotometrical analysis of LDH release (photometer Multiskan FC, Thermo Scientific™) expressed respect to total LDH induced by massive osmotic shock.

Cytosolic and mitochondrial calcium changes in ischaemia-reperfusion

The effect of aging on cytosolic calcium handling during ischaemia and reperfusion was investigated in isolated cardiomyocytes incubated with 5 µmol/l of the acetoxymethyl ester of the calcium-sensitive fluorochrome fluo-4 (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C, washed and subsequently submitted to transient IR on the stage of the microscope. During IR, cells were excited at 488 nm with an Ar/Kr laser confocal system (Yokogawa CSU10, Nipkow spinning disk) set on an Olympus IX70 (VoxCell Scan, Visitech, Tyne and Wear, UK) at 60X oil immersion objective lens, and changes in 505 nm emission were captured with a CCD digital camera (ORCA Hamamatsu, Japan) and monitored

throughout time in cytosolic regions of interests previously defined within the cells. Cell fluorescence was analysed in background-subtracted images using commercially available software (VoxCell Scan, Visitech, UK). To analyse the changes in mitochondrial calcium, a cold-warm protocol was used to reduce cytosolic compartmentation of the dye. Briefly, isolated cardiomyocytes were incubated with 5 µmol/l of the acetoxymethyl ester of the mitochondrial calcium-sensitive fluorochrome rhod-2 (Molecular Probes) for 60 min at 4°C (this step is required to inhibit AM cleavage from cytosolic unspecific esterases and to improve mitochondrial uptake and retention of the fluorochrome), washed and post-incubated for additional 30 min at 37°C. Loaded cells were submitted to transient 15 min ischaemia-10 min reperfusion while being excited at 560 nm. Changes in 580 nm emission were monitored throughout time in mitochondrial regions previously defined within the cells. In all experiments, perfusion was initiated with 2 min normoxic buffer to obtain baseline fluorescence. Changes in cytosolic and mitochondrial calcium were expressed as arbitrary units of fluorescence (a.u.) respect to the initial normoxic value.

Mitochondrial membrane potential (Δψm) during ischaemia-reperfusion

For the analysis of Δψm, isolated cardiomyocytes from young and old mouse hearts were incubated with 10 µmol/l of the membrane-permeant form of JC-1 (Molecular Probes) for 8 min at 37°C, washed and subsequently submitted to transient IR on the stage of the microscope. During IR period, cells were excited at 488 nm using an Ar/Kr laser confocal system, and simultaneous changes in 525 nm and 590 nm emission wavelengths were monitored throughout time. Capture speed was set at 1 image/30 seconds (s) to avoid phototoxicity. The fluorescence ratio obtained from both emissions was normalised respect to the baseline normoxic value.

Mitochondrial permeability transition pore and ROS production during reperfusion.

To investigate the effect of age on the occurrence of mitochondrial permeability transition pore (mPTP) opening and ROS production during IR, isolated cardiomyocytes were simultaneously incubated with 5 µmol/l of the acetoxymethyl ester of calcein (Molecular Probes), 15 min at 37°C, and MitoSoxTM (Molecular Probes), 10 min at 37°C, washed and post-incubated with control buffer supplemented with 1 mmol/l $CoCl_2$ for additional 10 min, to quench cytosolic fluorescence (18). Cells were subsequently submitted to IR in a microchamber placed on the stage of the microscope and were alternatively excited at 488 nm (calcein) and 561 nm (MitoSox) with the Ar/Kr laser confocal system. In all perfusion buffers, 0.5 mmol/l CoCl2 was present to prevent cytosolic fluorescence contribution to mitochondrial fluorescence quantification. Decline in calcein fluorescence was indicative of mPTP opening, whereas increase in MitoSox fluorescence corresponded to mitochondrial ROS production.

Isolation of subsarcolemmal and interfibrillar heart mitochondria

To obtain subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria, fresh cardiac ventricles were minced and homogenized in iced cold buffer A (in mmol/l: 290 sucrose, 5 MOPS at pH 7.4, 2 EGTA and 0.2% deffated albumin) using a Potter-Elvehjem device. Nuclei and other cell debris were pelleted in an initial centrifugation step at 750 g (5 min, 4°C). The supernatant was centrifuged at 5,000 g (5 min, 4°C) and SSM were precipitated in the resulting pellet. IFM were obtained by treating the initial 750 g pellet with 1 ml isolation buffer B (in mmol/l: 100 KCl, 5 MOPS at pH 7.4, 2 EGTA and 0.2% deffated albumin) and 3 mg/ml proteinase K for 1min. Interfibrillar homogenate was centrifuged at 750 g (5 min, 4°C). After discarding the pellet, supernatant was centrifuged at 5,000 g (5 min, 4°C) to precipitate the IFM. Mitochondrial protein concentration and citrate synthase activity were measured by colourimetric assays.

Mitochondrial calcium retention capacity

To investigate the effect of aging on mitochondrial calcium tolerance, a concentration of 0.2–0.4 mg/ml of SSM or IFM from young and old mouse hearts was suspended in the assay buffer (in mmol/l: 200 sucrose, 6 MOPS, 5 KH_2PO_4, 1 $MgCl_2$, 10 succinate, 0.001 rotenone) and plated in a microplate fluorometer (Max GeminiXS, Molecular Devices) in the presence of 2 µmol/l of the hexapotassium salt impermeant calcium sensitive dye calcium-Green™ 5N. Consecutive pulses of 4 µmol/l calcium were added to the mitochondrial suspension, and mitochondrial calcium uptake was detected as decay of fluorescence emission at 532 nm (Ex: 506 nm), recorded every 15 s during 2 min for each calcium pulse. Calcium was added repeatedly until total mitochondrial calcium retention capacity was exceeded, a phenomenon that could be detected by the increase in buffer fluorescence reflecting the occurrence of mPTP. In parallel experiments, mitochondrial calcium retention capacity was assessed in the presence of either 1 µmol/l cyclosporin (CsA, to inhibit mPTP) or 10 µmol/l Ru360 (to inhibit mitochondrial calcium uniporter).

Differential quantitative mitochondrial proteomics

Peptides of mitochondrial FoF1ATP synthase complex were identified and quantified by differential high-throughput proteomic analysis performed by stable isotopic labelling in SSM and IFM isolated from young and old mouse hearts, using a previously described protocol (19). Changes in the abundance and composition of cysteine-thiol redox status of FoF1 ATP synthase peptides were quantified using de GELSILOX methodology (20). Analyses of samples by LC-MS/MS were performed as previously described (19). Functional protein classification was done using the Gene Ontology database. Proteomics results were analysed as previously described (21).

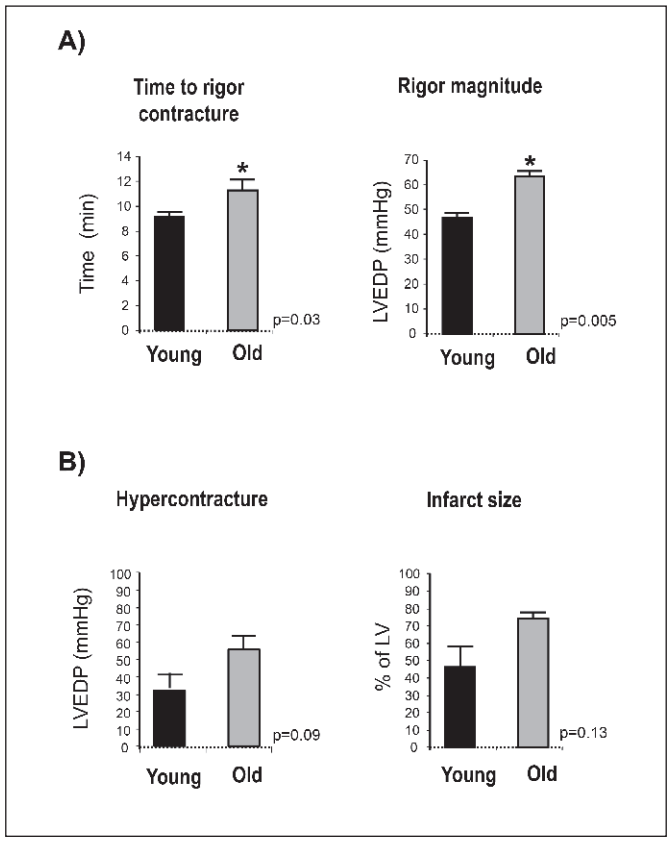

Figure 1: Ischaemia and reperfusion in isolated perfused hearts. A) Time to the onset of ischaemic rigor contracture and magnitude of rigor contracture, as manifested by peak left ventricular end-diastolic pressure (LVEDP) during ischaemia in Langendorff-perfused hearts from young and old mice. B) Reperfusion-induced hypercontracture, as manifested by peak LVEDP during reperfusion, and infarct size, expressed as % of the left ventricle, in Langendorff-perfused hearts from young and old mice. Data correspond to mean ± SEM of 3–5 hearts per group.

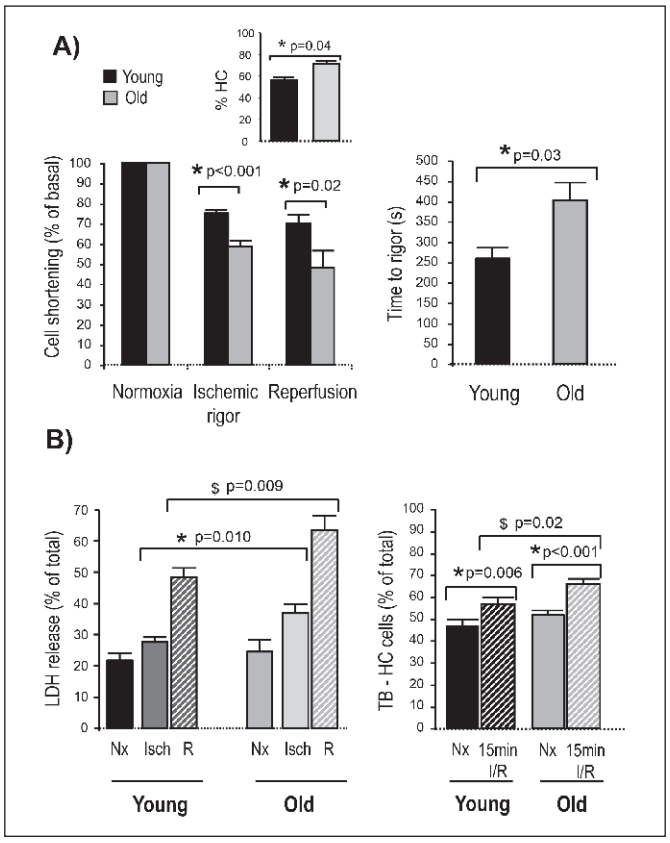

Figure 2: Effect of aging on ischaemia-reperfusion injury in isolated cardiomyocytes. A) Left panel: Morphological changes associated with ischaemia and reperfusion. The inset shows the percentage of hypercontracted cells during reperfusion respect to total cell number. Right panel: time to rigor contracture in young and old cardiomyocytes. Mean ± SEM of n>10 cells per group. B) Cell death during ischaemia and reperfusion, determined by LDH release respect to total cell LDH content (left panel), and percentage of hypercontracted cells during reperfusion that develop membrane disruption, as determined by trypan blue (TB) positive staining (right panel) in young and old cardiomyocytes. Mean ± SEM of 3 replicates per group. Isch: ischaemia; R: reperfusion; I/R: ischaemia and reperfusion.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). For comparisons between two groups with normal distributions, two-tailed Student's t-test for independent or paired samples was used. For comparisons between more than two groups with normal distribution, one-way ANOVA and planned contrasts was used. Differences in temporal evolution were assessed by repeated-measures factorial ANOVA. When samples did not follow a normal distribution, the non-parametric Mann-Whitney U or Kruskal-Wallis tests were used for each design as needed. Differences of $p<0.05$ were considered statistically significant. In experiments involving isolated cardiomyocytes, cells were derived from $n\geq 3$ independent isolation procedures. All statistical analyses were performed with SPSS v.15 software.

Results

Ischaemia and reperfusion injury in aging hearts

In isolated Langendorff-perfused mouse hearts, there were no differences in LV function between groups at baseline. Previous morphometric analysis of the hearts of these mice by echocardiography showed no age-related hypertrophy, but a trend towards increased LV end-diastolic volume in old mice (27). Ischaemia resulted in a complete cessation of contraction and a progressive increase in LVEDP reflecting development of ischaemic rigor. Hearts from old mice showed a significant delay in the time to ischaemic rigor (►Figure 1A, left panel) and an increase in its magnitude (►Figure 1A, right panel). During reperfusion, there was a trend towards age-related increased hypercontracture, defined as maximal rise in LVEDP, and infarct size (►Figure 1B).

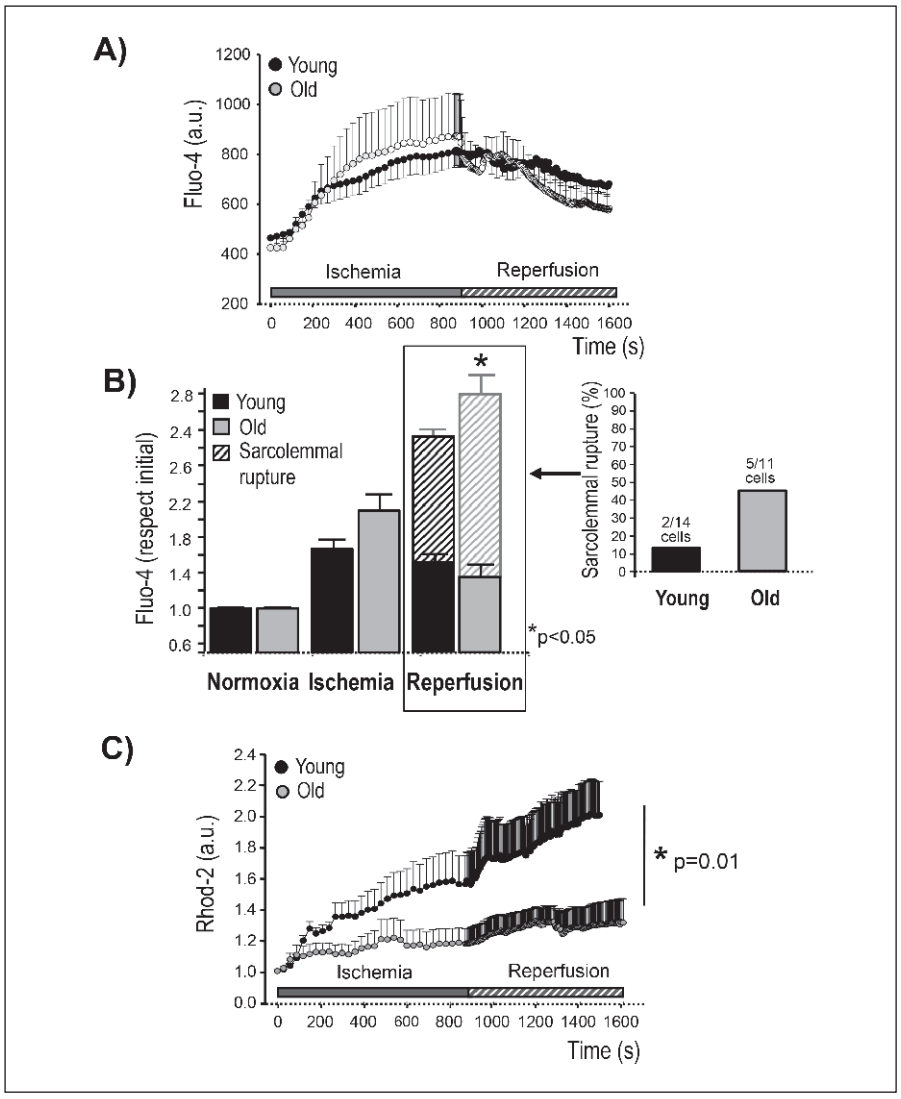

Figure 3: Cytosolic and mitochondrial calcium handling during ischaemia-reperfusion in isolated cardiomyocytes. A) Cytosolic calcium kinetics throughout ischaemia and reperfusion in cardiomyocytes from young and old mouse hearts. Data are expressed as mean ± SEM of n= 6–12 cells per group. a.u.: arbitrary units. B) Final degree of cytosolic calcium overload, respect to the initial normoxic value, obtained the end of ischaemic period and after 10 min of reperfusion. Faded bars correspond to cytosolic calcium level immediately before sarcolemmal rupture. Inset shows the percentage of cells experiencing reperfusion-induced sarcolemmal rupture in this series. Data are expressed as mean ± SEM of n=11–14 cells per group. C) Mitochondrial calcium kinetics throughout ischaemia and reperfusion in cardiomyocytes from young and old mouse hearts. Data are expressed as mean ± SEM of n= 6–11 cells per group. a.u.: arbitrary units.

Ischaemia and reperfusion injury in isolated aging cardiomyocytes

After 15 min of ischaemia, rigor cell shortening was more pronounced with aging. While young cardiomyocytes shortened around 25% respect to their initial length, cardiomyocytes from aging hearts shortened more than 40% (►Figure 2A, left panel). Paradoxically, the time at which rigor contracture developed –an accepted marker of severe ATP depletion- was significantly delayed with aging (►Figure 2A, right panel). During reperfusion, cardiomyocytes experienced an additional cell shortening coincident with energy restoration, usually accompanied by massive morphological distortion (hypercontracture). The mean length of reperfused cardiomyocytes was significantly shorter in the aging group (►Figure 2A, left panel).

Reperfusion-induced sarcolemmal disruption

During the first minutes of reperfusion, a proportion of isolated cardiomyocytes developed sarcolemmal rupture and death, quantified by the release of LDH to the extracellular medium. Reperfusion-induced cell death was significantly increased in cardiomyocytes from aging hearts (►Figure 2B, left panel). The rate of sarcolemmal rupture associated to hypercontracture, as detected by trypan blue positive staining, was significantly increased in cardiomyocytes from aging hearts (►Figure 2B, right panel).

Cytosolic and mitochondrial calcium handling is impaired in aging cardiomyocytes during ischaemia-reperfusion

Fluo-4 loaded isolated cardiomyocytes submitted to transient ischaemia experienced an increase in cytosolic calcium concentration (►Figure 3A). During the first minutes of reperfusion, cytosolic calcium overload was partially corrected in surviving cells

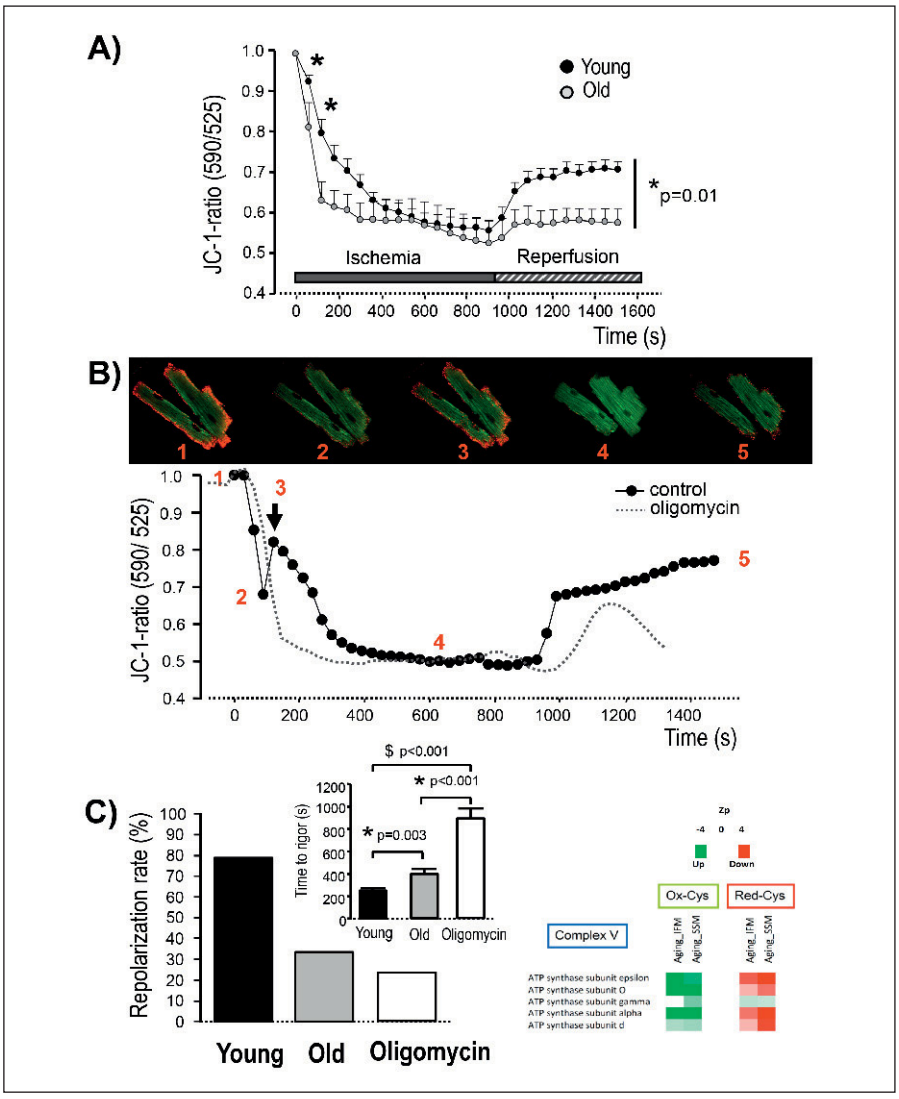

Figure 4: Δψm, FoF1 ATP synthase reversal and cysteine oxidation. A) Changes in Δψm during ischaemia and reperfusion respect to the initial normoxic value, quantified by JC-1 ratiofluorescence, in isolated cardiomyocytes from young and old mouse hearts. Mean ± SEM of n=6–8 cells per group. B) Confocal imaging sequence of a JC-1 loaded pair of young cardiomyocytes and the corresponding fluorescence monitorisation throughout ischaemia and reperfusion, to illustrate the initial decline of Δψm during the first minutes of ischaemia (images 1 and 2), the transient repolarisation wave (image 3, arrow), the end-ischaemic degree of mitochondrial depolarisation (image 4) and the partial recovery of Δψm during the first minutes of reperfusion (image 5). Grey dotted line corresponds to Δψm in an oligomycin-treated cell, in which transient Δψm repolarisation during ischaemia is prevented. C) Percentage of cells experiencing a transient repolarisation wave during ischaemia among cardiomyocytes from young and old mouse hearts, and cardiomyocytes from young mouse hearts treated with 10 µmol/l of oligomycin to inhibit mitochondrial ATPase. The inset shows the time at which rigor contracture developed in the same groups of cells. D) Quantitative redox proteomics using GELSILOX of the cysteine containing peptides detected in mitochondrial FoF1 ATP synthase (complex V) in subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) from aging hearts. Increase in the peptide-containing oxidised cysteine is shown in green and correlates with decrease in the peptide-containing reduced cysteine (in red) in different subunits of FoF1 ATP synthase.

from both groups of ages. The number of cells presenting sarcolemmal rupture at reperfusion was higher in the aging group (►Figure 3B) and cytosolic calcium immediately before reperfusion-induced membrane rupture was significantly higher in aging cells (►Figure 3B). The kinetics of mitochondrial calcium uptake in rhod-2 loaded cardiomyocytes subjected to IR disclosed an impaired ability of mitochondria from old hearts to uptake and accumulate calcium both during ischaemia and reperfusion (►Figure 3C). The fact that mitochondrial calcium is reduced in the presence of higher cytosolic calcium concentration in aging cells denotes that there is no cross-interference between the two markers.

Aging is associated with altered Δψm during ischaemia and reperfusion

During ischaemia, isolated cardiomyocytes developed a progressive decline of Δψm, quantified by JC-1 ratiofluorescence. Mitochondrial membrane depolarisation was accelerated in old cardiomyocytes (►Figure 4A). Upon reperfusion, there was a rapid although incomplete recovery of Δψm in young cardiomyocytes, which was significantly depressed in the aged cells (►Figure 4A). Immediately before maximal ischaemic depolarisation, a rapid and transient recovery of Δψm took place in 80% of the control young cells (►Figure 4B). This repolarisation wave corresponded to the reversal of FoF1 ATP synthase because it could be prevented by the addition of 10 µmol/l of the specific ATP synthase inhibitor oligomycin (►Figure 4C). By contrast, and despite faster mitochondrial depolarisation, only 30% of aging cardiomyocytes experienced a reversal of ATP synthase (►Figure 4C). The delay in rigor contracture development observed in aging cells could be reproduced with oligomycin, suggesting that it is secondary to the inability of the ATP synthase to revert to its ATPase mode. Second generation proteomics combined with GELSILOX analysis demonstrated an increase in the number of oxidised cysteine residues in the mitochondrial ATP synthase of the aging hearts (►Figure 4D), without changes in its expression levels (data not shown).

Effect of age on mPTP and ROS production during reperfusion

Reperfusion triggered mitochondrial calcein release, reflecting the occurrence of mPTP opening, in cardiomyocytes from both groups of ages (►Figure 5A). The extent of mitochondrial permeabilisation was greater in aged cardiomyocytes (►Figure 5A). Concomitantly, there was a narrow and high peak of mitochondrial ROS production, detected by MitoSox fluorescence, during the first minutes of reperfusion. This peak of ROS appeared to be associated with reactivation of mitochondrial respiratory activity, as it overlapped with restoration of Δψm and was significantly attenuated in aged cells (►Figure 5B). The proportion of mitochondrial calcein released in relation to maximal ROS production during the first minutes of reperfusion, an index of the mPTP sensitivity to ROS, was significantly increased in cardiomyocytes from aged hearts (►Figure 5C). These data indicate a reduced threshold of mitochondria from aged cells to undergo membrane permeabilisation.

Aging reduces mitochondrial tolerance to calcium overload

Isolated mitochondria exposed *in vitro* to consecutive external calcium pulses eventually developed CsA-sensitive mPTP when the threshold of their calcium retention capacity was exceeded, as determined by extramitochondrial calcium by CG5N fluorescence (►Figure 6A). Mitochondrial calcium uptake was prevented by Ru-360, indicating that it occurred through the mitochondrial calcium uniporter (►Figure 6A). Subsarcolemmal mitochondria (SSM) showed a less calcium retention capacity than interfibrillar mitochondria (IFM) regardless of age (►Figure 6B). However, aging importantly impaired calcium tolerance in IFM population (►Figure 6B).

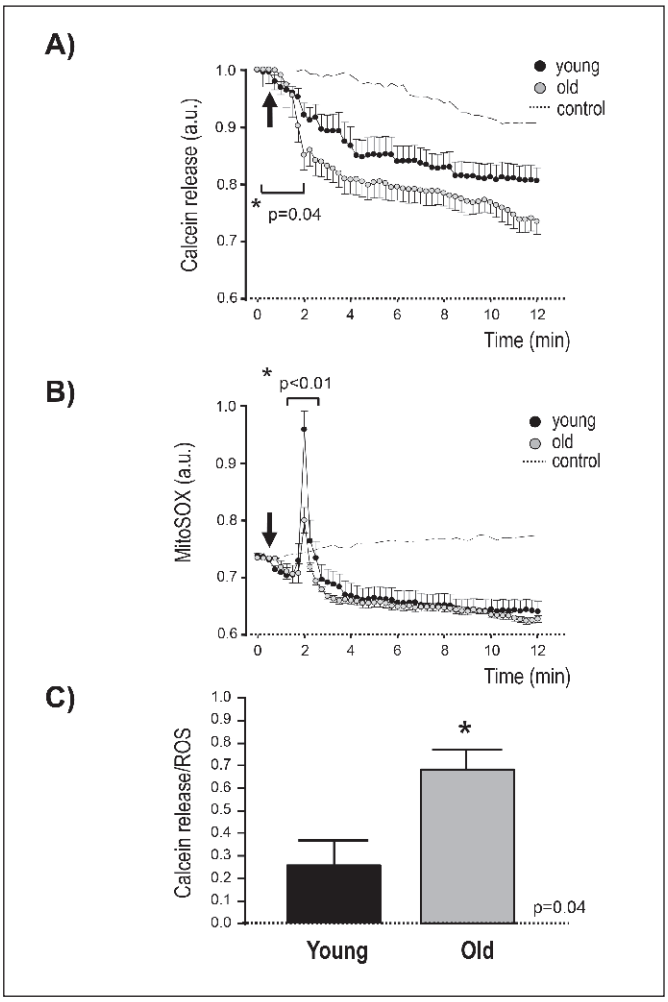

Figure 5: mPTP opening and mitochondrial ROS production during reperfusion. A) Mitochondrial calcein fluorescence decay, reflecting mPTP opening, during reperfusion in isolated cardiomyocytes from young and old mouse hearts. Arrow points the onset of reperfusion. Control cells were not submitted to previous ischaemia. B) Reperfusion-induced mitochondrial ROS production in isolated cardiomyocytes from both groups of age. Arrow points the onset of reperfusion. Control cells were not submitted to previous ischaemia. C) Ratio between mitochondrial calcein release and mitochondrial ROS production in the first minutes of reperfusion in isolated cardiomyocytes from both groups of age, as an index of the susceptibility of mitochondria to undergo permeabilisation in response to ROS. Data correspond to mean ± SEM of n=7–12 cells per group.

Discussion

The present study provides evidence that altered FoF1 ATP synthase function contributes to the reduced tolerance to ischaemia in aging hearts. Altered FoF1 ATP synthase activity is associated with oxidative damage of this protein complex and causes more rapid dissipation of Δψm, reduced mitochondrial calcium uptake and delayed ATP exhaustion marked by the delayed occurrence of ischaemic rigor contracture. Mitochondria from old cardiomyocytes are more prone to undergo mPTP during reperfusion or during external calcium exposure *in vitro*. Because recent studies suggest that dimmers of ATP synthase form the mPTP, it is tempting to speculate that impaired ATP synthase activity observed in aging hearts contributes to both altered energy metabolism and increased susceptibility to mPTP.

Ischaemia and reperfusion in the aging heart: energy exhaustion

During ischaemia, cytosolic ATP levels progressively decline because ATP expenditure is not adequately compensated by its regeneration through mitochondrial oxidative phosphorylation. Arrest of oxidative phosphorylation results in mitochondrial depolarisation by a dual mechanism: 1) interruption of H^+ extrusion to the intermembrane space, and 2) dissipation of Δψm mainly through proton-driven ATP synthase, although other uncoupling

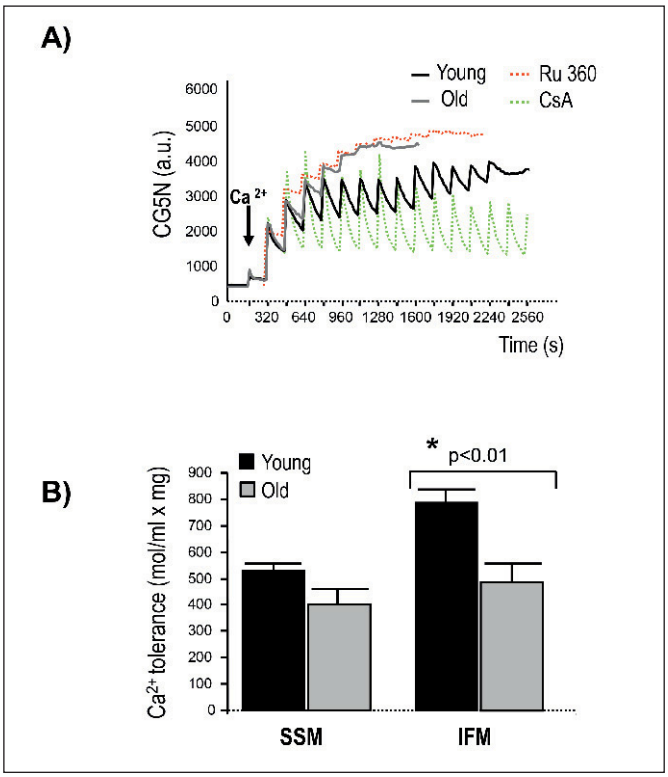

Figure 6: Effect of aging on calcium retention capacity in cardiac mitochondria. A) Calcium uptake and accumulation in mitochondria isolated from young and old mouse hearts and submitted to consecutive external calcium pulses of 4 µmol/l, quantified by changes in extramitochondrial CG5N fluorescence. In a subset of experiments either 10 µmol/l Ru360 (inhibitor of the mitochondrial calcium uniporter) or 1 µmol/l CsA (inhibitor of mPTP) was added during calcium exposure. B) Quantification of total calcium tolerance in subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) from both groups of age before membrane permeabilisation takes place. Data correspond to mean ± SEM of five replicates per group.

proteins may play a role (22). Importantly, when Δψm declines beyond certain level, ATP synthase reverses to proton-pumping ATPase, in a futile attempt to maintain mitochondrial intermembrane H^+ gradient (23). Reversal of ATP synthase during ischaemia converts mitochondria into major cellular ATP consumers (23) and accelerates ATP fall to the critical threshold of 50–100 µmol/l, in which rigor-type calcium-independent actin-myosin interaction develops (24). Up to 50 % of cellular ATP is consumed by reverse mode of the mitochondrial F1-ATPase (25). Rigor-type contracture, manifested as an abrupt cell shortening in unrestrained isolated cardiomyocytes and increased resting tension in intact myocardium, is an ATP-consuming response by itself, and may propagate from cell-to-cell through gap junctions (26).

Aging cardiomyocytes have preserved mitochondrial membrane potential and respiratory activity under resting conditions (27). However, during oxygen deprivation they develop a paradoxical response, consisting of an acceleration of mitochondrial membrane depolarisation and a concomitant delay in the development of rigor contracture. This response was consistently observed in isolated cardiomyocytes and intact hearts submitted to global ischaemia, and could be explained by failure of FoF1 ATP synthase to revert to the ATP consuming mode that precipitates rigor shortening during ischaemia. The observation that oligomycin –an FoF1 ATPase inhibitor- when present during ischaemia prevents the occurrence of the transient repolarisation wave in JC-1 loaded cardiomyocytes and delays rigor development, mimicking the effect of aging, supports that this is indeed the case. The failure of ATP synthase to revert its activity may also explain the increased rate of mitochondrial depolarisation in aging cardiomyocytes. Although the exact mechanism of this failure cannot be established with the present data, quantitative proteomics revealed increased cysteine oxidation at different subunits of ATP synthase complex in aging hearts, including subunit rotation γ and oligomycin-senstive subunit Fo, without changes in their abundance. Interestingly, the extent of cysteine cross-linking as a result of disulfide bond formation and S-glutathionylation has been shown to have important functional consequences, and to be negatively correlated with the hydrolytic activity of ATP synthase (28).

Mitochondrial calcium handling during ischaemia-reperfusion

The acceleration of mitochondrial membrane depolarisation observed in aging cardiomyocytes during ischaemia may have important consequences on cellular calcium handling. Mitochondria exhibit a striking ability to accumulate enormous amounts of calcium (29), crucial for buffering cytosolic calcium in conditions in which sarcoplasmic reticulum (SR) calcium uptake through SERCA ATPase is not thermodynamically favoured, like during ischaemia (30). Moreover, the proxidative status present during ischaemia and reperfusion has been described to promote calcium leak from sarcoplasmic reticulum by ryanodine receptors (31, 32), increasing even more the concentration of calcium at the SR-mitochondria microenvironment. We have previously demonstrated that aged cardiomyocytes have abnormal spontaneous spark behaviour, consistent with altered RyR gating properties, although resting cytosolic calcium, sarcoplasmic reticulum content or mitochondrial calcium unirporter activity is similar to those observed in young cardiomyocytes (27). On the other hand, there is solid evidence indicating that calcium can be significantly overloaded in restricted subcellular areas before significant changes can be detected in the cytosol (33) and that mitochondrial calcium uptake is indeed more dependent on the calcium levels reached within these microdomains than on bulk cytosolic calcium concentration (33–35). Calcium uptake and accumulation by mitochondria requires Δψm, a condition that is fully met during the initial phase of ischaemia, prior to maximal Δψm dissipation (17, 36). Importantly, the driving Δψm force for mitochondrial calcium uptake is not depressed in aging cells under normoxic conditions (27). Mitochondrial calcium uptake has been demonstrated to actively participate in cytosolic calcium control both under normoxia and ischaemia (17, 37), alleviating calcium overload and improving cell survival upon reperfusion (17). This may explain why pharmacological o genetic inhibition of mitochondrial calcium uptake confers no protection against mPTP opening or necrosis in reper-

fusion (17, 38). It is therefore plausible that depressed mitochondrial calcium uptake secondary to accelerated mitochondrial depolarisation during ischaemia, and impaired Δψm recovery during reperfusion, significantly aggravates cytosolic calcium overload and hypercontracture in aging cells. Defective communication between SR and mitochondria during aging may further contribute to this effect by further impairing mitochondrial calcium uptake (27).

Previous observations indicated an age-related increase in diastolic calcium during ischaemia and early reperfusion that may account for the increased sensitivity to injury (39, 40). The mechanisms responsible for impaired calcium handling in aging myocardium are far from being elucidated. It has been proposed that a decline in SR proteins that participate in calcium removal plays a causative role (41), although other studies found no changes in the decay of calcium transient in aging, suggesting normal SERCA activity (39). Some studies describe changes in relaxation times compatible with reduced SERCA participation and compensatory increase in sarcolemmal Na^+/Ca^{2+} exchanger activity (42), while others advocate post-translational modifications, like increased oxidation of SERCA and phospholamban (43) or changes in the phosphorylation status of SR cycling proteins secondary to reduced CaM kinase II expression (44). To our knowledge, this is the first evidence indicating that depressed mitochondrial calcium uptake during ischaemia and reperfusion can be causally involved in the aggravation of cytosolic calcium overload of senescent cardiomyocytes.

Mitochondrial ATP synthase oxidation and mPTP opening

Our results demonstrate an enhanced susceptibility to mPTP opening in mitochondria from aging hearts. This observation was obtained both in intact cardiomyocytes submitted to ischaemia/reperfusion and in isolated mitochondria exposed *in vitro* to calcium overload. The results in intact cardiomyocytes indicate that mPTP opening occurs more easily during reperfusion in aging cells but do not rule out the possibility that this depends on more intense triggering conditions. However, the fact that isolated mitochondria, in which the contribution of cytosolic environment and SR interaction is excluded, exhibit less calcium tolerance suggests that sensitivity to mPTP opening is intrinsically increased with aging. The increased susceptibility to mPTP opening associated with aging is specifically manifested in interfibrillar mitochondria, in full agreement with previous observations (45). Other studies have demonstrated an enhanced susceptibility of senescent mitochondria to undergo mPTP and proposed several mechanisms, including excess of membrane cardiolipin oxidation (46), increased mitochondrial calcium content (47) or impairment of cytosolic cardioprotective signalling pathways, mainly Akt/GSK-3 beta (48), or several posttranslational modifications such as acetylation (49). Our experiments indicate that the increased rate of mPTP opening in aging may be independent of cytosolic signalling pathways, as it occurs in normoxic isolated mitochondria, but also that it takes place in cells with lower mitochondrial calcium uptake, and is not associated with increased ROS production during the initial phase of reperfusion. All these observations suggest an intrinsic alteration of mPTP in older hearts.

The long-standing concept that mPTP forms at the contact sites of voltage-dependent anion channel (VDAC) and adenine nucleotide translocator (ANT) has been challenged by evidences indicating that both molecules are dispensable for pore formation in genetically modified mitochondria (50, 51). Recent studies propose dimerisation of ATP synthase as the molecular entity conforming pore structure (52). In our experiments, mitochondria from aging hearts exhibited an increased cysteine oxidation in several subunits of ATP synthase, and this structural modification was correlated with a functional failure of the molecule during ischaemia, resulting in increased rate of H^+ dissipation and higher rates of mPTP opening, despite lower driving force for mitochondrial calcium uptake. These findings further are consistent with the view that ATP synthase may directly participate in mPTP opening, and suggest a new link between bioenergetic failure, calcium handling and membrane permeabilisation in mitochondria from aging hearts.

Recent proteomics surveys have revealed that lysine acetylation is a widespread cellular modification that is particularly abundant in mitochondrial enzymes and proteins, with known implications in aging and longevity. MS-based proteomics approaches led to the identification of thousands of novel acetylation sites (53, 54). The reversible and nutrient-sensitive nature of these acetyl modifications has strongly implicated mitochondrial NAD+-dependent deacetylases (sirtuins) in energy homeostasis. Thus, mice lacking the predominant mitochondrial deacetylase SIRT3 exhibit depleted cardiac ATP levels (55). Since mitochondria and metabolism are sensitive targets for damage during IR injury, the role of SIRTs in cardioprotection is of particular interest. Recently, it has been demonstrated that decreased SIRT3 may enhance the susceptibility of cardiac-derived cells and adult hearts to IR injury and may contribute to IR injury in the aged heart (56). The downstream cardioprotective mechanism of SIRT3 remains unclear but it has been related to the acetylation of CypD lysine 166, a residue that lies adjacent to the ciclosporin-A binding pocket of this protein, suggesting that its acetylation might regulate the mPTP (49). SIRT3 may also enhance antioxidant defense via deacetylation and activation of MnSOD (57) and regulate OxPhos by deacetylating specific OxPhos subunits (55). Overall, acetylation of cardiac mitochondria could be associated with an inability of the heart to respond adequately to cardiac stress. Our proteomics analysis found a negligible number of acetylated peptides in mitochondria from aging hearts (0.26% of the total number of identified peptides vs 3% of oxidized Cys-containing peptides), in agreement with previous reports (58).

Conclusions and implications

Our data demonstrate that aged cardiomyocytes exhibit more pronounced bioenergetic failure, more severely altered cytosolic and mitochondrial calcium, and reduced tolerance to the stress im-

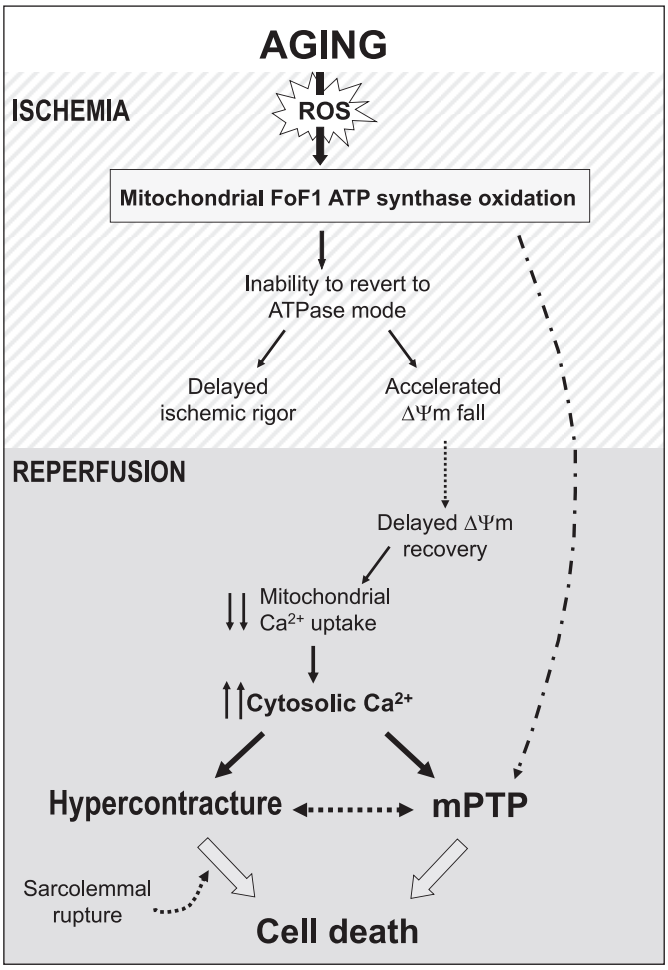

Figure 7: Diagram with a proposed pathophysiological mechanism.

posed by IR, by mechanisms that converge into higher mPTP opening and cell death. These findings could be related to the observed increased oxidation of some critical subunits of the FoF1 ATP synthase molecule. ►Figure 7 summarises the proposed pathophysiological mechanism. Importantly, advanced age is associated with partial loss in the efficiency of some cardioprotective interventions, like ischaemic pre- and postconditioning or drug-induced cardioprotection. It has been proposed that abrogation of cardioprotection may be related with deficient activity of some signal transduction pathways, like depressed mitochondrial Cx43 or PKC translocation (59, 60) or reduced expression/phosphorylation of STAT3 in aged myocardium (61), among other potential mechanisms. Characterisation of these mechanisms should help to identify pharmacologic targets in the elderly and to refine therapeutic strategies specifically addressed to this group of patients.

Conflicts of interest
None declared.

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