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HAL Id: inserm-00388555

https://inserm.hal.science/inserm-00388555

Submitted on 30 May 2020

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Summary

Aging triggers several abnormalities in muscle glycolytic fibers including increased proteolysis, reactive oxygen species (ROS) production and apoptosis. Since the mitochondria are the main site of substrate oxidation, ROS production and programmed cell death, we tried to know whether the cellular disorders encountered in sarcopenia are due to abnormal mitochondrial functioning. Gastrocnemius mitochondria were extracted from adult (6 months) and aged (21 months) male Wistar rats. Respiration parameters, opening of the permeability transition pore and ROS production, with either glutamate (amino acid metabolism) or pyruvate (glucose metabolism) as a respiration substrate, were evaluated at different matrix calcium concentrations. Pyruvate dehydrogenase and respiratory complex activities as well as their contents measured by Western blotting analysis were determined. Furthermore, the fatty acid profile of mitochondrial phospholipids was also measured. At physiological calcium concentration, state III respiration rate was lowered by aging in pyruvate conditions (−22%), but not with glutamate. The reduction of pyruvate oxidation resulted from a calcium-dependent inactivation of the pyruvate dehydrogenase system and could provide for the well-known proteolysis encountered during sarcopenia. Matrix calcium loading and aging increased ROS production. They also reduced the oxidative phosphorylation. This was associated with lower calcium retention capacities, suggesting that sarcopenic fibers are more prone to programmed cell death. Aging was also associated with a reduced mitochondrial superoxide dismutase activity, which does not intervene in toxic ROS overproduction but could explain the lower calcium retention capacities. Despite a lower content, cytochrome c oxidase displayed an increased activity associated with an increased n–6/n–3 polyunsaturated fatty acid ratio of mitochondrial phospholipids. In conclusion, we propose that mitochondria obtained from aged muscle fibers display several functional abnormalities explaining the increased proteolysis, ROS overproduction and vulnerability to apoptosis exhibited by sarcopenic muscle. These changes appear to be related to modifications of the fatty acid profile of mitochondrial lipids.
Key words: aging; calcium; fatty acid profile of mitochondrial lipids; mitochondria; muscle atrophy; permeability transition pore; reactive oxygen species.

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Introduction

Between 20 and 80 years old, individuals lose approximately 40% of the skeletal muscle mass of their legs (Wanagat *et al.*, 2001). This phenomenon, called sarcopenia, makes elderly people more fragile and alters their capacities to fight efficiently against severe diseases. Several biochemical processes are involved in aging-associated muscular atrophy. Sarcopenia mainly affects glycolytic fibers (Thompson, 1994), causing atrophy (Reynolds *et al.*, 2002) via increased proteolytic activity (Yarasheski, 2003; Zinna & Yarasheski, 2003). It also makes them overproduce reactive oxygen species (ROS) (Ji, 2002; Capel *et al.*, 2004; Fulle *et al.*, 2004). Finally, it destroys them through apoptosis (Pollack & Leeuwenburgh, 2001; Pollack *et al.*, 2002). All these processes could result from abnormal mitochondrial functioning, since the mitochondria are the main site of substrate oxidation, ROS production and the initiation of programmed cell death.

Aging is often associated with a lack of physical activity, a phenomenon known to favor the so-called 'immobilization atrophy' characterized by loss of contractile force, proteolysis, autophagy (Appell, 1990), as well as apoptosis (Smith *et al.*, 2000; Jin *et al.*, 2004; Siu & Alway, 2004). Immobilization atrophy mainly concerns oxidative fibers, but it also affects glycolytic

fibers. It is associated with an increase in intracellular calcium and the calcium channel blocker nifedipine completely prevents it (Soares *et al.*, 1993). Aging-related muscle atrophy is also thought to display an increased cellular calcium concentration through which apoptosis occurs. First, aging is associated with cellular calcium accumulation in different organs including the brain (Khachaturian, 1989; Ying, 1996). Second, insulin resistance develops with aging and is characterized by calcium influx into the cell (Resnick, 1999). Third, ryanodine receptors are over-represented in aged muscles (Bastide *et al.*, 2000) and calcium uptake by sarcoplasmic reticulum is depressed (Carmeli *et al.*, 2002). Since both types of muscle atrophy are accompanied by a rise of cytosolic calcium, it is questionable why immobilization atrophy is associated with higher apoptosis in old rats as compared with their adult counterparts (Siu *et al.*, 2005).

An answer to this paradox could be a different aging-related sensitivity of viable mitochondria to the effect of numerous stimuli, including the rise in intracellular calcium. Changes in membrane lipid composition could intervene. Oxidative stress is particularly involved in aging (Harman, 1956; Pollack *et al.*, 2002) and the mitochondrial genome endures numerous ROS attacks inducing deletions/mutations (Eimon *et al.*, 1996; Pallotti *et al.*, 1996) that trigger the emergence of ragged red cells with a COX^-/SDH^+ phenotype (Lopez *et al.*, 2000). A high level of exposure to ROS during a long lifespan could modify some characteristics of mitochondrial function (enzyme activity, antioxidant properties, membrane lipid composition, etc.) and these organelles might react differently when facing specific stresses. All the abnormalities involved in sarcopenia (ROS overproduction, cell death and proteolysis) could be explained by calcium-induced changes in mitochondrial functioning. ROS overproduction has been observed after matrix calcium loading in isolated cardiac mitochondria (Grijalba *et al.*, 1999). Cell death can occur through increased mitochondrial calcium content and opening of the permeability transition pore (Green & Reed, 1998). Finally, proteolysis could result from a change in oxidative substrate selection, since calcium activates the pyruvate, iso-citrate and α-ketoglutarate dehydrogenases (Cox & Matlib, 1993) and probably modifies the expression of other mitochondrial enzymes.

The purpose of this study was to evaluate the aging-related changes in mitochondrial functioning to find out if they can partly explain the abnormalities observed in the sarcopenic gastrocnemius. The activities and expression of several enzymes including pyruvate dehydrogenase (PDH), respiratory chain complexes and super-oxide dismutase (SOD) were evaluated in the muscle of 6- and 21-month-old rats. *In vivo* mitochondrial calcium concentrations were determined. Since cytosol calcium loading probably occurs during aging, mitochondrial function was analyzed in the presence of increasing calcium concentrations. The measurements included the opening of the permeability transition pore, the selection of oxidative substrate by measuring the mitochondrial respiration parameters with either pyruvate or glutamate (involved in glucose or amino acid metabolism, respectively) and, finally, mitochondrial ROS production. Furthermore, the fatty acid profile of mitochondrial phospholipids was determined.

Results

General data

The animal body weights were not altered by aging (Table 1). Conversely, the gastrocnemius weight was significantly decreased in aged animals (–28% as compared to adult rats). The amount of extracted mitochondria depended on the available muscle mass, but the extraction yield was similar in adult and aged animals.

Effects of calcium and aging on glutamate respiration

The respiration parameters of adult and aged mitochondria were determined with glutamate in three media differing in their free-calcium concentration (Fig. 1). In mitochondria from adult rats, with a low free-calcium concentration, the state III respiration rate (panel A) and respiratory control index (RCI, panel C) were high, whereas the state IV respiration rate (panel B) was low. The effect of increasing the medium free-calcium concentration on the state III respiration rate had two phases. Amplifying the calcium concentration from 0.43 to 2.67 μM significantly increased state III oxygen consumption (19%, $P < 0.01$). However, when the free calcium was further increased (from 2.67 to 19.1 μM), the state III respiration rate was reduced (–50%, $P < 0.001$). The state IV respiration rate was raised (108%, $P < 0.001$) when the free-calcium concentration was increased from 0.43 to 2.67 μM and remained at a similar elevated value with the highest free-calcium concentration. The RCI dropped gradually with increasing free-calcium concentrations, reflecting the changes in both state III and state IV respiration rates. Finally, the efficiency of oxidative phosphorylation (ADP:O ratio) (panel D) was decreased when the free-calcium concentration

	Adult	Aged	ANOVA
Animal weight (g)	638 ± 14	639 ± 15	NS
Gastrocnemius weight (g)	3.51 ± 0.09	2.51 ± 0.12	$P < 0.001$
Gastrocnemius relative weight (%)	0.553 ± 0.018	0.392 ± 0.015	$P < 0.001$
Extracted mitochondria (mg proteins)	8.7 ± 0.4	6.6 ± 0.5	$P < 0.01$
Isolation yield (mg proteins per g muscle)	1.25 ± 0.05	1.30 ± 0.07	NS

Table 1 Influence of aging on characteristics of animal morphology and mitochondrial extraction

The number of experiments was 10 per group.
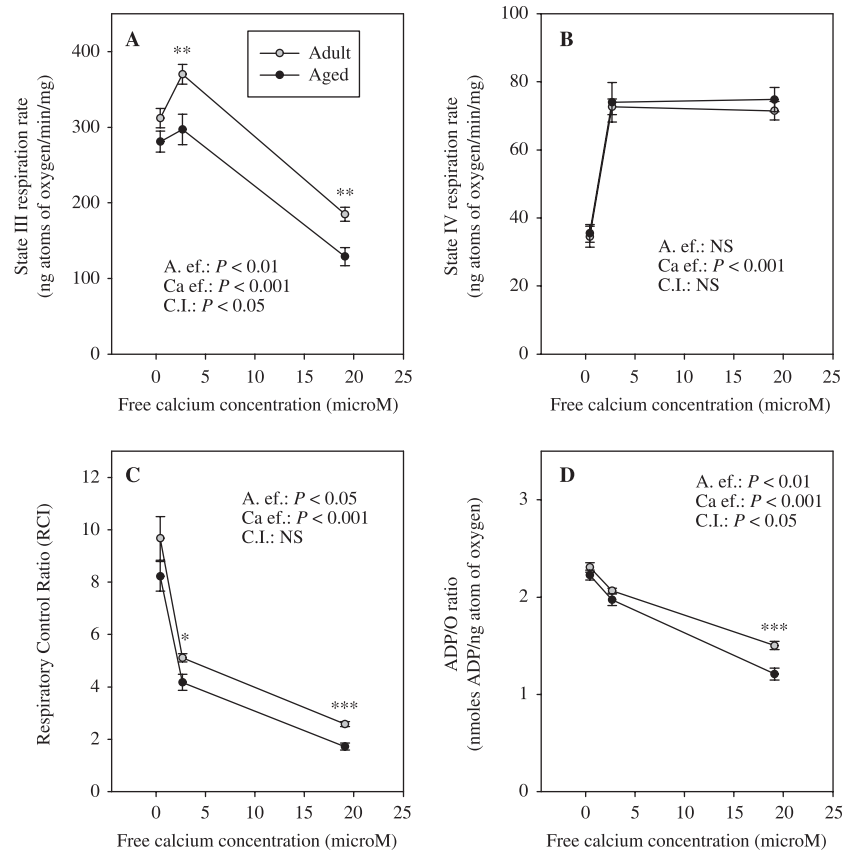NS, not significant.

Fig. 1 Influence of aging on respiration parameters [panel A: state III respiration rate; panel B: state IV respiration rate; panel C: respiratory control ratio (RCI); panel D: ADP:O ratio] of mitochondria from adult and aged rats with glutamate 5 mM + malate 1 mM as respiratory substrate. The number of experiments was 10 per group. A. ef., aging effect; Ca ef., free-calcium effect; CI, cross-interaction; NS, not significant. The stars indicate a significant difference between adult and aged animals at the same free-calcium concentration ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$).

was raised (−10 and −35% as compared to the value measured at the lowest free-calcium concentration, $P < 0.001$).

Aging had a pronounced effect on respiration parameters, but its influence depended on the free-calcium concentration. When free-calcium concentration was 0.43 μM, aging did not modify the parameters of oxidative phosphorylation. However, differences appeared with increased free-calcium concentrations. As evidenced by the significant cross-interaction, aging suppressed the calcium-induced stimulation of state III respiration rate observed when the free-calcium concentration was raised from 0.43 to 2.67 μM. Aging decreased this respiration parameter when the free-calcium concentration was equal to (−21%) and higher than (−30%) 2.67 μM. The state IV respiration rate was not altered by aging and the RCI was decreased (−18 and −19% at calcium 2.67 and 19.1 μM, respectively). The reduction of ADP:O ratio was more pronounced, as evidenced by the significant cross-interaction and the low value obtained at the highest free-calcium concentration (−19% as compared to the mitochondria extracted from adult animals).

Effects of calcium and aging on pyruvate respiration

Figure 2 shows the effects of calcium and aging on pyruvate oxidation. Increasing the free-calcium concentration regularly reduced the state III respiration rate (−14 and −36% as compared to calcium 0.48 μM, $P < 0.05$ and $P < 0.001$, respectively,

panel A). The ADP:O ratio (panel D) decreased by −25% ($P < 0.001$) when the free-calcium concentration was raised from 0.48 to 9.18 μM.

Aging also had interesting effects on pyruvate oxidation. At calcium 0.48 μM, it decreased the state III respiration rate (−22%) compared to the adult, whereas this was not the case with glutamate. Moreover, the RCI (panel C) was also reduced (−24%) at this calcium concentration. The glutamate respiration rate to pyruvate respiration rate ratio was higher in mitochondria from aged rats than in their adult counterparts (1.30 ± 0.06 vs. 1.11 ± 0.03, respectively, $P < 0.01$). The difference was maintained for higher calcium concentrations (data not shown). At calcium 9.18 μM, the rate of state III respiration for pyruvate was even slightly lower (−15%) than that measured for glutamate at calcium 19.1 μM, indicating that mitochondria were more sensitive to the deleterious effect of calcium when they oxidized pyruvate. In contrast with the glutamate conditions, aging had no influence on the ADP:O ratio.

Effect of aging on pyruvate dehydrogenase activity and content as well as *in vivo* mitochondrial calcium concentration

Figure 3A shows that aging reduced the active PDH activity (−30%) without modifying total activity. The last statement was confirmed by the lack of change in PDH content determined
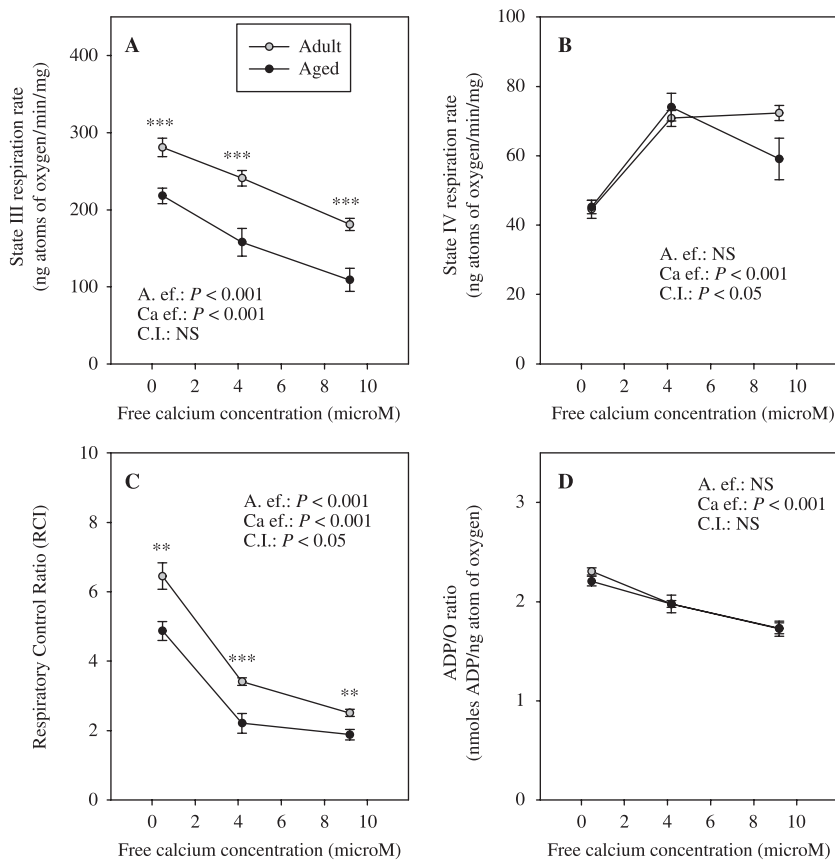
Fig. 2 Influence of aging on respiration parameters (panel A: state III respiration rate; panel B: state IV respiration rate; panel C: RCI; panel D: ADP:O ratio) of mitochondria from adult and aged rats with pyruvate 5 mm + malate 1 mm as respiratory substrate. The number of experiments was 10 per group. A. ef., aging effect; Ca ef., free-calcium effect; CI, cross-interaction; NS, not significant. The stars indicate a significant difference between adult and aged animals at the same free-calcium concentration (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

by Western blot (Fig. 3B,C). *In vivo* mitochondrial calcium content was evaluated after mitochondrial extraction under conditions such that influx of the divalent cation into and its efflux from the organelles were prevented. In the gastrocnemius, calcium content tended to decrease (−16%, $n = 6$ per group) with aging (1.98 ± 0.22 vs. 1.67 ± 0.31 nmol mg^{-1} in adult and aged mitochondria, respectively), but the difference was not significant. In the soleus, however, it tended to be increased (41%, $n = 6$ per group) in the aged animals (3.59 ± 1.05 nmol mg^{-1} as compared to 2.55 ± 0.41 vs. in the adult group, not significant).

Effect of free-calcium concentration and aging on ROS production, SOD activity and respiratory chain complex activities

ROS production was determined during glutamate oxidation (Fig. 4A). Increasing the free-calcium concentration from 2.67 to 19.1 μm elevated the ROS production (146%). Aging also promoted this production (59%). As evidenced by the non-significant cross-interaction, the effect of calcium was similar in mitochondria from adult and aged rats. However, when the ratio of ROS production at calcium 19.1 μm to ROS production at calcium 2.67 μm was calculated (Fig. 4B), a significant difference was observed between adult and aged rats. This ratio was higher in old animals (59%) as compared to adult ones, indicating that the effect of calcium was more pronounced in 21-month-old

rats. Figure 5 shows the activity of manganese-superoxide dismutase (Mn-SOD) of isolated mitochondria. Aging significantly reduced Mn-SOD activity (−33%).

The activities of NADH cytochrome c reductase (complexes I + III), succinate cytochrome c reductase (complexes II + III) and succinate dehydrogenase (complex II) as well as the content of complex I determined by immunoblotting were not changed by aging (data not shown). Conversely, the activity of cytochrome c oxidase (Fig. 6A) was increased (34%), whereas its content estimated by immunoblotting of the subunit IV (Fig. 6B,C) was significantly reduced with aging (−35%).

Effect of aging on mitochondrial capacities of calcium retention

When glutamate was used as a substrate (Fig. 7A), the calcium-retention abilities were approximately 120 μm and roughly similar in adult and aged animals. With pyruvate (Fig. 7B), these capacities were strongly decreased (−84% as compared to glutamate in adult animals), and, interestingly, further reduced by aging (−35%).

Effects of aging on the fatty acid profile of mitochondrial phospholipids

Aging modified the fatty acid composition of the isolated mitochondria (Table 2). It decreased the proportion of stearic acid
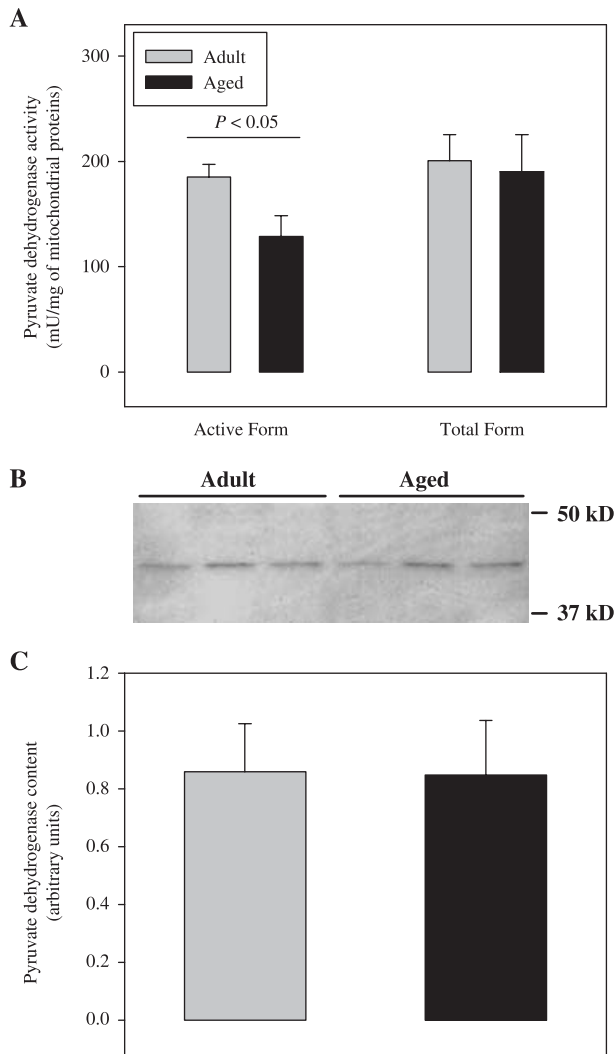
A

B

C

Fig. 3 Influence of aging on pyruvate dehydrogenase (PDH) activity (A**)** and content (B and C) determined by immunoblotting of isolated gastrocnemius mitochondria. The number of experiments was six per group.
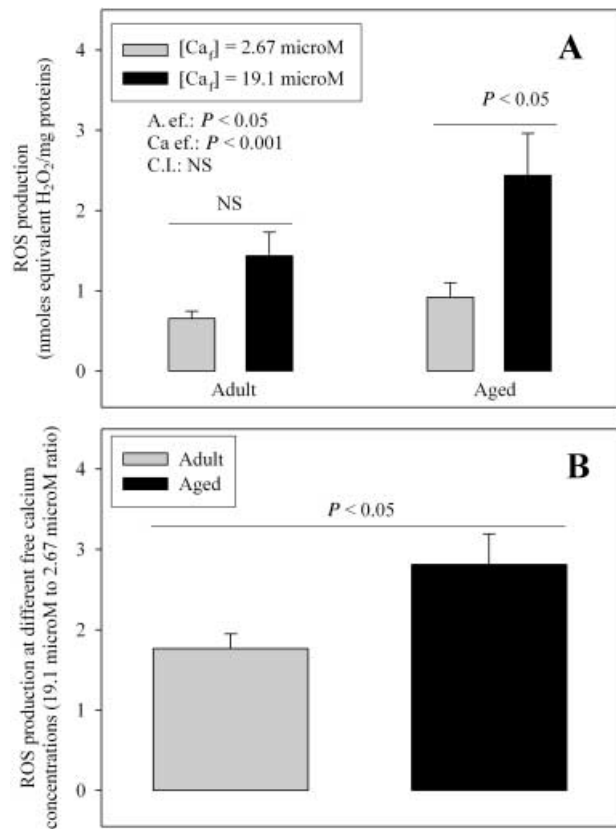

Fig. 4 Effect of aging and free-calcium concentration on the production of reactive oxygen species (ROS) in glutamate respiring mitochondria. In panel (A), the results are expressed in nanomoles hydrogen peroxide (H_2O_2) produced per milligram proteins, since the ROS-induced changes in fluorescence was normalized to that measured in response to a known amount of H_2O_2. In panel (B), the results are expressed as the ratio between ROS production measured at two different free-calcium concentrations (19.1 μM per 2.67 μM). The number of experiments was 10 per group. A. ef., effect of aging; Ca ef., effect of the free-calcium concentration; CI, cross-interaction; NS, not significant.

Fig. 5 Effect of aging on the activity of manganese- superoxide dismutase (Mn-SOD). The number of experiments was six per group. NS, not significant.

Discussion

The purpose of this study was to determine if aging induces alterations of mitochondrial functioning that could partly

(−8%), thus contributing to reduce the percentage of saturated fatty acids (−3%). Conversely, it raised the proportion of oleic acid (10%) and total monounsaturated fatty acids (11%). The proportion of total polyunsaturated fatty acids was not modified, but the nature of these fatty acids was changed. As indicated by the significant increase in the n–6 polyunsaturated fatty acid (PUFA)/n–3 PUFA ratio (30%), n–6 PUFA was raised (7%) and n–3 PUFA was reduced (−18%). In the n–6 PUFA family, only linoleic acid was significantly increased (9%). In the n–3 PUFA series, C22:5 n–3 was increased (−19%) and C22:6 n–3 was decreased (−23%). However, since the proportion of C22:6 n–3 was approximately seven times higher than that of C22:5 n–3, the decrease in C22:6 n–3 predominated on the increase in C22:5 n–3. The C22:6 n–3 to C22:5 n–3 ratio was reduced by aging (−36%), suggesting retro conversion from C22:6 n–3 to C22:5 n–3.

Fig. 6 Effect of aging on the activity (A) and on the content determined by immunoblotting (B and C) of cytochrome c oxidase. The number of experiments was six per group.

Table 2 Effect of aging on the fatty acid profile of mitochondrial phospholipids

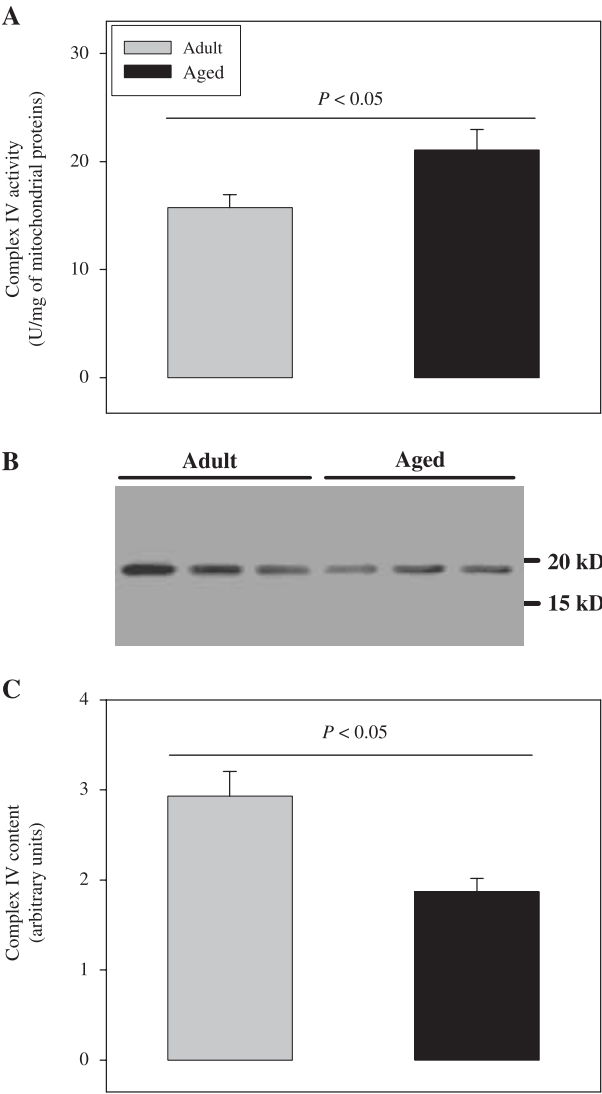|	Adult	Aged	ANOVA
C16:0	19.94 ± 0.14	20.04 ± 0.45	NS
C17:0	0.53 ± 0.03	0.52 ± 0.02	NS
C18:0	15.42 ± 0.24	14.22 ± 0.25	$P < 0.01$
C22:0	0.46 ± 0.02	0.49 ± 0.02	NS
SFA	36.35 ± 0.20	35.27 ± 0.20	$P < 0.01$
C16:1	0.63 ± 0.08	0.75 ± 0.04	NS
C18:1	6.79 ± 0.14	7.49 ± 0.19	$P < 0.05$
MUFA	7.42 ± 0.22	8.23 ± 0.22	$P < 0.05$
C18:2 n–6	27.34 ± 0.35	29.72 ± 0.49	$P < 0.01$
C20:4 n–6	13.92 ± 0.31	14.5 ± 0.29	NS
C22:4 n–6	0.27 ± 0.01	0.27 ± 0.01	NS
C22:5 n–6	0.33 ± 0.03	0.24 ± 0.03	NS
n–6 PUFA	41.86 ± 0.50	44.73 ± 0.45	$P < 0.01$
C18:3 n–3	0.07 ± 0.05	nd	NS
C20:5 n–3	0.23 ± 0.01	0.20 ± 0.07	NS
C22:5 n–3	1.81 ± 0.12	2.16 ± 0.06	$P < 0.05$
C22:6 n–3	11.92 ± 0.15	9.17 ± 0.16	$P < 0.001$
n–3 PUFA	14.04 ± 0.17	11.54 ± 0.12	$P < 0.001$
Total PUFA	56.16 ± 0.38	56.37 ± 0.52	NS
n–6 PUFA/n–3 PUFA	2.98 ± 0.07	3.88 ± 0.05	$P < 0.001$
C22:6 n–3/C22:5 n–3	6.67 ± 0.42	4.26 ± 0.15	$P < 0.01$

The number of experiments was five per group.
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, not detected; NS, not significant.

explain the abnormalities encountered in muscles of aged rats (increased ROS production, proteolysis and cellular death).

Experimental model

The gastrocnemius muscle was chosen since this mixed muscle is partly composed of glycolytic fibers and is sensitive to sarcopenia (Thompson, 1994). Indeed, when we determined the gastrocnemius weight of 6- and 21-month-old rats, we observed that the muscle mass was decreased by 28% in the aged animals, despite
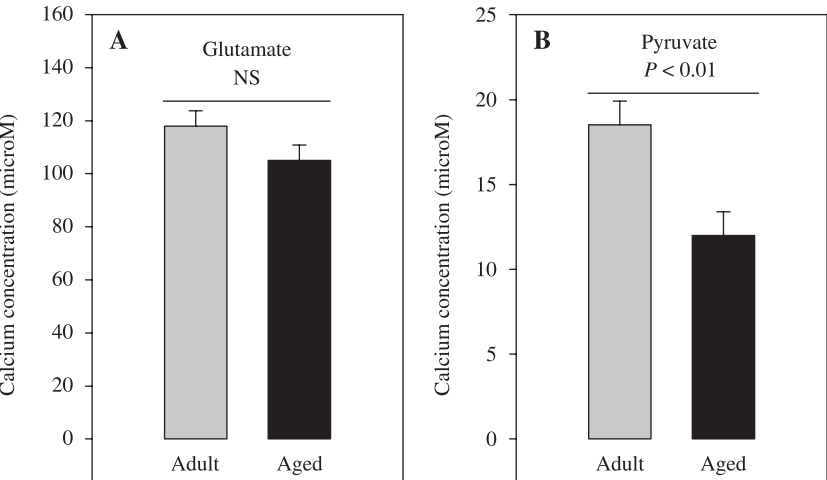

Fig. 7 Effect of aging on mitochondrial ability to retain calcium during glutamate (panel A) and pyruvate (panel B) oxidation. The number of experiments was 10 per group. NS, not significant.

a similar body weight average. The soleus (> 90% of oxidative fibers) weight was not changed (0.316 ± 0.015 and 0.282 ± 0.020 g in adult and aged animals, respectively, $n = 6$, not significant). This indicates that aging triggered a clear atrophy of glycolytic fiber-enriched muscles while preserving oxidative fiber-rich muscles. Recently, Phillips & Leeuwenburgh (2005) and Dirks & Leeuwenburgh (2006) have reported that aging is associated with an increased circulating TNF-α. They showed that this increase is responsible for the activation of different signaling cascades in type 1 and type 2 fibers. Type 1 fibers preferentially develop the inflammatory signaling pathway through the transcription factor NF-κB and thus survive. In contrast, type 2 fibers follow the pathway leading to TNF-α-induced apoptosis. This partly explain why aging triggers an enrichment of mixed muscles with type 1 fibers (Siriett *et al.*, 2006). In our study, mitochondria were extracted from mixed (white plus red) gastrocnemius in order to obtain a sufficient amount of the organelles. Aging should have thus enriched the final preparation with mitochondria obtained from type 1 fibers and this could partly explain its effect on mitochondrial functioning. However, mitochondria extracted from type 1 fibers display lower oxidative capacities in the rat as has been demonstrated when comparing the functioning of isolated mitochondria obtained from soleus and tibialis anterior muscles (Capel *et al.*, 2004). A similar conclusion has been drawn from comparisons of white and red gastrocnemius (Molnar *et al.*, 2004). In our experiment, we did not determine the effect of aging on the gastrocnemius fiber type composition. However, if aging had substantially increased the proportion of mitochondria obtained from type 1 fibers in our preparation, the oxidative phosphorylation with glutamate as substrate would have been reduced when the free-calcium concentration of the respiration medium was low. This was not the case, indicating that our results mainly reflected changes in mitochondrial function rather than alterations of muscle fiber composition with aging. Furthermore, the results obtained on *in vivo* matrix calcium content also support this idea. Indeed, aging increased matrix calcium in mitochondria obtained from type 1 fibers (soleus), whereas it decreased this parameter in those extracted from the gastrocnemius muscle.

Intact and damaged mitochondria are present in the muscle and it is almost certain that the isolation procedure did not extract a representative population of all mitochondria. Since the mitochondria are extracted according to their density, it is likely that the more-damaged mitochondria (swollen mitochondria, mega mitochondria and disrupted mitochondria) were excluded. It is well known that aging favors the formation of COX[−], SDH[+] and COX[−]/SDH[+] fibers in the rat (Aspnes *et al.*, 1997). In our experiment, analysis of the mitochondria extracted from old gastrocnemius displayed a decreased COX subunit IV content, probably reflecting altered transcription from nuclear DNA. However, COX activity was increased, suggesting a compensatory mechanism to increase energy production. Since COX activity is dependent on its lipid environment, changes in COX activity could result from changes in the activity of enzymes involved in phospholipid synthesis. This may lead the cell to establish a specific polyunsaturated fatty acid profile less prone to nonspecific oxidation triggered by ROS attack. The most damaged mitochondria were probably not extracted during our purification procedure. The extraction yield expressed as the amount of mitochondrial protein extracted per gram of muscle was, however, similar in adult and aged rats. It indicates that the percentage of damaged mitochondria was low, maybe because they were rapidly eliminated through autophagy. A better extraction yield might have been obtained by using a specific mitochondrial enzyme marker in muscle homogenate, since the mitochondrial content of the muscle might be modified by aging. However, Pastoris *et al.* (1991) did not observe any noticeable change in the activity of gastrocnemius citrate synthase with aging. Moreover, the structural homogeneity of the organelles extracted from the gastrocnemius of adult and aged rats does not signify that their biochemical behavior is similar.

The mitochondria extracted from old muscles can present different sensitivities to stressing agents, which would explain why all fibers do not die at the same moment and also why sarcopenia develops slowly. Changes in the lipid composition of mitochondrial membranes could contribute to such differential sensitivities. In the present experiment, we evaluated the effects of aging on the fatty acid profile of phospholipids of gastrocnemius mitochondria. Briefly, aging triggered an increase in monounsaturated fatty acids to the detriment of saturated fatty acids. The proportion of PUFAs was not modified by aging, but a clear increase in n–6 PUFAs occurred at the expense of n–3 PUFAs. N–3 PUFAs are the most unsaturated PUFAs and the most susceptible to auto-oxidation through ROS attack. Since aging is accompanied by oxidative stress (Harman, 1956; Pollack *et al.*, 2002), it is possible that n–3 PUFA is partly eliminated and substituted by more stable PUFAs such as linoleic acid (two double bonds instead of six for C22:6 n–3). It might also be possible that the cells resist ROS attack by modifying the expression of genes involved in the synthesis of enzymes participating in phospholipid formation. Whatever the mechanism, the increased n–6 PUFAs to n–3 PUFAs ratio of membrane phospholipids would decrease membrane fluidity, but the loss would be partly compensated for by the increase in monounsaturated fatty acids at the expense of saturated fatty acids.

All these modifications could alter mitochondrial function, since PUFAs are important constituents of functional domains in the inner membrane. The changes in phospholipid fatty acid composition could alter the membrane physical properties, particularly membrane fluidity, which in turn could modify the activity of membrane-bound enzymes. Paradies *et al.* (1999) showed that cardiac ischemia/reperfusion was associated with oxidative stress, loss of mitochondrial cardiolipin and fall in COX activity. It is well known that cardiolipin plays an important role in enzyme activity, due to its localisation in microdomains. Cardiolipin is rich in linoleic acid. In our study, we saw an aging-induced increase in phospholipid linoleic acid proportion to the detriment of n–3 polyunsaturated fatty acid. This could reflect a rise in cardiolipin content and would explain why COX activity was increased by aging despite a reduced subunit IV content.

Effect of aging on respiration substrate selection

Aging had several impacts on mitochondrial function. We examined function at different free-calcium concentrations, including one in the physiological range (0.43 and 0.48 μM for glutamate and pyruvate, respectively) and two high concentrations (2.67 and 19.1 μM for glutamate, and 4.18 and 9.18 μM for pyruvate) that could supervene in pathological conditions. Elevations in calcium concentrations can obviously occur *in vivo* and can be responsible for muscle atrophy, since the calcium channel blocker nifedipine completely abolishes immobilization-induced soleus weight loss (Soares *et al.*, 1993). At the physiological free-calcium concentration, aging reduced pyruvate oxidation, without modifying glutamate degradation. Several investigators already observed similar glutamate oxidation in adult and old mixed muscles (Barrientos *et al.*, 1996; Capel *et al.*, 2004), but pyruvate degradation was less studied. Pyruvate and glutamate follow a different pathway towards α-ketoglutarate in the Krebs cycle. After this step, the metabolic pathway (Krebs cycle and oxidative phosphorylation) is common for the two substrates. Pyruvate enters the mitochondria where it is oxidatively decarboxylated by PDH to acetylCoA. Recently, Drew *et al.* (2003) described lower ATP production from pyruvate by gastrocnemius mitochondria of 26-month-old rats as compared to their 12-month-old counterparts. They attributed this phenomenon to a lower matrix ATP content. However, if such a decrease had occurred in our study, glutamate oxidation would have been also reduced at the physiological free-calcium concentration. This was not the case, which suggests involvement of another phenomenon.

We demonstrated that the active form of PDH was lowered by aging, whereas its total activity was unchanged. This explains the aging-related decrease in the state III respiration rate observed with pyruvate. Pyruvate dehydrogenase is activated by a phosphatase and inactivated by a kinase whose activities are regulated by calcium, ATP, NADH and acetylCoA (Cox & Matlib, 1993). Decreased calcium as well as increased ATP, NADH and acetylCoA contribute to reduce the active form. We did not measure ATP, NADH and acetylCoA, but according to Drew *et al.* (2003), ATP is decreased in mitochondria obtained from old rat gastrocnemius. Instead of inactivating the PDH system, this should activate it. This clearly demonstrates that the PDH system in old animals is regulated by other factors whose inhibitory activity predominates. Mitochondrial calcium could be decreased by aging. We measured *in vivo* mitochondrial calcium and found that it tended to be reduced (–16%). A small reduction of mitochondrial calcium could have contributed to a large decrease in active PDH and concomitant reduction of pyruvate oxidation. We do not know if the reduced matrix calcium results from PUFA-related decrease in ΔΨ and calcium entry through the calcium uniport or activation of the inner membrane Na/Ca exchange.

Protein degradation is inversely related to muscle mass in aged mice (Reynolds *et al.*, 2002). In the rat, proteolysis has been measured in 24-month-old animals by evaluating urine excretion of 3-methylhistidine (Mosoni *et al.*, 1999). It is increased in these old animals as compared to 12-month-old rats. We did not measure proteolysis in our study. However, Kimball *et al.* (2004) reported that muscle weight loss occurs from the 18th month of life in the rat. Surprisingly, these authors showed that gastrocnemius protein anabolism was stimulated while muscle mass was dropping. This suggests occurrence of intense muscular proteolysis from the 18th month of life in the rat. The higher relative contribution of glutamate oxidation as compared to pyruvate degradation observed in our 21-month-old animals could contribute to the increased proteolytic activity observed in the aged muscle. Interestingly, soleus weight was preserved by aging. In this muscle, we did not measure PDH activity, but the high *in vivo* mitochondrial calcium concentration observed in 21-month-old rats suggests that this activity was either maintained or even increased. The glutamate oxidation rate to pyruvate oxidation rate should thus be maintained, which would partly explain the maintenance of soleus weight.

Effect of aging on mitochondrial function at increasing calcium concentrations

Depending on the concentration used, calcium had several impacts on mitochondrial function. It first reduced the respiration of NADH-linked substrate (glutamate and pyruvate) and favored ROS production. This was achieved without complete opening of the permeability transition pore (PTP) as it occurred at higher calcium concentrations, but the state IV respiration rate was increased and the RCI decreased, suggesting a proton leak and a partial opening of the PTP. On the one hand, the decreased RCI and ADP:O ratio with excess calcium have already been described in isolated cardiac mitochondria (Sentex *et al.*, 1999; Meynier *et al.*, 2003). In these organelles, it is due to calcium-induced stimulation of isocitrate and α-ketoglutarate dehydrogenases. As a result, succinate accumulates in the mitochondrial matrix, which contributes to stimulate ROS production, to open the PTP in a population of 'sensitive' mitochondria and to favor proton leak. On the other hand, the decrease in oxidative phosphorylation observed before the complete PTP opening might be due to inhibition of complex I. Indeed, calcium inhibits respiration of NADH-linked substrates by direct blockade of complex I in isolated hepatic mitochondria (Fontaine & Bernardi, 1999). It could also be due to a partial PTP opening in a sensitive mitochondrial subpopulation with NADH release from the matrix (Fontaine *et al.*, 1998; Batandier *et al.*, 2004). From our results, we cannot conclude which hypothesis is valid, but both could contribute.

Aging amplified all these phenomena. It further reduced the respiration rate, whatever the substrate used. It favored the decrease in ADP:O ratio with glutamate as a substrate, but not with pyruvate. It amplified ROS production, which cannot be explained by the reduction of Mn-SOD [such a decrease should contribute to reduce hydrogen peroxide (H_2O_2) production]. On the contrary, the reduced mitochondrial SOD activity should favor the release of the highly toxic hydroxyl ion. This ROS would damage the mitochondrial DNA, thus reducing the expression

of mitochondrial DNA-made enzymes such as COX subunits. Decreased content and increased activity of COX could alter electron transport, favor ROS production and encourage loss of metabolic efficiency. Finally, aging facilitated complete PTP opening when pyruvate was used as a substrate, maybe because of the low rate of oxidative phosphorylation and a resulting drop of proton gradient. The mitochondria from aged gastrocnemius were thus weakened when facing a calcium stress as compared to their adult counterparts. The resulting release in cytochrome c and loss in energy production might induce cell death by apoptosis and/or oncosis (Green & Reed, 1998). Skeletal muscle of 24-month-old rats displays apoptosis (Dirks & Leeuwenburgh, 2004). However, we have not examined this in the case of our 21-month-old rats. Our data on isolated mitochondria suggest that the muscle cells of 21-month-old rats are more prone to apoptosis than those of their adult counterparts. A change in cellular and mitochondrial calcium could occur with aging through insulin-resistance or other aging-related pathology (Resnick, 1999). In our study, excess calcium loading at the mitochondrial level triggered ROS overproduction. This was also demonstrated in cardiac mitochondria (Grijalba *et al.*, 1999). The phenomenon could alter some key elements in mitochondrial functioning. Polyunsaturated fatty acids of inner membrane phospholipids could be subjected to peroxidation (Ochoa *et al.*, 2003), releasing toxic compounds such as 4-hydroxynonenal (Aldini *et al.*, 2002). Proteins could directly endure ROS attack, activity loss (Squier & Bigelow, 2000) and accelerated proteolysis (Levine *et al.*, 1996). Mitochondrial DNA could also be subjected to the action of ROS, leading to deletions/mutations (Eimon *et al.*, 1996; Pallotti *et al.*, 1996). All these changes could contribute to make the mitochondria more fragile when facing the deleterious action of calcium with aging.

In conclusion, aging has several noticeable effects on mitochondrial functioning of gastrocnemius muscle. This is probably related to a change in the PUFA profile of mitochondrial lipids in the face of increased production of toxic ROS. The altered PUFA profile in turn probably amplified the deleterious action of calcium (facilitation of ROS production and PTP opening as well as reduction of energy synthesis); it also favored glutamate oxidation as compared to pyruvate degradation through a mechanism involving reduced matrix calcium and PDH activity. All these modifications can partly explain the abnormalities encountered during sarcopenia. During the aging process, multiplication of different mechanical, biochemical and oxidative stresses could contribute to transient increases in mitochondrial calcium, thus favoring ROS overproduction and mitochondrial damage which in turn would facilitate proteolysis and cell death.

Experimental procedures

Animal care

In these experiments, the care and use of laboratory animals followed the European Union recommendations. Thirty-two male Wistar rats (Janvier, Le Genest Saint Isle, France) were used. Sixteen animals were aged 6 months (adult group) and 16 were aged 21 months (aged group) at the beginning of the experiments. The rats were housed in individual cages in an animal facility with controlled temperature, dark/light cycle and air humidity degree. They were fed with standard commercial pellets (A04, Safe, Gannat, France) *ad libitum* with free access to water. After 1 week of stabilization, the experiments began and lasted no more than 4 weeks. Ten animals of each group were used to evaluate the respiration parameters, ROS production, activity of respiratory chain complexes and calcium retention capacities. The other six rats of each group were used for the determination of *in vivo* calcium concentration, PDH and SOD activities as well as protein expression.

Chemicals

All products were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). The fluorescent probes for detection of free calcium (calcium Green 5N) and reactive oxygen species (DCFDA) were purchased from Molecular Probes (Invitrogen SARL, Cergy Pontoise, France).

Mitochondria preparation

After anesthesia with thiobarbital (10 mg per 100 g of body weight), the gastrocnemius muscle of both legs was collected, weighed and transferred in the cold. A medium containing sucrose 150 mM, KCl 75 mM, Tris–HCl 50 mM, KH_2PO_4 1 mM, $MgCl_2$ 5 mM, EGTA-Tris 1 mM, and lipid-free serum albumin 0.2%, pH 7.4, was used for mitochondrial extraction. Mitochondria were prepared according to Fontaine *et al.* (1998).

Mitochondrial respiration

Mitochondrial respiration was determined at 25 °C in a 1.2 mL chamber with a Clarke oxygen electrode related to an oxygen monitor (YSI, Fisher Bioblock Scientific, Illkirch, France). The chamber was totally filled with the respiration buffer and closed after addition of mitochondria (0.25 mg proteins mL^{-1}). The respiration substrate (either glutamate 5 mM + malate 1 mM or pyruvate 5 mM + malate 1 mM) was added, followed by the phosphorylation substrate (ADP, 242 μM) 90 s later (this duration allowed a complete matrix calcium loading). The parameters of oxidative phosphorylation (state III and IV respiration rates, respiratory control index and ADP:O ratio) were calculated according to Chance & Williams (1956) and Estabrook (1967). Oxygen consumption was determined in three media differing in their free-calcium concentration. The low-free calcium medium was composed of sucrose 250 mM, Pi-Tris 10 mM, and EDTA 2 mM, pH 7.3. The free-calcium concentration of this medium was evaluated with a fluorescent indicator (calcium Green 5N) using calibration solutions (mixture of calcium and EDTA) calculated according to Fabiato & Fabiato (1979). The calcium concentrations were 0.43 and 0.48 μM with glutamate 5 mM + malate 1 mM, and pyruvate 5 mM

+ malate 1 mM, respectively. The second respiration medium was similar to the first one except that the EDTA concentration was 5 μM. This induced the free-calcium concentration to 2.67 and 4.18 μM with glutamate and pyruvate, respectively. Finally, the third respiration medium was obtained by adding excess calcium to the second medium. The added calcium concentrations were chosen to be lower (10 and 40 μM for pyruvate and glutamate, respectively) than that necessary to trigger a complete opening of the permeability transition pore. The corresponding free-calcium concentrations were 9.18 and 19.1 μM, respectively.

When glutamate was used as a respiration substrate, the respiratory control index was 9.67 ± 0.83 and 8.22 ± 0.57 in mitochondria from adult and aged rats (no significant difference), respectively, at the lowest free-calcium concentration. This indicates the similarity and the good quality of the mitochondrial preparations used.

Measurement of enzyme activities

The active and total forms of PDH were measured according to Coore *et al.* (1971). Mn-SOD activity was determined with the RANSOD kit from Randox Laboratorie (Montpellier, France) in isolated mitochondria after inhibition of residual Cu,Zn-SOD activity by sodium cyanide. The activities of respiratory chain complexes (NADH cytochrome c reductase, succinate cytochrome c reductase, succinate dehydrogenase and cytochrome c oxidase) were evaluated as described by Veitch & Hue (1994).

Immunoblotting

Mitochondrial contents in PDH, cytochrome c oxidase and complex I were determined by Western blot. Mitochondrial proteins along with molecular weight markers were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting on polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated 3 h in blocking buffer (NaCl 150 mM, nonfat dried milk 10%, Tween 20 0.1%, Tris 50 mM, pH 7.5), and then incubated overnight at 4 °C with either anti-PDH antibody (MitoSciences, Eugene, OR, USA, 1 μg mL^{-1}), anticytochrome c oxidase subunit IV antibody (Molecular Probes, 0.5 μg mL^{-1}) or anticomplex I 39 kDa subunit antibody (Molecular Probes, 1 μg mL^{-1}). Membranes were washed three times in NaCl 150 mM, Tris 50 mM, pH 7.5 and then incubated 1 h with appropriate horseradish peroxidase-linked secondary antibodies (Amersham Biosciences, Orsay, France). Protein contents were detected by enhanced chemiluminescence (Amersham Biosciences) with exposure on autoradiography films (Kodak X-OMAT). Band densities were quantified by software analysis using Scion Image (Scion Corporation, Frederick, MD, USA). To allow consistency between experiments, a small aliquot of the same sample was systematically loaded on every gel and the generated signal was used as one arbitrary unit to express the results.

In vivo calcium content

On the day of sacrifice, rats were anesthetized using a mixture of ketamine (120 mg kg^{-1}) and xylazine (6 mg kg^{-1}) solubilised in NaCl (0.9%). Radioactive calcium (^{45}Ca, 0.925 MBq dissolved in 0.5 mL of 0.9% NaCl – Amersham Biosciences) was injected in the left saphenous vein. Anesthesia was maintained for 45 min to allow ^{45}Ca to diffuse in the organism so that specific activity of the tracer became uniform in every cell compartment. Gastrocnemius and soleus muscles of both legs were collected and weighed. Muscle mitochondria were prepared according to Pepe *et al.* (1999). Extraction medium (pH 7.4) contained sucrose 250 mM, HEPES 5 mM, EDTA 2 mM, and also diltiazem 30 μM and ruthenium red 3.2 μM to prevent calcium entering the mitochondria through the calcium uniport and escaping from the mitochondrial matrix through Na/Ca exchange. Ruthenium red was prepared according to Luft (1971). Mitochondrial calcium was extracted with perchloric acid (0.6 M, final concentration). After homogenisation, the extract was centrifuged (10 000 *g*, 5 min) and the supernatant transferred in a scintillation vial containing 10 mL of Ultima Gold (Perkin Elmer, Courtaboeuf, France). ^{45}Ca radioactivity was measured (2000CA, Packard Instrument, Rungis, France) for 1 h and corrected for quenching and chemo luminescence. Calcium-specific activity was determined in the plasma by evaluating calcium radioactivity as mentioned above. The amount of calcium in the plasma was determined by atomic absorption photometry (Perkin Elmer 3300 – absorption wave length set at 423 nm) after perchloric acid extraction and addition of lanthane chloride (0.1%) in the extract. The specific activity of calcium was considered to be similar in the plasma and mitochondrial compartment after 45 min of diffusion in the organism (McCormack & Denton, 1984). Mitochondrial calcium content was calculated as the ratio of mitochondrial radioactivity to plasma-specific activity and expressed in nanomole per milligram mitochondrial protein.

Calcium retention capacities

The mitochondrial capacities of calcium retention were determined according to Walter *et al.* (2000), using calcium Green 5N as a fluorescent indicator of free calcium in the medium. The excitation and emission wavelengths were adjusted to 505 and 535 nm, respectively. The assay was carried out at 25 °C in the second respiration medium in the same conditions as those used for respiration measurements. Five minutes after the addition of the medium, substrate (glutamate 5 mM + malate 1 mM or pyruvate 5 mM + malate 1 mM), calcium Green 5N and mitochondria were successively added. A first calcium pulse (2.5 and 10 μM for pyruvate and glutamate, respectively) was added, leading to an increase in the fluorescence due to the presence of free calcium in the medium. Thereafter, the mitochondria absorbed the calcium and the fluorescence decreased to its previous value. Other calcium pulses were performed until all the matrix calcium was released from the mitochondria (opening of the permeability transition pore) and the calcium concentration remained high in the medium.

Oxygen-free radical production

ROS production was measured according to Walter *et al*. (2000) using the DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) probe. In the presence of ROS, the de-acetylated form of DCFDA (DCF) becomes fluorescent (506 and 521 nm for excitation and emission wavelengths, respectively). The assay was performed under the same conditions as those used for respiration measurements, except that the mitochondrial concentration was 0.75 mg proteins mL^{-1}. The second respiration medium was used and glutamate 5 mM + malate 1 mM, pig liver esterase (25 IU), mitochondria and DCFDA (8 μL) were successively added. The fluorescent measurement was recorded for 15 min and calcium (40 μM) was added. The measurement was then recorded for another 15-min period. Pyruvate was not used, since it is known to have a ROS scavenging activity (Varma *et al*., 1998). The results were expressed as nanomoles H_2O_2 produced per milligram proteins, using a calibration curve established with known amounts of H_2O_2.

Fatty acid profile of isolated mitochondria

The fatty acid composition of gastrocnemius mitochondria was determined as previously described (Demaison & Grynberg, 1991). The lipids were extracted from the mitochondria according to Folch *et al*. (1957). The phospholipids were separated from nonphosphorus lipids using a Sep-pack cartridge (Juaneda & Rocquelin, 1985). After transmethylation, the fatty acid methyl esters were separated and analyzed by gas chromatography on a DB wax capillary column.

Statistical analysis

The results are presented as mean ± SEM. In general, the data were analyzed with one-way analysis of variance describing the effect of aging (A. ef.). The effect of calcium on the functioning of mitochondria from adult and aged rats was determined with an analysis of variance with repeated measures, since this effect was tested in the same mitochondrial preparation. The analysis of variance described the effect of aging (A. ef.), of free calcium (Ca ef.) and the cross-interaction between the two factors. When necessary, a Fisher's protected least significant difference (LSD) test was used to compare the means. A P value lower than 0.05 was considered significant.

Acknowledgments

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