Translational Article

The Mitochondria’s Role in the Aging Process

Original Article

Sexual Dimorphism in the Alterations of Cardiac Muscle Mitochondrial Bioenergetics Associated to the Ageing Process

Bartomeu Colom,1,3 Jordi Oliver,1,2 and Francisco J. Garcia-Palmer1,2

1Grup de Metabolisme Energètic i Nutrició, Departament de Biologia Fonamental i Ciències de la Salut, Institut Universitari d’Investigació en Ciències de la Salut (IUNICS), Universitat de les Illes Balears, Palma de Mallorca, Spain.
2CIBERobn Fisiopatología Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III, Palma de Mallorca, Spain.
3Present address: Centre for Microvascular Research, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK.

Address correspondence to Bartomeu Colom, PhD, Centre for Microvascular Research, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK. Email: b.colom@qmul.ac.uk

Received July 29, 2013; Accepted January 16, 2014

Decision Editor: Rafael de Cabo, PhD

Abstract

The incidence of cardiac disease is age and sex dependent, but the mechanisms governing these associations remain poorly understood. Mitochondria are the organelles in charge of producing energy for the cells, and their malfunction has been linked to cardiovascular disease and heart failure. Interestingly, heart mitochondrial content and functionality are also age and sex dependent. Here we investigated the combinatorial effects of age and sex in mitochondrial bioenergetics that could help to understand their role on cardiac disease. Cardiac mitochondria from 6- and 24-month-old male and female Wistar rats were isolated, and the enzymatic activities of the oxidative-phosphorylative complexes I, III, and IV and ATPase, as well as the protein levels of complex IV, β-ATPase, and mitochondrial transcription factor A (TFAM), were measured. Furthermore, heart DNA content, citrate synthase activity, mitochondrial protein content, oxygen consumption, and H2O2 generation were also determined. Results showed a reduction in heart mitochondrial mass and functionality with age that correlated with increased H2O2 generation. Moreover, sex-dependent differences were found in several of these parameters. In particular, old females exhibited a significant loss of mitochondrial function and increased relative H2O2 production compared with their male counterparts. The results demonstrate a sex dimorphism in the age-associated defects on cardiac mitochondrial function.

Key Words: Mitochondria—Sex—Aging—Energy metabolism—Heart
Although mortality by cardiovascular disease (CVD), including cardiovascular disease, has been falling in recent years, it still remains the leading cause of death in developed countries. The incidence of cardiac disease is age and sex dependent, with increased risk in older individuals and in males compared with that in females. This sex-associated protection exists in different species and has been attributed to the hormonal milieu, as it disappears after menopause when the cardiovascular risk of women can even exceed that of age-matched men (1–4). However, conflicting results on preventing CVD in postmenopausal women after hormone replacement trials suggest that other factors might be involved (5). Overall, the mechanisms responsible for the effects of age and sex on the incidence of CVD remain poorly understood.

The heart is a highly metabolic demanding tissue that relies on mitochondria, which are particularly abundant in this organ, to adequately provide the energy required to function properly. Mitochondria mainly generate energy as ATP through a series of sequential chemical reactions taking place in the oxidative-phosphorylation system (OXPHOS), comprised by the protein complexes I–V (6). The entire process needs to be finely regulated because a small percentage of electrons are diverted to reactive oxygen species (ROS) production, particularly in the complexes I and III, which can potentially cause oxidative damage to macromolecules and cellular components overall affecting the functionality of the tissue (7,8).

Alterations in mitochondrial bioenergetics have been associated to CVD and heart failure, which could be caused by lack of mitochondria-supplied ATP or by increased ROS generation by malfunctioning mitochondria (9–11). Accordingly, defects in cardiac mitochondrial OXPHOS function and increased ROS production as well as tissue oxidative damage have been reported during ageing and linked to heart failure (12–15) although this is still the subject of debate (16). Importantly, it has also been suggested that the role of sex in heart disease could be linked to a sexual dimorphism in cardiac energy mitochondrial metabolism (3). Supporting the above, we recently identified sex-dependent differences in rat cardiac muscle mitochondrial biogenesis and function, where female mitochondria were found to be more differentiated and functionally efficient compared with male mitochondria (17). These sex-related differences in mitochondrial features are not specific from cardiac muscle because they have also been described in other tissues such as skeletal muscle, brown adipose tissue, and liver (18–20). Sex hormones could be responsible for these effects because they have been reported to directly regulate several mediators of the mitochondriogenesis program (21). In addition, cardiac muscle from females has also been reported to generate less mitochondrial ROS and exhibit lower oxidative damage than males (17), in agreement with their higher cardioprotection.

On the whole, the role of ageing and sex on the incidence of cardiac disease could be related to their effects on heart mitochondrial performance; however, to our knowledge, the combined effects of sex and age on heart mitochondrial function have to this date not been investigated in a systematic manner. Therefore, we examined the effects of age and sex on heart mitochondrial content and their functional properties. In particular, we investigated the oxygen consumption, enzymatic activity, and content of several proteins with a key role in mitochondrial energy metabolism and the mitochondrial H$_2$O$_2$ generation of cardiac muscles from 6- and 24-month-old rats of both sexes. The results demonstrated a sex-dependent decrease in both cardiac mitochondrial content and function with age. Specifically, old females exhibited a further loss of mitochondrial mass and function as well as increased H$_2$O$_2$ generation compared with their male counterparts. Collectively, our findings demonstrate a sexual dimorphism in the alterations of mitochondrial function during ageing.

**Methods**

**Animals and Sample Collection**

Animal experiments were approved by our institutional ethics committee and conform to the Directive 2010/63/EU of the European Parliament. Six- and 24-month-old male and female Wistar rats were housed individually in wire-bottom cages and acclimated at 22°C with a 12-hour light/dark cycle, with free access to water and pelleted standard diet (A04, Panlab, Barcelona). Animals were sacrificed by decapitation at the start of the light cycle, and the hearts were quickly removed, washed, and cut into pieces. Tissue homogenizing and mitochondria isolation were performed as previously described (17).

**Total DNA and Mitochondrial Protein Content**

Total DNA was measured in homogenates using the diaminobenzoic acid method (22). Mitochondrial protein content was measured in isolated mitochondrial preparations by the method of Bradford (23).

**Mitochondrial Oxygen Consumption**

Mitochondrial oxygen consumption was measured in isolated mitochondria using a Clark-type O$_2$ electrode (Oxygraph, Hansatech, UK) at 37°C as previously described (17). A final concentration of 0.2 mg of mitochondrial protein/mL of respiratory medium was used. Mitochondrial respiratory state 4 was assayed with pyruvate/malate 2.5 mM or succinate 5 mM (plus rotenone 2 µM) as substrates. State 3 activation was also measured with both substrates by addition of ADP 0.5 mM to the medium. Mitochondrial viability was assessed by measuring the respiratory control ratio (RCR = oxygen consumption at state 3/state 4).

**Enzymatic Activities**

Enzymatic activities of complexes I, III, and IV and ATPase from the OXPHOS system were measured in mitochondrial preparations, whereas citrate synthase (CS) activity was measured in both mitochondrial preparations and tissue homogenates as described previously (17). All enzymatic activities were performed using a 96-well microplate spectrophotometer (Biotek instruments Inc., Vermont).

**Mitochondrial H$_2$O$_2$ Production Rates**

Mitochondrial H$_2$O$_2$ generation was assayed as described before (17) by measuring the increase in fluorescence of Amplex Red reagent (Molecular probes). Mitochondria (0.2 mg protein/mL) were added to the same medium used for respiration and supplemented with horseradish peroxidase 0.1 U/mL (Sigma) and Amplex Red reagent 50 µM. State 4 and state 3 were induced as for respiration (see the Mitochondrial Oxygen Consumption section). Maximal H$_2$O$_2$ generation rates of complexes I and III were measured in presence of rotenone 2 µM for complex I or rotenone 2 µM + antimycin A 5 µM for complex III. Assays were performed at 37°C for 30 minutes in a 96-well microplate fluorimeter (FLX800, Biotek instruments Inc., Vermont). Blanks without mitochondrial sample were used to correct for spontaneous fluorescence of Amplex Red. The H$_2$O$_2$ production rates were calculated using a standard curve of H$_2$O$_2$ generated by addition of β-D(+)glucose 14 mM in presence of glucose oxidase.

**Western Blotting**

Thirty micrograms of protein were loaded into sodium dodecyl sulfate–polyacrylamide gels (14%) in order to estimate the protein content of complex IV subunit IV (antibody MS407, Mitosciences),
F1-\(\beta\)-ATPase (antibody C-20, Sc-16690; Santa Cruz Biotechnology), and mitochondrial transcription factor A (TFAM; provided by Dr. Hidetoshi Inagaki). Samples were run in parallel for direct comparison between groups. Western blot was performed using standard techniques (17). Blots were quantified by photodensitometric analysis (Kodak-1D Image Analysis Software).

Statistics
Data analysis was performed using GraphPad Prism 4 (San Diego, CA). Results are expressed as mean ± SEM. Statistical significance was assessed by two-way ANOVA with Bonferroni post hoc test or Bivariate linear regression (Figure 2E and F).

Results
Animal and Tissue Weights
Total body and heart weights were higher in male than in female rats and increased with age in both sexes (Figure 1A and B, respectively). However, the heart weight-to-body weight ratio was significantly higher in young females compared with that in their male counterparts (Figure 1C). This sex dimorphism has been reported before, and although the exact mechanism how this occur remains unclear, it seems to be dependent on the female hormonal milieu (17, 24). In accordance, although the sex hormone serum levels decreased with age in both sexes (data not shown), only old, compared with young, females exhibited a significant reduction in the heart relative weight (Figure 1C).

Heart Cellular Content and Mitochondrial Mass
Cardiac tissue undergoes a gradual loss of myocytes during ageing (25). We herein examined whether the hearts from old animals experienced a sex-dependent reduction in cellular content by measuring the DNA levels of tissue homogenates. Results indicated that hearts from young female rats exhibited higher cellular content per gram of tissue than their male counterparts (Figure 1D). The same profile was found in old animals although the differences did not reach statistical significance. Both sexes showed a similar decrease in heart cellular content with age; specifically, a reduction of −46.7% and −41.1% was found in old males and females, respectively, compared with the content in young groups.

We next investigated whether the sex- and age-dependent differences in cell content also affected heart mitochondrial mass. For this purpose, total mitochondrial protein content and tissue homogenate CS activities were measured (Figure 2A and B, respectively). The results expressed per gram of tissue showed a significant decrease in both parameters with age, whereas no significant differences were found between sexes (mitochondrial protein content was −61.7% and −60.9%, and CS activity was −52.7% and −59.7% in old—compared with content and activity from young—males and females, respectively), suggesting a lower mitochondrial content in hearts from old—compared with heart from young—animals. However, when results were normalized per DNA content neither total mitochondrial protein nor homogenate CS activity exhibited significant differences between groups (Figure 2C and D, respectively) although, interestingly, old females showed a nonsignificant

![Figure 1](https://academic.oup.com/biomedgerontology/article-abstract/70/11/1360/2605531)
29.8% less mitochondrial protein than old males. These results suggested that the reduction of cardiac mitochondrial mass with ageing was caused by a decrease in cardiac cellular content rather than in mitochondrial content per cell, which was confirmed by the positive correlations found between tissue levels of mitochondrial protein and CS activity versus total DNA (Figure 2E and F, respectively).

All together, the results demonstrated a sex-independent loss of heart mitochondrial mass with age proportional to the reduction in cardiac cells.

Mitochondrial Oxygen Consumption and OXPHOS Activities

Mitochondrial bioenergetic machinery is the primary energy source in cardiac muscle, and defects or deficiencies in this system have been related to heart failure (9–11). We investigated the effects of sex and age in heart mitochondrial function by measuring mitochondrial oxygen consumption using different substrates (pyruvate/malate or succinate) in absence (state 4) or presence (state 3) of ADP, and the enzymatic activity of the complexes I, III, and IV and ATPase.

Respiratory control ratios measured from isolated mitochondria showed no significant differences between groups confirming the proper integrity of mitochondrial preparations (Figure 3A). Cardiac muscle mitochondrial oxygen consumption expressed per gram of tissue was similar between sexes in all conditions investigated, with the exception of a significant decrease in young female—compared with male—mitochondria when succinate was used as substrate in state 3 (Figure 3B). Mitochondrial oxygen consumption was significantly reduced with age both in males and females in all conditions (with decreased percentage values around −50% to −70% depending on the substrates).
When expressed per gram of tissue, no significant differences between sexes were found in the activities of the complexes I, III, and IV (Supplementary Figure 1), whereas the ATPase activity was 34.9% higher in young females compared with that in their male counterparts. On the other hand, the activity of all four complexes was significantly lower in the hearts from old—compared with hearts from young—animals. Of note, the decreased enzymatic activity in old groups was consistently more pronounced in females than in males. In particular, the percent loss of activity in old compared with the activity in young animals was −83.3% versus −79.7% for complex I, −87.3% versus −76.4% for complex III, −45.4% versus −33.9% for complex IV, and −84.9% versus −71.5% for ATPase in females and males, respectively. Collectively, the results demonstrated a significant loss of cardiac mitochondrial function during ageing with further reduced mitochondrial performance in old females than in old males.

Functionality of Cardiac Mitochondria

With the exception of complex IV, the age-associated loss of mitochondrial OXPHOS activity (average activity loss ~80.5%) was more pronounced than the age-related decrease in mitochondrial mass (average decrease ~58.7%; compare results in Figure 2A and B with results in Supplementary Figure 1). These differences suggested that not only the number of mitochondria per gram of tissue would be reduced with age but also that the functionality per unit of mitochondria could also be affected. To examine this possibility, the enzymatic activities of CS and the OXPHOS complexes measured in isolated mitochondria were calculated and expressed per milligram of mitochondrial protein. Results showed no significant differences between sexes in the specific activity (I.U/mg mitochondrial protein) of CS and the complexes I, III, and IV (Figure 4A–D). However, young females exhibited a significant 29.3% higher ATPase activity compared with that in age-matched males (Figure 4E). The specific activity of CS was not altered with age (Figure 4A), whereas complexes I and III and ATPase showed a significant activity loss in the old groups (Figure 4B, C, and E, respectively). Interestingly, complex IV specific activity was higher in mitochondria from old—compared with that in mitochondria from young—animals although the difference did not reach statistical significance (Figure 4D). These results would suggest that the functionality of cardiac mitochondria decreased with age besides the reduction in mitochondrial mass. Of note, similar results were obtained when enzymatic activities were corrected per milligram of DNA (data not shown), confirming that the decreased heart mitochondrial function with age was not only due to a reduction in cellular and mitochondrial content but also to a loss of enzymatic activity per unit of mitochondria. Of importance, the age-dependent decrease in complex III (Figure 4C) and ATPase (Figure 4E) activities was more pronounced in females than in males (~71.7% vs ~51.7% for complex III and ~65.7% vs ~55.3% for ATPase in females and males, respectively), demonstrating a sex-dependent effect in the alteration of mitochondrial function associated with age.

The ratio between ATPase/complex IV activities is a good indicator of the mitochondrial efficiency to produce energy because it relates the phosphorylative with the oxidative mitochondrial capacity. Mitochondria from young females showed a 36.4% higher ATPase/complex IV activity ratio than males (Figure 4F), suggesting that young female mitochondria are more efficient than their male counterparts in agreement with previous studies (17,18). However, age caused a significant reduction in this parameter in both sexes that was exacerbated in old compared with young females (72.6% vs 61.5% decrease in old females and males, respectively).

Together the results demonstrate that the decreased cardiac mitochondrial function with age was not only due to a reduction in the content of these organelles per gram of tissue but also to a loss of function per unit of mitochondria. Furthermore, the age-dependent loss of mitochondrial function affected old females in a greater extent than males.

Mitochondrial Content of β-ATPase, Complex IV-Subunit IV, and TFAM

OXPHOS complexes IV and ATPase are the main regulators of mitochondrial function. Therefore, we next investigated whether the sex- and age-dependent differences in mitochondrial function described earlier could be linked to changes in the content of these proteins. Representative blots and the quantification of heart mitochondrial β-ATPase and complex IV-subunit-IV (COX-IV) protein levels (expressed as a.u/mg mitochondrial protein) are shown in Figure 5. COX-IV content was lower in young female than in males, whereas β-ATPase levels showed nonsignificant differences between sexes (Figure 5B and C, respectively). Old animals of both sexes exhibited a significant decrease in COX-IV protein levels, whereas only female β-ATPase content was reduced with age. Overall, these results revealed sex- and age-related differences in the mitochondria protein levels of these key bioenergetic enzymes.

TFAM is a mitochondrial transcription factor that has been identified as a key regulator of mitochondrial content and biogenesis. In this study, we found no significant differences between sexes in the protein content of TFAM in young animals (Figure 5D). However, old females showed a reduction in the expression levels of TFAM compared with that in both old males and young females. Collectively the results demonstrate a sex- and age-dependent
regulation in the expression levels of heart mitochondrial OXPHOS proteins and TFAM that could account for the differences in mitochondrial function between groups.

Mitochondrial Hydrogen Peroxide Generation

Mitochondrial functional defects such as the ones described in this study could be associated to increased ROS generation and consequently contribute to cardiac disease. This encouraged us to examine the role of sex and age in the generation of mitochondrial H$_2$O$_2$. Complexes I and III from the mitochondrial respiratory chain are the main free radical generator sites within cardiac muscle cells (8,26,27), and using appropriate combinations of respiratory chain substrates and inhibitors, it is possible to measure the generation of H$_2$O$_2$ from each of them separately. Following this approach, we found no significant differences between groups in the basal generation of H$_2$O$_2$ from any complex or in the maximal H$_2$O$_2$ production by complex I (Figure 6A). However, complex III H$_2$O$_2$ production significantly increased in old animals from both sexes. These results were surprising given that the activities of both complexes I and III decreased in old compared with the activities in young groups (Figure 3B and C). Interestingly, when the maximal H$_2$O$_2$ production of complexes I and III was normalized by their enzymatic activity, both complexes showed a significantly higher H$_2$O$_2$ generation in old compared with the same parameter in young groups (Figure 6B and C, respectively). Of importance, the relative-to-the-activity H$_2$O$_2$ production by complex III was significantly higher in old females compared with the same parameter in age-matched males (Figure 6D).

Collectively, the results show a sex- and age-dependent dimorphism in the relative H$_2$O$_2$ generation by mitochondrial respiratory chain complexes I and III.

**Discussion**

Mitochondria are the organelles in charge of producing the energy necessary for the cells and are particularly important for the proper
Figure 5. Quantification of ATPase, complex IV, and TFAM protein content. (A) Representative Western blots of cardiac muscle ATPase (subunit-β), complex IV (subunit-IV, COX-IV), and mitochondrial transcription factor A (TFAM) from young (6 months) and old (24 months) male and female rats. (B–D) Quantification of the densitometry values for COX-IV (B), β-ATPase (C), and TFAM (D) analyzed by Western blot. Values ($n = 5–6$) are expressed as mean ± SEM (net intensity/milligram mitochondrial protein) and are compared with young males as 100%. versus males ($p < .05$), versus young ($#p < .05$, ##$p < .01$, ###$p < .001$).

Figure 6. (A) Effect of sex and age on cardiac mitochondrial $H_2O_2$ generation. $H_2O_2$ production was measured in isolated mitochondria. Maximal $H_2O_2$ generation of complexes I and III was assayed using pyruvate/malate 2.5 mM + rotenone 2 µM (for complex I), or succinate + rotenone 2 µM + antimycin A 5 µM (for complex III). (B and C) The $H_2O_2$ generation versus enzymatic activity was measured for both complexes I (B) and III (C). Values ($n = 6$) are expressed as mean ± SEM. versus males (**$p < .01$), versus young (*$p < .05$, **$p < .01$, ***$p < .001$).
function of highly metabolic tissues such as the heart. Sex differences in cardiac mitochondrial features as well as loss of mitochondrial function with age have been reported before and associated to CVD and heart failure. However, to our knowledge, no studies to date have examined the combined effects of age and sex in mitochondrial bioenergetics. To address this fundamental issue, we have analyzed a number of heart mitochondrial bioenergetic parameters in 6- and 24-month-old rats of both sexes. The findings provide clear evidence for a previous unidentified sex dimorphism in the age-associated defects in cardiac mitochondrial function, highlighting the need for further exploration of the mechanisms responsible for these effects that could help to understand and potentially lead to the development of new therapies to diminish the risk of cardiac disease associated to sex and age.

Histological examination of human hearts by Olivetti and coworkers (25) in the early 90s indicated that loss of cardiac myocytes contribute to the development of myocardial dysfunction and failure in the elderly adults. In this study, we initially investigated the effect of age and sex in rat cardiac cell content. Our findings showed a sex-independent age-associated decrease of ~43.9% in the average cardiac cell content, in line with the reduction of cardiac myocytes found by Olivetti and coworkers (25) in old compared with young individuals. These results prompted us to examine whether the decrease in total cell content by age was associated to a reduction in heart mitochondria that could compromise the cardiac tissue performance (28).

For this purpose, we measured the total mitochondrial protein content and CS activity of heart homogenates as indicators of mitochondrial mass. Both parameters decreased with age in a sex-independent manner, but, interestingly, when expressed per milligram of DNA, these differences disappeared indicating that the loss of mitochondrial mass with age occurs in parallel to a loss in cardiac cell number. These results are in line with two separate studies showing no differences in mitochondrial DNA content related to nuclear DNA content in old compared with the contents in young rat cardiac tissue (29,30).

The age-associated decrease in tissue mitochondria was accompanied by a reduction in mitochondrial functional properties measured as oxygen consumption as well as activity and content of the OXPHOS complexes. Interestingly, the differences in mitochondrial function were more pronounced than those predicted from the loss of tissue cells, indicating that ageing caused not only a decrease in mitochondrial content but also a loss of mitochondrial bioenergetic capacity per unit of mitochondria. These data are consistent with a number of studies reporting that mitochondria from old animals and also humans exhibit reduced functionality compared with young ones (31–34). In addition, our data provide evidence pointing to a sex-dependent effect on the age-associated decrease of mitochondrial function. To be specific, when compared with males, female mitochondria exhibited a further age-associated decrease in the activity and in some cases the content of the OXPHOS complexes as well as in the ratio between ATPase/complex IV activities, a parameter that links the phosphorylative and oxidative capacities of mitochondria, rendering a good indication of the potential capacity of mitochondria to generate energy in form of ATP. Altered mitochondrial function could then partly explain the increased risk of cardiac disease in postmenopausal women. Of importance, it has been recently reported that old female rats lose their resistance to heart ischemia-reperfusion injury compared with young animals (35). The latter seems to be dependent on the hormonal status because female resistance to ischemia-reperfusion injury was also abolished in young ovariectomized females, a procedure known to alter mitochondrial function (35,36).

Changes in tissue mitochondrial content and function, as the ones described in the present study, can be regulated through mitochondrial biogenesis (37). This includes both mitochondrial proliferation (ie, increase in mitochondrial population) and differentiation (ie, increase in functionality of preexisting mitochondria) and requires the coordinated contribution of nuclear and mitochondrial genomes. Mitochondrial biogenesis is regulated by a number of key proteins including TFAM, a mitochondrial transcription factor that controls the expression of mitochondrial proteins codified by mtDNA (38). TFAM protein levels can be finely regulated on demand under different physiological and pathological conditions. As an example, orchestrated changes in TFAM expression and mitochondrial biogenesis occur in the different conceptus tissues (embryo, visceral yolk sac, and placenta) during organogenesis, a period when these tissues undergo profound adaptations to adjust to the varying energy requirements (39–41). In this study, the protein content of TFAM was found to be decreased by age in a sex-dependent manner. Specifically, no differences were found in old—compared with young—male cardiac levels of TFAM, in agreement with a previous study (30). However, old females exhibited a significant reduction in TFAM protein levels compared with that in young animals. Several studies have highlighted the critical role of TFAM for the proper function of the heart because genetically modified rodents depleted or overexpressing TFAM develop severe cardiomyopathies or ameliorate mitochondrial deficiencies and cardiac failure after myocardial infarction, respectively (42–44). The sex-dependent effect in the age-associated decrease in TFAM levels could be related to the altered hormonal milieu because the expression of mitochondrial biogenesis—signaling factors including TFAM has been shown to be influenced by sex hormones in vitro (21).

The alterations in the mitochondrial bioenergetic machinery described earlier could be linked to increased generation of free radicals. The latter might occur when the fine-tuned OXPHOS controlling the electron flux in the respiratory chain is disrupted by these alterations and the electrons accumulate in the system, a process that inevitably results in increased ROS production (7). Our findings demonstrated a significant increase in maximal complex III radical generation with age, whereas basal ROS production from complex I or III was not affected. We have previously reported a strong direct correlation between the activities and the ROS generation by complexes I and III (17); however, in this study, we found that enzymatic activities of both complexes were reduced in the old groups without an associated decrease in H2O2 generation. This could be due to a malfunctioning of the complexes with age, which would explain the impaired activity and increased free radical production. Indeed, defects in the ubiquinol binding site in the vicinity of heme b of cytochrome b have been found during ageing and linked to increased ROS production from complex III (45).

Our findings also revealed an increase in the relative H2O2 generation by complexes I and III in old animals, which was probably due to the reduced activity of the complexes with age. Interestingly, female mitochondria exhibited a further increase in complex III relative H2O2 generation with age compared with that in mitochondria of males. The hormonal milieu might play an important role in the sex effect on radical production because mitochondria from ovariectomized female rats have been shown to increase ROS generation, which can be reversed by estrogen treatment (36,46). These results could help to understand the increase in cardiovascular risk after menopause (2–4) because the altered H2O2 production in mitochondria from old females could lead to increased oxidative stress contributing to impair their cardiac function (47,48).
It has been hypothesized that interventions to decrease mitochondrial ROS generation could potentially affect the risk of CVD (49). Therefore, the development of mitochondrial-targeted drugs to modulate mitochondrial function and ROS production could have important implications for the treatment or prevention of age- and sex-associated CVD (10,43,49–52). However, a better understanding of the mechanisms regulating mitochondrial function and ROS generation in different physiological and pathophysiological conditions will be necessary to identify novel targets for cardiac disease and treatment.

In summary, this study provides conclusive evidence demonstrating a sex dimorphism in the age-associated alterations of cardiac mitochondrial function. In particular, our results indicate that hearts from old female rats exhibit impaired mitochondrial bioenergetics accompanied by increased relative mitochondrial H2O2 generation compared with their male counterparts. The enhanced age-related loss of mitochondrial function and relative ROS production in female hearts could be linked to the increase in cardiac risk described in postmenopausal women, which emphasizes the need for more detailed investigations into the occurrence, mechanisms, and implications of this phenomenon that could help to identify mitochondrial-targeted therapeutic strategies to tackle this issue.

**Supplementary Material**

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/.

**Funding**

This work was supported by the Dirección General de Investigación y Gestión del Plan Nacional de I+D+i (SAF2010-21792) and Fondo de Investigaciones Sanitarias of the Spanish Government (PIO60293). B.C. was supported by a Fellowship from the Spanish Government (FPU).

**Acknowledgment**

We are grateful to Dr. Hidetoshi Inagaki for kindly providing the TFAM donor for superoxide formation by complex III of heart mitochondria.

**References**

28. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria.


