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Caloric restriction and gender modulate cardiac muscle mitochondrial H_2O_2 production and oxidative damage

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Abstract

Objective: Gender and diet have an important effect in cardiovascular disease and other aging-associated disorders, whose initiation and/or worsening seem to be delayed in females from different species and in animals subjected to caloric restriction (CR). The aim of the present study was to investigate whether cardiac muscle bioenergetic mitochondrial features could be responsible for these beneficial effects.
Methods: Fifteen-month-old male and female Wistar rats were fed *ad libitum* or subjected to 40% CR for 3 months. Cardiac mitochondrial function (citrate synthase activity, oxygen consumption), activity of complexes I, III, IV and ATPase of the OXPHOS system, antioxidant activities (MnSOD, GPx), mitochondrial DNA and protein content, mitochondrial H_2O_2 production, heart oxidative damage, complex IV and ATPase content and efficiency, as well as protein levels of mitochondrial transcription factor A (TFAM) and peroxisome-proliferator-activated receptor-gamma co-activator 1 alpha (PGC1α) were measured.
Results: Female and CR rats exhibited lower cardiac mitochondria content, which were more efficient and generated less H_2O_2 than in males and *ad libitum* fed animals, with their consequent lower heart oxidative damage.
Conclusion: Higher mitochondrial differentiation becomes a metabolic adaptation to increase energy efficiency, as what happens in female and CR rats. This adaptation is associated with their lower mitochondrial free radical production and oxidative damage, which could help to understand the mechanism by which these animals exhibit a lower incidence of aging-related disorders, including cardiovascular disease.
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Keywords: Energy metabolism; Gender; Mitochondria; Oxidative phosphorylation; Oxygen radicals

1. Introduction

The link between gender and cardiovascular disease is well documented both in humans and animal models [1–3]. The protection of females against cardiovascular risk is largely attributed to the sex-related hormonal milieu [2], but the specific molecular mechanisms underlying the sex differences remain poorly understood. In this way, dysfunctions in the mitochondrial respiratory chain can led to initiation and/or worsening of cardiac failure, showing the importance of oxidative–phosphorylative processes for the normal function of the heart [4]. The role of mitochondria in cardiovascular disease would involve the generation of reactive oxygen species (ROS), leading to increased oxidative damage [5]. Gender could also have an effect on ROS production, since liver and neurons from female rats exhibit lower mitochondrial H_2O_2 production and oxidative damage compared to males [6]. Furthermore, previous studies in our laboratory performed in rat skeletal muscle, liver and brown adipose tissue, point to a clear gender difference in mitochondrial energy metabolism [7–10], which could be related to a different ROS production. However, there are no similar works performed in cardiac muscle which could relate mitochondrial features with the different incidence of heart diseases between genders.

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Caloric restriction (CR) is the only intervention known to be able to increase lifespan and delay the initiation and propagation of aging associated diseases in several species [11]. It is believed that to achieve this anti-aging phenomenon, CR decreases mitochondrial ROS generation followed by lower oxidative damage in restricted animals [12], although the exact mechanism by which this happens is yet to be determined.

In a previous report, we found that females are more efficient than males in adapting to energy deficient situations [13], thus, the same processes involved in decreasing ROS production by CR could also be related to the gender differences in this parameter. We propose that these mechanisms would be linked to the mitochondrial OXPHOS system, since it is the main ROS generator within the cell [14], and differences in their activities and content have been reported in several tissues both between genders [7–10] and in food restricted animals [15,16].

Taking this background into account, the objective of the present work was to investigate the role of mitochondrial oxidative–phosphorylative machinery in the oxidant production and oxidative damage in rat cardiac muscle of both genders fed *ad libitum* (AL) and calorie restricted, which could be related to the lower incidence of aging-related disorders, including cardiovascular diseases, in females and restricted animals. To tackle this aim, 15-month-old male and female rats were fed *ad libitum* or subjected to 40% CR for 3 months, and several parameters related to mitochondrial content, function, as well as free radical oxidative stress were determined.

2. Materials and methods

The experimental protocols were approved by our institutional ethics committee, and conform with the EU rules (86/609/EEC) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Materials

Routine chemicals were supplied by Sigma-Aldrich (St. Louis, USA), Panreac (Barcelona, Spain) and Amersham Pharmacia Biotech (Little Chalfont, UK). Real-time PCR reagents and Oligonucleotide primer sequences were supplied by Roche Diagnostics (Basel, Switzerland).

2.2. Animals and diet

15-month-old Wistar rats were divided into four experimental groups (6–7 animals per group). Two groups of males and females were fed *ad libitum* with pelleted standard diet (A04, Panlab, Barcelona). Another two groups of males and females were subjected to 40% food restriction for three months. Animals were housed individually with free access to water in wire-bottom cages and acclimated at 22 °C with a 12-h light/dark cycle. Caloric restricted animals were fed on a daily basis at the beginning of the dark cycle and the amount of food was weekly updated compared to AL group.

2.3. Sacrifice and isolation of samples

Animals were sacrificed and the heart was quickly removed, washed and cut into pieces. Heart mitochondria were isolated as follows: samples were homogenized with a Teflon/glass homogenizer in 25 mL of isolation buffer (mannitol 220 mM, sucrose 70 mM, EDTA 1 mM, Tris–HCl 10 mM, pH 7.4)+25 mg of fatty acid free BSA. Nuclei and cell debris were first removed by centrifugation at 500 ×*g* for 10 min at 4 °C. Supernatants were centrifuged twice at 8000 ×*g* for 10 min 4 °C and mitochondrial pellets were resuspended in isolation buffer.

2.4. Total protein, total DNA, mitochondrial protein and mitochondrial DNA

Total DNA was measured in homogenates using the diaminobenzoic acid method [17]. Mitochondrial and total protein were measured by the method of Bradford [18] in mitochondrial preparations and homogenates respectively. Mitochondrial DNA was measured in pre-treated homogenates by real-time PCR as previously described [19] (primers sequence: 5′-TACACGATGAGGCAACCAAA-3′; 5′-GGTAGGGGGTGTGTTGTGAG-3′).

2.5. Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured using a Clark-type O_2 electrode (Oxygraph, Hansatech, UK) at 37 °C. Respiration medium consisted of KCl 145 mM,

Table 1
Body and heart weights of male and female *ad libitum* fed and caloric restricted rats

	Males		Females		Statistics
	Ad libitum	Restricted	*Ad libitum*	Restricted	
Initial weight (g)	559±18	559±38	363±8	362±16	G
Final weight (g)	579±17	392±17 [a]	350±14 [b]	240±15 [a, b]	G, R, GxR
Heart weight (g)	1.16±0.03	0.92±0.07	0.80±0.03	0.66±0.05	G, R
Relative heart weight (g/kg)	2.01±0.07	2.31±0.06	2.30±0.05	2.75±0.10	G, R

Data represent the mean±SEM of six to seven independent experiments. Significant differences are from two-way ANOVA ($p<0.05$) and Student's t-test *post hoc* analysis. G, effect of gender; R, effect of food restriction; GxR, interactive effect.

[a] Compared to *ad libitum*.

[b] Compared to males.

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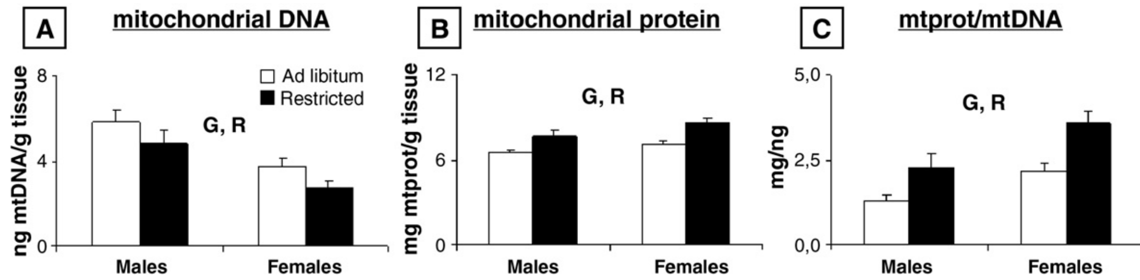

Fig. 1. mtDNA and protein content of cardiac muscle mitochondria. (A) Mitochondrial DNA (mtDNA) was measured by real-time PCR in heart homogenates. (B) Mitochondrial protein (mtprot) was determined in cardiac mitochondrial preparations. (C) Ratio between mitochondrial protein and DNA content. Data represent the mean ± SEM of six to seven independent experiments and significant differences are from two- way ANOVA ($p<0.05$). G, effect of gender; R, effect of food restriction.

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Hepes 30 mM, KH_2PO_4 5 mM, $MgCl_2$ 3 mM, EGTA 0.1 mM, BSA 0.1% at pH 7.4. 0.3 mg of mitochondrial protein per millilitre of medium was used. Mitochondrial respiratory state4 was assayed with pyruvate/malate 2.5 mM or succinate 5 mM (plus rotenone 2 μM) as substrates. State3 activated respiration was measured by adding ADP 0.5 mM to the medium.

2.6. Mitochondrial H_2O_2 production rates

H_2O_2 production was assayed in mitochondria by measuring the increase in fluorescence (530 nm excitation, 590 nm emission) due to the reaction of Amplex Red reagent (Molecular probes) with H_2O_2 in the presence of horseradish peroxidase. Assays were performed at 37 °C during 30 min in a 96-well microplate fluorimeter (FLx800, Bio-tek instruments). Mitochondria (0.2 mg protein/mL) were added to the same medium used for respiration and supplemented with horseradish peroxidase 0.1 U/mL and Amplex Red reagent 50 μM. State4 was induced by addition of pyruvate/malate 2.5 mM or succinate 5 mM. All the measurements with succinate were determined in the presence of rotenone 2 μM to prevent the reversal electron flux to complex I. State3 was measured using the same substrates than state4 plus addition of ADP 0.5 mM. Maximal H_2O_2 generation rates of complexes I and III were measured as described previously [15], using pyruvate/malate + rotenone 2 μM for complex I, and succinate + antimycin A 5 μM for complex III. The rate of H_2O_2 production was calculated using a standard curve of H_2O_2 generated by addition of β-D(+)glucose 14 mM in the presence of glucose oxidase.

2.7. Enzymatic activities

The enzymatic activities of complexes I [20], III [21], IV [22] and ATPase [20] of the OXPHOS system were measured in mitochondrial preparations. Glutathione peroxidase (GPx) and mitochondrial superoxide dismutase (MnSOD) were determined in homogenates and mitochondria respectively [23,24]. Citrate synthase activities were assayed in homogenates [25]. All the activities were performed in a 96-well microplate spectrophotometer (Biotek instruments, USA).

2.8. Protein content by western blotting

Samples were loaded into polyacrylamide SDS-PAGE gels to estimate the protein content of complex IV subunit II (K-20, Sc-23984; Santa Cruz Biotechnology) and subunit IV (MS407, Mitosciences), F1-β-ATPase (C-20, Sc-16690; Santa Cruz Biotechnology), TFAM (antiserum provided by Dr. Hidetoshi Inagaki), PGC1α (K-15, Sc-5816; Santa Cruz Biotechnology) and Pan-Actin Ab5 (ACTN05, MS-1295-PO; Neomarkers). Proteins were electrotransferred onto a nitrocellulose filter (Biorad, California, US). A ponceau S staining was performed in order to check correct loading and electrophoretic transfer before incubating with the corresponding antibodies or

Table 2
Cardiac muscle citrate synthase activity and mitochondrial oxygen consumption in male and female *ad libitum* fed and caloric restricted rats

		Males		Females		Statistics
		Ad libitum	Restricted	*Ad libitum*	Restricted	
Citrate synthase activity (I.U./g tissue)		712 ± 40	639 ± 97	716 ± 67	728 ± 137	NS
Pyruvate/malate (nmols O_2/mg mitoch prot·min)	S4	28.2 ± 3.1	31.4 ± 1.2	26.4 ± 3.4	31.2 ± 3.3	NS
	S3	164 ± 23	193 ± 4	152 ± 25	144 ± 14	NS
Succinate (nmols O_2/mg mitoch prot·min)	S4	76.2 ± 10.3	94.2 ± 7.2	65.6 ± 8.1	74.2 ± 8.3	NS
	S3	162 ± 17	160 ± 11	155 ± 21	159 ± 20	NS

Date represent the mean ± SEM of 4–6 (citrate synthase activity) and 6–7 (oxygen consumption) independent experiments. The statistical analysis was two-way ANOVA ($p<0.05$). Citrate synthase activity was measured in heart homogenates and expressed as I.U. (μmol/min) per gram of tissue. Oxygen consumption was measured using a Clark-type O_2 electrode and is expressed as nmols O_2/mg mitochondrial protein·min. A final concentration of 0.3 mg of mitochondrial protein was used for the measurements. Mitochondrial respiratory state 4 (S4) was assayed with pyruvate/malate 2.5 mM or succinate 5 mM (plus rotenone 2 μM) as substrates. State 3 (S3) activated respiration was also measured by addition of ADP 0.5 mM to the medium. NS, not significant.

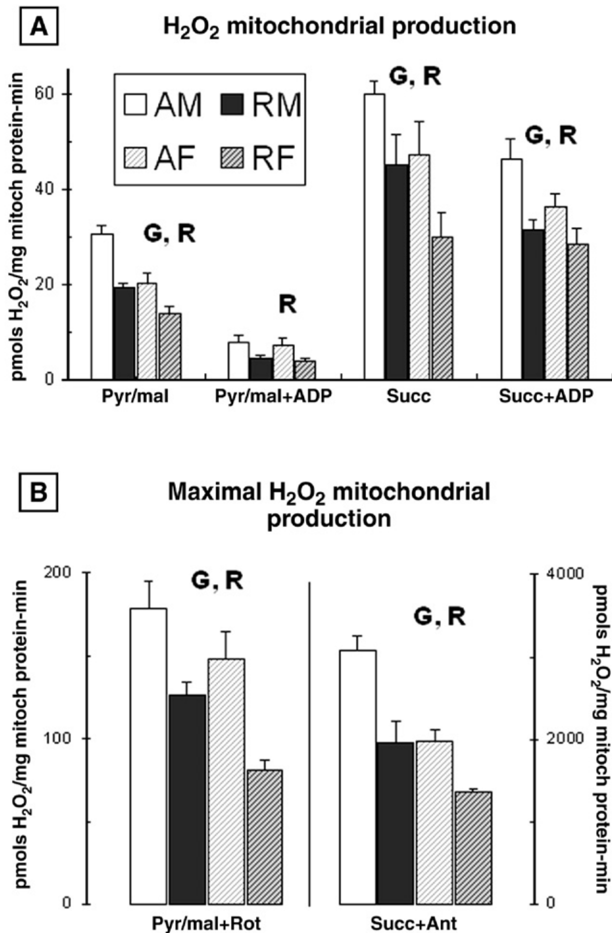

Fig. 2. Effect of gender and caloric restriction on cardiac muscle mitochondrial H_2O_2 production. (A) Measurements were assayed in isolated mitochondria using pyruvate/malate or succinate (plus rotenone) in the absence (state 4) and the presence (state 3) of ADP. (B) Maximum H_2O_2 generation of complex I and III was assayed using specific inhibitors (rotenone and antimycin A respectively). Data represent the mean ± SEM of five to six independent experiments and significant differences are from two-way ANOVA ($p<0.05$). G, effect of gender; R, effect of food restriction. AM, fed *ad libitum* males; RM, restricted males; AF, fed *ad libitum* females; RF, restricted females.

antiserum. Immunoblot development was performed using an enhanced chemiluminescence western blotting analysis system (Amersham, UK). Film blots were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software).

2.9. Protein carbonyl content

Protein carbonyl content of heart homogenates was measured by western blot using the Oxyblot™ Protein Oxidation Detection Kit (S7150, Chemicon Int).

2.10. Statistics

Results are expressed as mean ± SEM. Differences between groups were assessed by two-way ANOVA, followed by Student's *t*-test *post hoc* analysis ($p<0.05$) only when an interactive effect between CR and gender was shown. Bivariate linear regression was used in Fig. 6. Statistical analyses were performed with SPSS 13.0.

3. Results

3.1. Animal and tissue weights

Body and heart weights were lower in females compared to males as expected, whereas the heart-to-body weight was higher in females (Table 1). This gender dimorphism in the rat heart relative size has been described formerly and related to the different hormonal milieu [26,27].

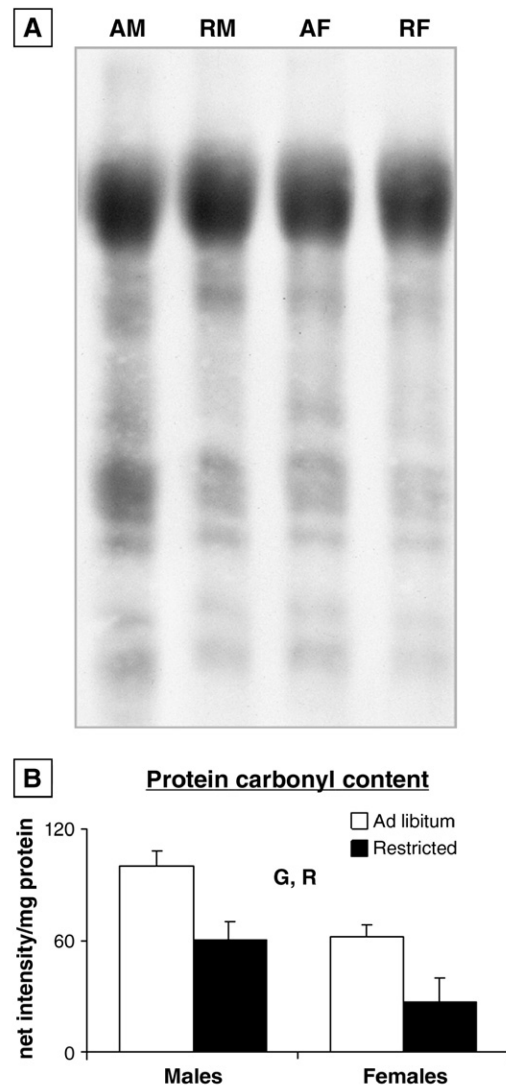

Fig. 3. Effect of gender and caloric restriction on cardiac muscle oxidative damage. (A) Representative blots of protein carbonyl groups in heart homogenates from male and female *ad libitum* fed (AM, AF) and food restricted (RM, RF) rats. (B) Heart protein carbonyl content per mg of total protein. The intensities of the whole lanes were used to quantify the protein carbonyl content. Data are expressed as net intensity per mg of protein setting *ad libitum* fed males as 100% ± SEM ($n=5$). Significant differences are from two-way ANOVA ($p<0.05$). G, effect of gender; R, effect of food restriction.

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Table 3
Cardiac muscle mitochondrial OXPHOS activities in male and female *ad libitum* fed and caloric restricted rats

	Males		Females		Statistics
	Ad libitum	Restricted	*Ad libitum*	Restricted	
Complex I (I.U./mg mitoch prot)	0.57±0.02	0.45±0.02	0.54±0.04	0.38±0.02	R
Complex III (I.U./mg mitoch prot)	14.1±1.4	5.7±0.9 [a]	6.1±0.6 [b]	3.1±0.3 [a, b]	G, R, GxR
Complex IV (a.u./mg mitoch prot)	1.42±0.17	1.75±0.28	0.73±0.04	0.67±0.07	G
ATPase (I.U./mg mitoch prot)	5.41±0.52	4.12±0.28	4.82±0.58	3.54±0.35	R

Specific activities of complexes I, III, IV and ATPase of the OXPHOS (oxidative phosphorylation) system were assayed in mitochondrial preparations. Data represent the mean±SEM in I.U. (μmol/min·mg of mitochondrial protein, except complex IV activity, which is expressed in arbitrary units/min·mg of mitochondrial protein). *N* values were 4–6 for complexes I, III and ATPase, and 6–7 for complex IV. Statistics were analyzed by two-way ANOVA ($p<0.05$) and Student's *t*-test *post hoc* analysis. G, effect of gender; R, effect of food restriction; GxR, interactive effect.

[a] Compared to *ad libitum*.

[b] Compared to males.

Three months of CR decreased total body weight (−32.3% and −31.4% in males and females respectively), with significant differences with respect to AL groups from the 3rd week after starting the diet (data not shown). CR also decreased the heart weight with a −20.7% and −17.5% in males and females. On the contrary, the relative-to-body heart weights increased by CR due to the different percentage of loss weight between body and heart. This difference was caused by a higher selective weight loss of body fat by CR, in a similar way as we have previously described in younger rats [13].

3.2. Heart mitochondrial DNA and protein

Mitochondrial content and function are key parameters to understanding the energy metabolism of a tissue. In the present work, we found that females showed lower mtDNA and higher mtprotein per gram of heart than males (Fig. 1). These results would suggest that female cardiac muscle exhibits fewer mitochondria than males, but they would have a higher mass, as illustrated by the ratio between mtprotein/mtDNA. Differences between mitochondrial content and mass can be used as a maker of mitochondrial differentiation [8,28]. Our results in cardiac mitochondria agree with those found in other tissues [7–10], which show a more differentiated mitochondrial pool in females compared to males.

Animals subjected to CR showed a decrease in heart mtDNA content (Fig. 1), together with an increase in their mitochondrial differentiation (mtprotein/g tissue and mtprotein/mtDNA).

3.3. Mitochondrial functional parameters

Citrate synthase activity (I.U./g tissue) measured in heart homogenates and mitochondrial oxygen consumption (nmols O_2/mg mtprotein·min) were used as markers of mitochondrial function. Results are presented in Table 2 and show no significant differences between genders in citrate synthase activity or oxygen consumption at any of the conditions used in the experiment (*i.e.* pyr/mal or succinate as substrates in both resting and phosphorylating states 4 and 3).

Mitochondrial function was not altered by CR, since it did not modify cardiac muscle mitochondrial oxygen consumption or citrate synthase activity compared to AL animals (Table 2).

3.4. Mitochondrial H_2O_2 production rates and heart oxidative damage

In this work, we measured cardiac mitochondrial H_2O_2 generation using succinate and pyruvate/malate as substrates in both state 4 (absence of ADP) and state 3 (presence of ADP), and the results, expressed as pmols of H_2O_2/min·mg mitochondrial protein, are illustrated in Fig. 2A. Heart mitochondria from AL fed females compared to males generated less H_2O_2 in all conditions studied, although it did not reach statistical significance in state 3 with pyr/mal as substrate. To test whether the lower oxidant production in females was accompanied by a lower oxidative damage, we determined and compared the protein carbonyl groups in heart homogenates of male and female rats. As illustrated in Fig. 3, females showed fewer carbonyl groups than males, supporting

Table 4
Cardiac muscle antioxidant enzymatic activities in male and female *ad libitum* fed and caloric restricted rats

	Males		Females		Statistics
	Ad libitum	Restricted	*Ad libitum*	Restricted	
MnSOD (I.U./mg mitoch prot)	1.03±0.09	1.17±0.17	1.05±0.19	1.18±0.11	NS
GPx (I.U./mg homog prot)	1.05±0.07	0.86±0.05	0.74±0.02	0.54±0.05	G, R

Specific activities of superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) were assayed in cardiac muscle mitochondria and homogenates respectively and expressed in I.U. (μmol/min·mg of protein). Data represent the mean±SEM of 5 to 6 independent experiments. Values were analyzed by two-way ANOVA ($p<0.05$). G, effect of gender; R, effect of food restriction; NS, not significant.

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the idea that the attenuation of cardiac oxidative damage in females may result from their lower oxidant production. Animals subjected to CR showed a lower heart mitochondrial H_2O_2 generation in all conditions performed in the experiment, together with a decrease in their heart oxidative damage.

3.5. Maximal mitochondrial H_2O_2 generation and enzymatic activities of complexes I and III

As we found lower mitochondrial H_2O_2 production in heart of females than males and restricted rats, we decided to go further and investigate the role of respiratory chain complexes I and III in these differences, since they are the maximum H_2O_2 generator sites in cardiac mitochondria [14,29]. Using appropriate combinations of respiratory chain substrates and inhibitors it is possible to measure the maximal H_2O_2 production of both complexes separately [15]. Results in Fig. 2B show that maximal H_2O_2 production of heart mitochondrial complexes I and III was lower in females compared to males and was decreased by CR.

Furthermore, we decided to test whether the enzymatic activities of these complexes were responsible for their lower H_2O_2 production. Specific enzymatic activity of complex III was lower in females than males, and CR decreased the activities of both complexes (Table 3). These data confirm the role of complexes I and III in the lower oxidative stress and oxidative damage found in females and restricted animals.

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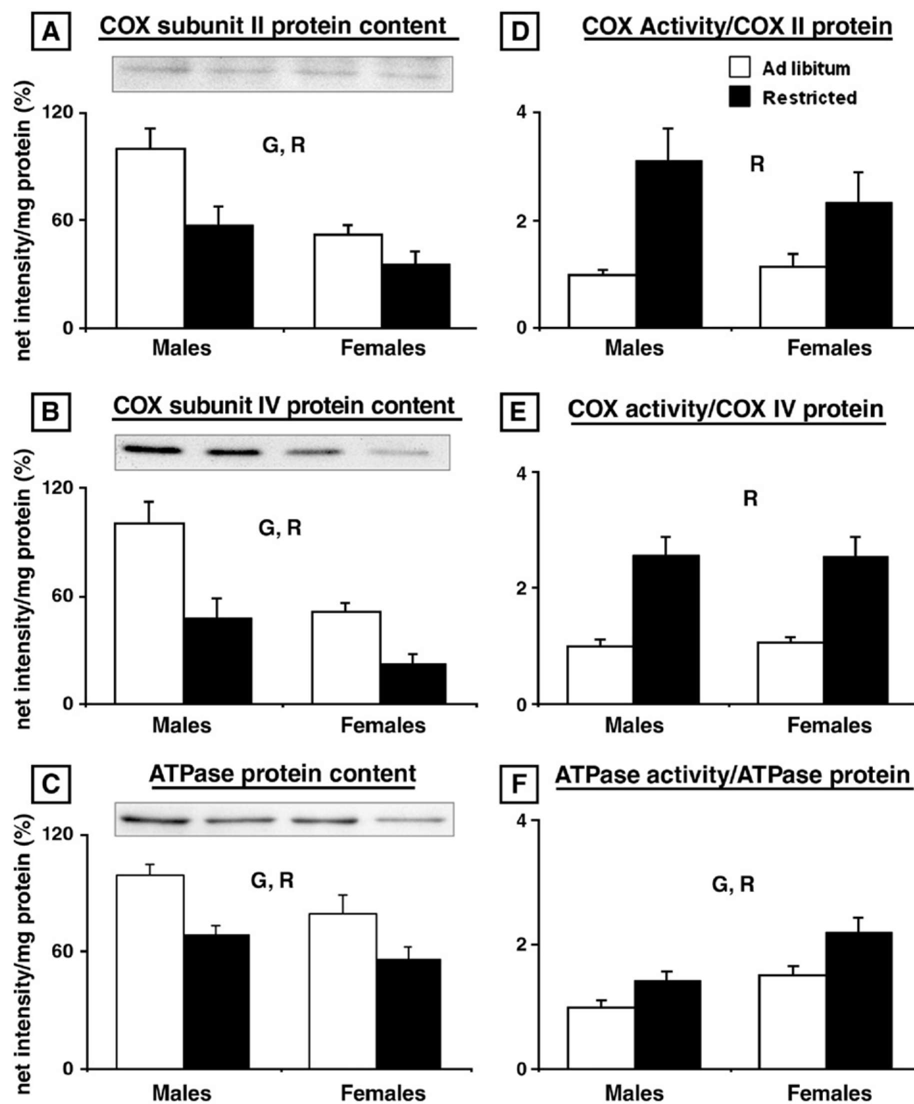


Fig. 4. Complex IV and ATPase protein content and efficiencies. Protein content of complexes IV (COX) subunits II (A), IV (B), and F_1-β-ATPase (C) was measured by western blot in cardiac mitochondria. Data are expressed as net intensity per mg of mitochondrial protein setting *ad libitum* fed males as 100%± SEM. *N* values were 5–6 for COX subunit II and 6–7 for COX subunit IV and ATPase. Enzyme efficiencies are expressed as the enzyme activity *vs* protein content estimated by western blot, taking *ad libitum* male as 1±SEM. (D) complex IV activity *vs* complex IV subunit II protein content; (E) complex IV activity *vs* complex IV subunit IV protein content; (F) ATPase activity *vs* F_1-β-ATPase protein content. Significant differences are from two-way ANOVA ($p<0.05$). G, effect of gender; R, effect of food restriction.

3.6. Antioxidant enzymatic activities

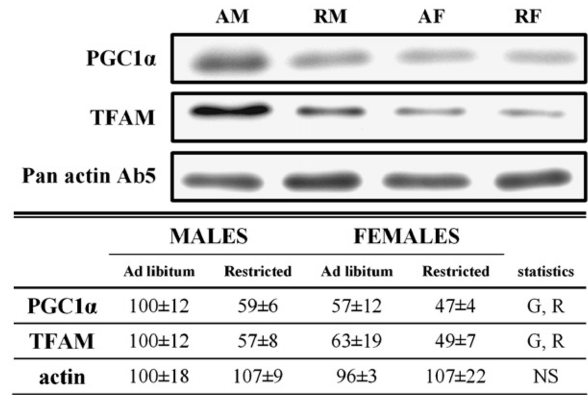
Oxidative damage can be caused by an increase in oxidant production, and also by a decrease in oxidant scavengers. The significance of antioxidant enzymes in the modulation of oxidative damage has been previously suggested [6,30], however, the controversy found in the literature regarding antioxidant activities has failed to describe any clear-cut overall pattern concerning the role of gender and CR in their regulation [12,31]. The present results in heart mitochondria demonstrated a lower GPx activity in females than males which decreased by CR, whereas no significant differences were found in MnSOD activities (Table 4).

3.7. Enzymatic activity, protein content and efficiency of complex IV and ATPase

Dysfunctions in any of the OXPHOS complexes could lead to impaired mitochondrial function and increased cardiovascular disease [32]. As we found lower heart enzymatic activities of complexes I and III by gender and CR, we decided to study the activities of complex IV and ATPase, which are the main regulators of mitochondrial function. Complex IV activities were lower in females than males and ATPase activities decreased in restricted animals (Table 3). We also measured the content of these enzymes, which were lower in females than males and reduced by CR, as illustrated in Fig. 4A and B (for complex IV subunits II and IV, respectively) and Fig. 4C (for ATPase). The lower OXPHOS complex contents and activities in females and restricted animals seem to be linked to their lower oxidative damage, but they could also cause impaired heart mitochondrial function. However, we did not find any difference in mitochondrial function between groups in this study (see above), which could be explained by an increase in efficiency of respiratory and phosphorylative machinery. Thus, the enzyme efficiency was calculated as the ratio between the enzymatic activity and its protein content measured by western blot. The results showed that phosphorylative efficiency was higher in females than males (Fig. 4F), and that CR increased both the oxidative and phosphorylative efficiency, since complex IV (Fig. 4D, for the complex IV subunit II; and 4E, for the complex IV subunit IV) and ATPase (Fig. 4F) efficiencies were higher than in fed AL rats. The increased OXPHOS efficiency could explain why there is no difference in mitochondrial function between groups, despite the lower complex IV and ATPase protein contents in females and restricted animals.

3.8. PCG1α and TFAM protein contents

PGC1α and TFAM have been identified as key regulators of mitochondrial content and functional capacity [33–35]. Along the same lines, we have previously reported a gender-related increased TFAM and mitochondrial content in rat liver and skeletal muscle of females [8,10]. The present



	MALES		FEMALES		
	Ad libitum	Restricted	Ad libitum	Restricted	statistics
PGC1α	100±12	59±6	57±12	47±4	G, R
TFAM	100±12	57±8	63±19	49±7	G, R
actin	100±18	107±9	96±3	107±22	NS

Fig. 5. Protein content of PGC1-α, TFAM and pan-actin Ab5. Protein content of PGC1-α, TFAM and pan-actin Ab5 (actin) was measured in heart homogenates. Figure shows representative blots of the different proteins from male and female *ad libitum* fed (AM, AF) and food restricted (RM, RF) rats. Values are expressed as net intensity per mg of protein setting *ad libitum* fed males as 100%±SEM of four to six independent experiments. Significant differences are from two-way ANOVA ($p<0.05$). G, effect of gender; R, effect of food restriction; NS, not significant.

results in cardiac muscle showed that PGC1α and TFAM protein content were lower in females compared to males and decreased in restricted animals (Fig. 5), in accordance with their lower mtDNA content and enzymatic oxidative–phosphorylative capacities.

4. Discussion

On the whole, our results show a lower mitochondrial hydrogen peroxide production in cardiac muscle from female rats and restricted animals, which entail lower heart oxidative damage compared to males and AL fed animals. Nevertheless, they also exhibit a lower mitochondrial content, without loss of mitochondrial functionality, due to their higher oxidative–phosphorylative efficiency, which indicates a greater mitochondria differentiation level in cardiac muscle of females and restricted animals.

4.1. Gender differences in ad libitum fed animals

Sex hormones have been postulated to be responsible for the lower incidence of cardiovascular disease in females than males, described both in humans and rodents [1–3]. Besides, there appears to be a close relationship between the initiation and/or worsening of these diseases and high free radical generation as well as oxidative damage in cardiac muscle [5,36]. According to the previous data, our results show lower heart oxidative damage in females compared to males, which is probably due to their lower mitochondrial H_2O_2 generation. Furthermore, cardiac muscle oxidative damage has been found to increase in castrated females [37], which could indicate that sex hormones may play a key role in regulating the production of these oxidant molecules. Accordingly, an increase in mitochondrial H_2O_2 generation

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has been reported in tissues such as liver and brain from ovariectomized female rats [6], and the H_2O_2 production reverted to the normal levels when these ovariectomized rats were treated with estrogens.

Respiratory chain complexes I and III are the main mitochondrial free radical generators within the cell [14,29], and their activities correlate directly with their radical production as illustrated in Fig. 6. These data agree with previous works that suggest a relationship between complex I content and mitochondrial ROS production [15,38]. On the other hand, our results would rule the antioxidant enzymes out as being responsible for the lower ROS production in females and restricted rats, as previously suggested [31]. All in all, our data would confirm that the lower oxidative damage in cardiac muscle of females is principally due to a lower ROS production by complexes I and III, and not to a higher antioxidant capacity.

The present data also show that cardiac mitochondrial differentiation is higher in females than males. This would be linked to a higher female mitochondrial functional efficiency, and also to a lower H_2O_2 generation, since the more efficient respiratory chain the less electron accumulation in their complexes, with the consequent decrease in free radical production [39]. The sex-related dimorphism in mitochondrial differentiation has been previously reported by our laboratory in other rat tissues such as liver [8], skeletal muscle [10], and brown adipose tissue [7,9]. However, contrary to these other tissues, cardiac muscle from females showed a lower mitochondrial content compared to males, although it did not imply a lower cardiac mitochondrial functionality. The latter was probably due to their higher phosphorylative efficiency, which allowed them to retain the oxygen consumption rate at the same level as males.

The lower mitochondrial content in females could also be related to their lower TFAM and PGC1α protein levels, which are key factors in the process of mitochondrial biogenesis [40]. Their content could be modulated by the different hormonal milieu between genders, since TFAM and PGC1α expression has been recently reported to be regulated by sex hormones in cultured brown adipocytes [41]. Regulation of these proteins is critical for the proper function of the heart, as TFAM and PGC1α genetically modified rodents develop severe cardiomyopathies [34,35].

4.2. Differences by caloric restriction diet

A decrease in free radical production by CR has been described in a wide range of species and tissues [11,12]. The present results agree with these reports and show a lower generation of H_2O_2 in heart mitochondria from restricted rats. As a result of this decreased oxidant production, these animals exhibit lower heart oxidative damage, which could be related to their longer life expectancy, since CR delays the initiation of different aging-related disorders like cardiovascular disease, among others [11,12].

In a similar way as what happened in the abovementioned differences between genders, the decreased H_2O_2 mitochondrial production by CR was related to the lower activity of their respiratory chain complexes I and III, and not to an increase in their antioxidant activity. Therefore, these results suggest that both complexes would be responsible for the decreased mitochondrial H_2O_2 generation in restricted animals, unlike other authors who point out complex I as the only cause of this lower radical production [42].

Cardiac muscle from restricted animals exhibited lower mtDNA content compared to fed AL, which is in accordance with their lower TFAM and PGC1α protein content. These differences were similar to those found between genders, and, as what happened in that case, the lower mitochondrial content was not accompanied by loss of mitochondrial functional capacity either. In agreement with this, no decrease in heart ATP production and content has been found between genders and restricted rats [43,44]. All these data could be explained by the presence in restricted animals of more differentiated cardiac muscle mitochondria, which would exhibit higher oxidative and/or phosphorylative efficiencies (as shown in Fig. 4D, E and F). The higher complex IV efficiency in response to CR has already been

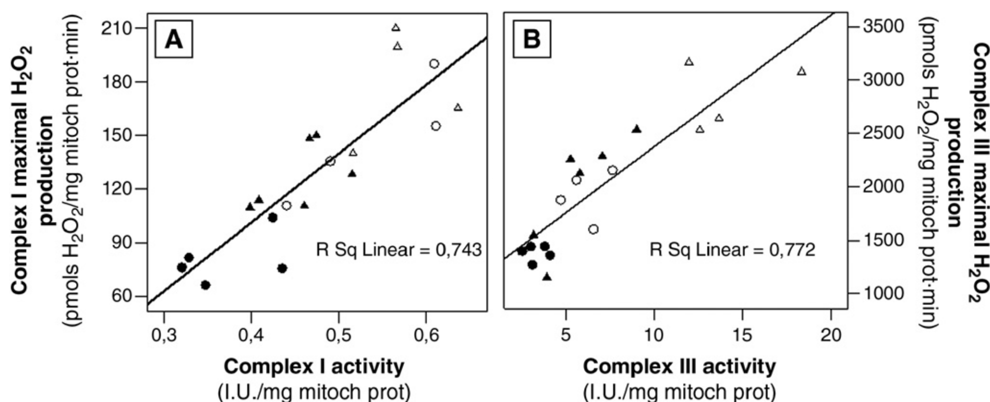

Fig. 6. Linear bivariate regression analysis. Bivariate linear regressions were calculated between maximal H_2O_2 production rates of complexes I (A) and III (B), measured separately, and their enzymatic activities. Open and closed triangles represent *ad libitum* and restricted males, open and closed circles represent *ad libitum* and restricted females.

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described in skeletal muscle [45,46], and is due to an increase in complex IV high-affinity binding sites. These changes would maintain the mitochondrial oxidative capacity of restricted animals by increasing the affinity of oxygen to complex IV. Furthermore, it would improve the electron flux through the respiratory chain, which would decrease the degree of reduction of its complexes, and, since this redox status is related to the free radical production [39], it would cause a decrease in the mitochondrial ROS generation. So, as a whole, this mechanism would lead to a lower cardiac muscle mitochondrial free radical production in rats subjected to CR, without a loss of mitochondrial functionality.

In summary, cardiac muscle from female and caloric restricted rats exhibits lower mitochondrial content, although more differentiated, without any loss of functionality, when compared to male and AL fed rats. These differences confer mitochondria from females and restricted animals the ability to generate fewer free radicals, which leads to lower heart oxidative damage in these animals. The results of the present work could be related to the lower incidence of cardiovascular disease in females than males, as well as to the delayed initiation of aging-associated disorders in animals subjected to caloric restriction.

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