ARTÍCULO

Reduction in mitochondrial membrane peroxidizability index and protein lipoxidation levels in the rat heart after ß-adrenergic receptor signaling interruption with the ß-blocker atenolol

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ABSTRACT

A new mammalian longevity model based on ß-adrenergic signaling interruption at the level of adenylyl cyclase has reported decreased bone and heart aging and mean and maximum longevity increases in AC5KO mice (1). We decided to mimic this model in male Wistar rats treated with the ß-blocker atenolol in the drinking water and to check if an oxidative stress decrease could be involved. Atenolol treatment did not modify heart mitROS generation rate and mitDNA oxidative damage but significantly decreased global peroxidizability index of mitochondrial membranes, as well as protein lipoxidation, probably mediated by changes in elongases and desaturases activities.

Keywords: Oxidative stress; β-blocker; Lipid damage.

RESUMEN

Reducción en el índice de peroxidizabilidad de la membrana mitocondrial y niveles de lipoxidación proteícos en el corazón de la rata después de la interrupción de la señalización del receptor betaadrenérgico con el betabloqueante atenolol

Un nuevo modelo de longevidad en mamíferos basado en la interrupción de la vía de señalización beta-adrenérgica a nivel de la adenilato ciclasa ha revelado una

ralentización del envejecimiento del corazón y el hueso de ratones AC5KO y un incremento de su longevidad media y máxima (1). Decidimos mimetizar este modelo en ratas Wistar utilizando atenolol en el agua de bebida para comprobar si un descenso de estrés oxidativo podría estar implicado. El tratamiento no modificó la tasa de generación de radicales y el daño oxidativo al ADN del corazón, pero si redujo el índice de peroxidizabilidad y la lipoxidación proteica de las membranas mitocondriales, probablemente debido a cambios en las actividades elongasas y desaturasas.

Palabras clave: Estrés oxidativo; β-bloqueante; Daño lipídico.

1. INTRODUCTION

The mitochondrial free radical theory of aging is currently supported by both experimental and comparative studies. From these comparative studies, there are two main factors that can contribute to explain the lower aging rate of long-lived species: a low generation rate of mitochondrial reactive oxygen species (mitROS) (2,3) and a low membrane fatty acid unsaturation degree (4,5).

Experimental manipulations like dietary or caloric restriction (CR), protein restriction and methionine restriction (metR) increase longevity in different kind of animals mainly decreasing the mitROS generation rate (6-9), whereas these manipulations show minor effects on the fatty acid unsaturation degree in most of these cases (10).

However, modulation of lipid biosynthesis contributes to stress resistance and longevity in C. elegans mutants, by reduction in lipid peroxidation substrates and shortening fatty-acid chain length to maintain membrane fluidity (11). The relationship between membrane fatty acid composition and longevity has been observed in all the animal models studied, including mammals, birds, rodents, honeybees and humans (12,13). A lower total number of double bounds of membrane fatty acids makes these molecules more resistant to lipid peroxidation. Highly unsaturated fatty acids like arachidonic acid (20:4n-6) and specially docosahexaenoic acid (22:6n-3) exhibit the highest sensitivity to ROS induced oxidative damage, their sensitivity increases as a function of the number of double bonds per fatty acid molecule (14,15) and long-lived animal species strongly avoid their presence in their tissue cellular membranes through tight homeostatic species-specific regulation. (5,13,16)

Different mammalian models of extended lifespan by gene-mutation have been recently described (17,18). Most of them are related to insulin/IGF-1-like signaling pathways (19), but there are others like ribosomal S6 protein kinase 1 (S6K1) (20) and AC5KO (1) that can also increase medium and maximum longevity.

In the AC5KO model, extension of lifespan in 129/SvJ-C57BL/6 mice has been obtained through the disruption of the β-adrenergic receptor signaling at the Type 5 adenylyl cyclase (AC5) level (1). This AC5KO knockout mouse showed increased mean and maximum longevity, from 25 to 33 months and from 33 to 37 months, respectively; and also showed improvements in the cardiomyopathy and bone deterioration related to aging. The disruption of AC5 preserved cardiac function in response to chronic pressure-overload and catecholamine stress, which is known to play a major role in heart failure development (21). These improvements seemed to be signaled in the cell due to an increase in the Raf/MEK/ extracellular signal-regulated kinase (ERK) pathway as suggested by increases in the amount of the p-MEK and p-ERK in various tissues, including heart, of AC5 KO mice (1). These mice also had higher levels of the antioxidant enzyme manganese superoxide dismutase (MnSOD) in heart, kidney and brain, suggesting that a decrease in oxidative stress could be involved in the mechanisms responsible for the aging delay effect.

Taking all these into account, it was interesting to test if the two main oxidative stress-linked factors related to longevity, the mitROS generation rate and the fatty acid unsaturation degree, were also lowered in animals with β-adrenergic receptor blocking. Atenolol is a second generation β-blocker with selectivity for cardiac β1 receptors. β1 receptors are predominantly located in cardiac tissue and primarily stimulated by norepinephrine; they are coupled to AC through a stimulatory G protein. From the different AC subtypes, AC5 is the main type in cardiac tissue. Therefore, blocking the β1-receptors can afford a fast and convenient model that could potentially mimic the phenotype of the AC5KO rodents. In the present study we tested the effect of the β1-selective blocker atenolol in Wistar rats (genetically heterogeneous animals, like human beings) to investigate if this drug also increases Raf/MEK/ERK signaling and whether or not the beneficial effects of β1-receptor blocking are also due to decreases in oxidative stress as it has been initially suggested.(1) We hypothesized that atenolol also increases Raf/MEK/ERK signalling in rats and that the mechanism responsible for its effects includes lowering one or both known factors related to longevity: mitROS production and the degree of fatty acid unsaturation. The possible beneficial effects of atenolol could be important because atenolol treatment in humans would be easier to implement than dietary restriction models.

In the present investigation, and for that purpose, we measured the mitochondrial ROS generation rate, mitochondrial oxygen consumption in states 4 (resting) and 3 (phosphorylating), the percent free radical leak (%FRL) in the respiratory chain, the respiratory complex I to IV amounts, and the content of the antioxidant enzyme MnSOD. We also studied the apoptosis-inducing factor (AIF) because it can stimulate apoptosis, but it is also required for the

assembly/maintenance of complex I (22), the respiratory complex at which mitROS generation is lowered in long-lived animals (23) as well as in rats subjected to dietary restriction (24). The marker of oxidative damage to mitochondrial DNA (mtDNA) 8-oxo-7,8-dihydro-2o-deoxyguanosine (8-oxodG) was analyzed by high-performance liquid chromatography (HPLC), and five oxidative damage markers of protein oxidation -the specific protein carbonyls glutamic and aminoadipic semialdehydes (GSA and AASA) indicating purely protein oxidative modification, the protein glycoxidation markers carboxyethyl lysine (CEL) and carboxymethyl lysine (CML), and the protein lipoxidation marker malondialdehyde lysine (MDAL) were measured in the rat heart by highly specific and sensitive mass spectrometry techniques. 8-oxodG in mtDNA (25) and MDA-lysine (26) are known to be correlated to longevity also and in the correct sense. We also measured the full fatty acid composition of heart mitochondria membranes to calculate the global degree of unsaturation, and because protein lipoxidation is secondarily influenced by lipid peroxidation, which strongly depends on membrane fatty acid unsaturation degree. Additionally, we estimated the elongase and desaturase activities to determinate their role in the acyl chain length and trying to clarify the mechanism responsible for putative changes in the fatty acid unsaturation level elicited by atenolol.

Finally, in our study we decided to analyze SIRT3 and SIRT5 in order to clarify if they could be involved in the signaling mechanisms resulting in the life-extending effect of ß-adrenergic receptor blocking. Sirtuin proteins initially identified in lower organisms seem to be also implicated in cellular signaling of longevity extension in mammals. Seven sirtuins (SIRT1-7) have been described in mammals (27) and they seem to have important roles in aging, stress resistance and metabolic regulation. In the present experiment, we have analyze SIRT3 and SIRT5, due to their localization at mitochondria. Beyond other metabolic actions, SIRT3 has been recently shown to control the levels of mitROS by multiple mechanisms , it is induced by DR and it has been proposed that these increases lower the rate of mitROS production (28). SIRT5 is located in the mitochondrial matrix and intermembrane space, and it can deacetylate cytochrome c, a protein of the mitochondrial intermembrane space with a central function in oxidative metabolism, as well as apoptosis (29). Most of the parameters measured in this investigation have not been assayed in the AC5KO longevity-extended model. Therefore, the present study can contribute to clarify whether mitochondrial or membrane oxidative stress is involved in the mechanism responsible for the increased life span elicited by β-adrenergic blockade.

2. MATERIALS AND METHODS

Animals and diets

Six week-old male Wistar rats were obtained from Harlan Laboratories (Unide, Italy). The animals were caged individually and maintained in a 12:12 (light-dark) cycle, 22ºC ± 2ºC and 50% ± 10% relative humidity at the animal house of the Complutense University. Sixteen male rats were fed ad libitum with a standard rodent diet (Panlab, Spain) and were divided into two groups of 8 animals each: CONTROL and ATENOLOL. The animals in the atenolol group had free access to a 1 g/L atenolol solution (Sigma, A7655) in the drinking water. Previous studies have used that dose of atenolol to cause blockade of β-adrenergic receptors in rats (30). The control group had free access to the same aliquot of drinking water without atenolol. The mean water intake per animal per day was not significantly different between both groups. After 2 weeks of treatment, the animals were sacrificed by decapitation. Hearts were immediately processed to isolate functional mitochondria, which were used to measure mitochondrial respiration and rates ROS generation rates. The remaining mitochondria were stored at -80ºC for posterior analyses. These experiments in Wistar rats were approved by the Animal Experimental Committee from the Complutense University. The present investigation conforms to the Directive 2010/63/EU of the European Parlament.

Isolation of functional mitochondria, oxygen consumption and ROS production

Mitochondria were obtained from fresh tissue by the procedure of Mela and Seitz (31) with modifications. After checking the functionality and phosphorylation capacity of the mitochondria (high respiratory control ratios) the rate of mtROSp was measured by the fluorometric method established at our laboratory (32). The oxygen consumption rate of heart mitochondria was measured at 37ºC in a water-thermostatized incubation chamber with a computer-controlled Clark-type O2 electrode (Oxygraph, Hansatech, UK).

Oxidative damage to mtDNA (8-oxodG)

Isolation of mtDNA was performed by the method of Latorre and cols (33) adapted to mammals (34). The isolated mitochondrial DNA was digested to deoxynucleoside level and the level of oxidative damage in mtDNA was estimated by measuring the amount of 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodG) referred to that of the non-oxidized base (deoxyguanosine, dG) by HPLC-EC as previously described (35).

Measurement of mitochondrial complexes I, II, III and IV, AIF, MnSOD, SIRT3 and SIRT5

The amounts of a) the mitochondrial respiratory chain complexes (I to IV), the complex I regulatory factor AIF, the mitochondrial biogenesis protein

indicators SIRT3 and SIRT5 and MnSOD were estimated using western blot analyses as previously described (36).

Oxidation-derived protein damage markers

GSA, AASA, CML, CEL and MDAL were determined as trifluoroacetic acid methyl esters (TFAME) derivatives in acid hydrolyzed delipidated and reduced mitochondrial protein samples by GC/MS (32) using a HP6890 Series II gas chromatograph (Agilent, Barcelona, Spain) with a MSD5973A Series detector and a 7683 Series automatic injector, a HP-5MS column (30-m x 0.25-mm x 0.25-μm), and the described temperature program (32). The amounts of product were expressed as μmoles of GSA, AASA, CML, CEL or MDAL per mol of lysine.

Fatty acid analyses and global fatty acid unsaturation indexes

Fatty acids from mitochondrial lipids were analyzed as methyl esters derivatives by gas chromatography (GC) as previously described (33). The following fatty acyl indices were also calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA) from n-3 and n-6 series (PUFAn-3 and PUFAn-6); and average chain length (ACL)=((Σ%Total$_{14}$ x 14) + (Σ% Total$_{16}$×16) + (Σ%Total$_{18}$×18) + (Σ%Total$_{20}$×20) + (Σ% Total$_{22}$×22) + (Σ% Total$_{24}$×24))/100. The density of double bonds in the membrane was calculated by the Double Bond Index, DBI = ((1×Σmol% monoenoic) + (2×Σmol% dienoic) + (3×Σmol% trienoic) + (4×Σmol% tetraenoic) + (5×Σmol% pentaenoic) + (6×Σmol% hexaenoic)). Finally, the membrane susceptibility to peroxidation was calculated by the Peroxidizability Index, PI= ((0.025×Σmol% monoenoic) + (1×Σmol% dienoic) + (2×Σmol% trienoic) + (4×Σmol% tetraenoic) + (6×Σmol% pentaenoic) + (8×Σmol% hexaenoic)).

Statistics

Data were analyzed by Student-t tests. The minimum level of statistical significance was set at $P< 0.05$ in all the analyses.

3. RESULTS

The mean body weight of the animals did not show significant differences between the two experimental groups at the beginning of the experiment (172 ±2.45 g in the control and 175 ±2.67 g in the atenolol group). No significant differences in body weight were observed after two weeks of treatment with atenolol either (299 ±4.09 g in the control and 301 ±4.75 g in the atenolol group). No significant differences in heart weight and food or water intake were found between the atenolol and the control groups (data not shown).

The rate of oxygen consumption from heart mitochondria was measured in the absence (state 4) and in the presence (state 3) of 500μM ADP, with complex I-

linked (glutamate/malate) and complex II-linked (succinate + rotenone) substrates (Table 1). No significant differences were detected with both substrates in the state 4 and 3 between atenolol and control groups. However, the rate of oxygen consumption was higher in the phosphorylating state than in the resting state with all the substrates used, indicating the good functionality and tight coupling of the mitochondria.

Table 1.- Rates of oxygen consumption of heart mitochondria from control or atenolol treated Wistar rats.

	CONTROL	ATENOLOL
Glutamate/malate (State 4)	60.8 ± 8.3	60.0±4.8
Glutamate/malate (State 3)	203.1±30.4	189.2±11.8
Glutamate/malate (RCI)	3.5±0.5	3.2±0.3
Succinate+rotenone (State 4)	135.9±9.4	135.1±11.3
Succinate+rotenone (State3)	357.3±22.7	364.2±31.9
Succinate+rotenone (RCI)	2.7±0.1	2.7±0.1

Values are means ± SEM (nmoles of O_2/ min ·mg protein) from 6-8 different samples per group. State 4: oxygen consumption in the absence of ADP. State 3: oxygen consumption in the presence of 500μM ADP. RCI: Respiratory control index.

The mitochondrial ROS (mitROS) generation rate showed a non-significant trend to decrease in the atenolol group with complex I-linked substrates (glutamate/malate) but not with succinate+rotenone (Table 2). Maximum rates of ROS generation were assayed in the presence of pyruvate/malate+rotenone (maximum complex I ROS production) and succinate+rotenone+antimycin A (maximum complex III ROS production). No significant differences between groups were found either for maximum complex I or complex III generation rates.

Table 2.- Basal and maximum rates of ROS production of heart mitochondria from control or atenolol treated Wistar rats in the presence of different substrates and inhibitors of the mitochondrial respiratory chain.

	CONTROL	ATENOLOL
Glutamate/malate	0.125±0.02	0.076±0.02
Succinate	1.08±0.24	1.37±0.40
Succinate+rotenone	1.34±0.25	1.43±0.26
Glutamate/malate+rotenone	2.67±0.47	2.98±0.51
Succinate+rotenone+Antimycin A	7.04±0.85	6.96±0.83

Values are means ± SEM (nmoles of H_2O_2/ min mg protein) from 6-8 different samples per group. Values with glutamate/malate+rotenone or succinate+rotenone+antimycin A estimate maximum mitochondrial ROS production from complex I and III respectively. The rest of the values represent basal rates of ROS generation at complex I (with glutamate/malate in forward and with succinate in reverse electron flow) or at complex III (with succinate, and with succinate+rotenone).

The free radical leak (FRL; the percentage of total electron flow in the respiratory chain directed to ROS generation) values of heart mitochondria did not show significant differences between the control and the atenolol group either with pyruvate/malate or succinate+rotenone as substrates (Table 3). Oxidative damage to mtDNA was estimated by measuring the amount of 8-oxodG referred to the amount of the non-oxidized deoxynucleoside (dG) (Table 3). In agreement with the lack of changes in FRL% and mitROS production, we did not observe significant differences in 8-oxodG between the control and the atenolol group.

Table 3.- Free radical leak (FRL%) at the mitochondrial respiratory chain and oxidative damage to mitochondrial DNA of heart mitochondria from control or atenolol treated Wistar rats.

	CONTROL	ATENOLOL
FRL % (glutamate/malate)	0.11±0.03	0.06±0.01
FRL % (succinate+rotenone)	0.56±0.13	0.58±0.10
8-oxodG in mtDNA	6.46±1.07	8.65±1.25

Values are means ± SEM (nmoles of H_2O_2/ min · mg protein) from 6-8 different samples per group. The FRL% is the percentage of the total electron flow in the respiratory chain directed to oxygen radical generation (see Materials and Methods for further details). It represents the efficiency of the mitochondria avoiding the univalent lateral leak of electrons out of the respiratory chain that generates ROS. The lower the FRL%, the higher such efficiency. 8-oxodG is a marker of steady-state oxidative damage to mtDNA and is expressed as 8-oxodG/10^5dG.

The amounts of the two complex I subunits (NDUFS3 and NDUFA9), complex II, III and IV were measured, as well as AIF (apoptosis inducing factor), SOD2 (superoxide dismutase), SIRT3 and SIRT5 (Figure 1). The NDUFA9 complex I subunit and MnSOD were significantly lower in the atenolol group. The other parameters did not show significant differences between experimental groups.

The markers of protein glycoxidation CEL and CML were significantly higher in the atenolol group (Figure 2). On the other hand, the lipoxidation-dependent marker of protein modification MDAL was significantly lower in atenolol treated animals, and the specific protein carbonyls GSA and AASA did not show significant differences (Figure 2).

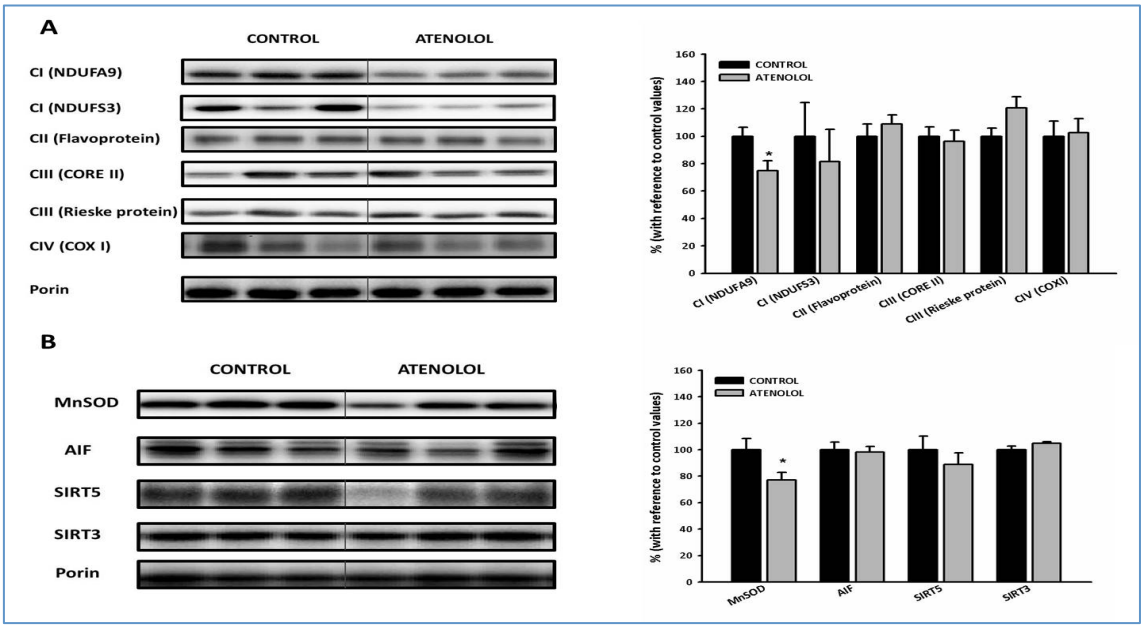

Figure 1.- Mitochondrial ETC complexes, MnSOD, AIF, SIRT3 and SIRT5 from control and atenolol treated Wistar rats. A, left: Representative immunoblots showing relative levels of mitochondrial complex subunits from complex I to IV of heart mitochondria from control and atenolol treated Wistar rats; right: The relative values were obtained by densitometric analyses. The intensity of each product was related to the control protein porin. B, left: Representative immunoblots showing relative levels of regulatory factors of mitochondrial biology of heart mitochondria from control and atenolol treated wistar rats; right: The relative values were obtained by densitometric analyses. The intensity of each product was related to the control protein porin. Values are means ± SEM from 6-7 diferent samples per group. Asterisks represent significant differences compared to the control group: * P<0.05.

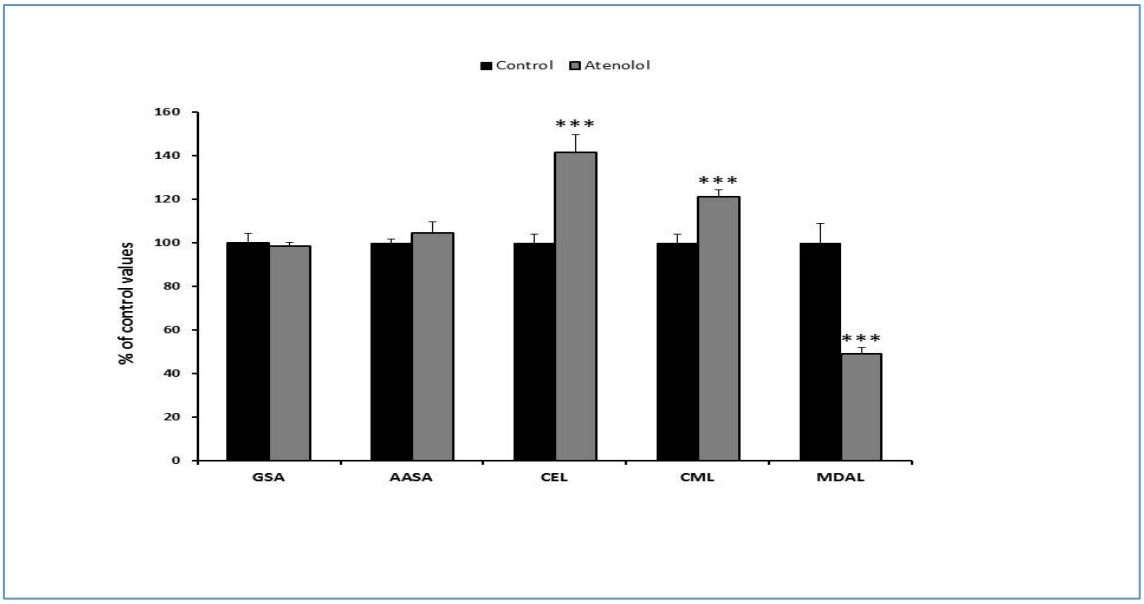

Figure 2.- Protein oxidation, glycoxidation and lipoxidation markers of heart mitochondria from control and atenolol treated Wistar rats. Values are means ± SEM from 8 different samples and are expressed as percentage from control group. Control values: 5276.91±238.38 (Glutamic SemiAldehyde, GSA); 229.67±4.31 (AASA, AminoAdipic SemiAldehyde, AASA); 605.79±23.36 (CarboxyEthyl-Lysine, CEL); 1558.30±62.06 (CarboxyMethyl-Lysine, CML); 584.47±51.77 (MalonDiAldehyde-Lysine, MDAL). Units: µmol/mol lysine. Asterisks represent significant differences between control and atenolol rats: *** P<0.001.

The fatty acid composition of heart mitochondria lipids and derived indexes were also measured (Table 4). The fatty acids 18:0, 20:4n-6, 22:0, 22:4n-6, 22:5n-6, 22:5n-3, 22:6n-3 and 24:0 were significantly lower and the fatty acid 18:2n-6 was significantly higher in the atenolol group. Due to these changes the acyl chain length decreased (ACL; 0.66% total decrease), the PUFAn-3 strongly decreased (27.61% total decrease) and the PUFAn-6 increased (12.31 % total increase). The final result was a strong decrease in the peroxidizability index (PI; 15.20% total decrease) and a non-significant trend to decrease the total number of double bonds (6.94% total decrease in the double bond index, DBI) in the atenolol group in relation to the control group ($P<0.01$ in PI; Figure 3).

The estimation of the desaturase and elongase activities is shown in Tables 5 and 6. The desaturase activity Δ9 (n-9) was higher, while Δ5 (n-6) was lower in the atenolol group. Integrated n-6 and n-3 desaturation and elongation activities were also lower in the atenolol group. The elongase activities ELOVL 1/3, ELOVL 5 (n-6), ELOVL 2/5 (n-6) and ELOVL 2/5 (n-3) were significantly lower in the atenolol group.

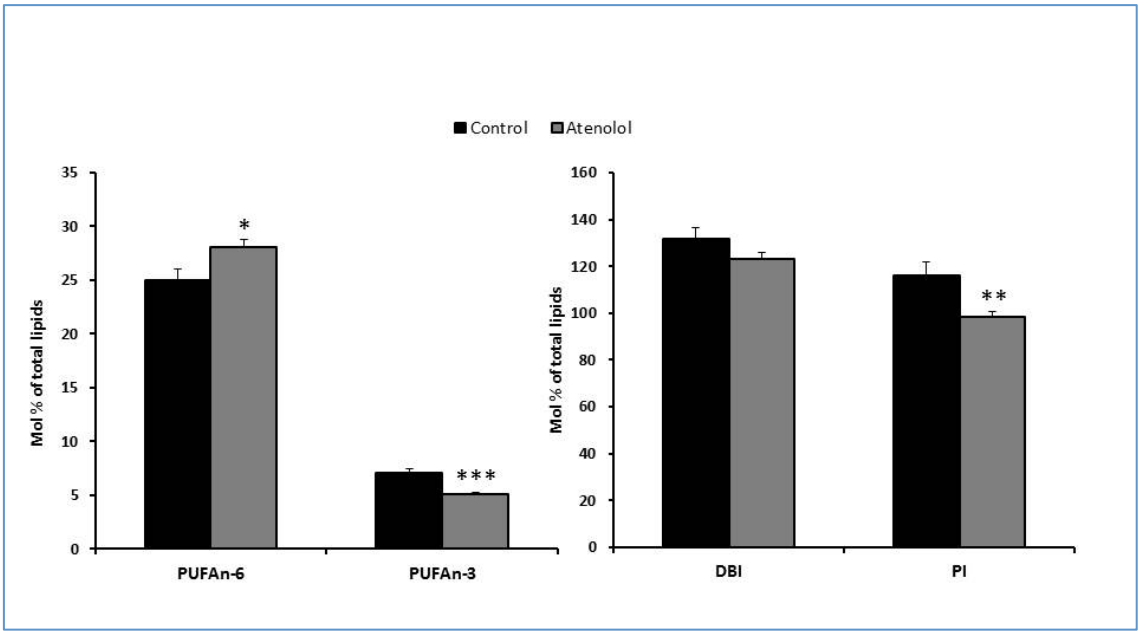

Figure 3.- PI and DBI (fatty acid unsaturation degree) and total PUFAn-6 and PUFAn-3 of heart mitochondria from control and atenolol treated Wistar rats. PUFA= polyunsaturated fatty acid; DBI=double bond index; PI= Peroxidizability index. Abbreviations and calculations of fatty acid classes and indexes are explained in the Materials and Methods section. Control values: 116.22±5.49 (PI); 131.93±4.70 (DBI); and 7.10±0.39 (for PUFAn-3) and 25.01±0.97 (for PUFAn-6), both expressed as mol %. Values are means ± SEM from 8 different samples per group. Asterisks represent significant differences compared to the control group: * $P<0.05$; ** $P<0.01$; ***$P<0.001$.

Table 4.- Fatty acyl composition (mol %) of total lipids of the heart mitochondria from control and atenolol treated Wistar rats.

	CONTROL	ATENOLOL
14:0	1.29±0.05	1.23±0.05
16:0	24.77±0.77	24.61±0.70
16:1n-7	1.14±0.05	1.18±0.04
18:0	25.45±0.22	24.28±0.22**
18:1n-9	14.29±0.31	14.65±0.24
18:2n-6	10.89±0.55	15.51±0.65***
18:3n-3	0.39±0.01	0.41±0.03
18:4n-3	0.13±0.01	0.13±0.01
20:0	0.18±0.01	0.18±0.01
20:1n-9	0.28±0.05	0.23±0.03
20:2n-6	0.93±0.03	1.01±0.03
20:3n-6	0.76±0.02	0.84±0.03
20:4n-6	11.61±0.48	10.16±0.26*
20:5n-3	0.14±0.01	0.15±0.01
22:0	0.24±0.02	0.19±0.01**
22:4n-6	0.34±0.03	0.23±0.02**
22:5n-6	0.35±0.03	0.20±0.01***
22:5n-3	1.04±0.05	0.88±0.04*
22:6n-3	5.52±0.43	3.69±0.15***
24:0	0.24±0.01	0.20±0.01**
ACL	18.02±0.04	17.90±0.02*
SFA	52.17±0.97	50.69±0.89
UFA	47.82±0.97	49.30±0.89
MUFA	15.71±0.37	16.06±0.29
PUFA	32.11±1.24	33.24±0.76

Values are means ± SEM from 8 different samples. ACL, acyl chain length, SFA, saturated fatty acids, UFA unsaturated fatty acids, MUFA, monounsaturated fatty acids, PUFA polyunsaturated fatty acids. Asterisks represent significant differences between the control and the atenolol group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table 5.- Estimation of desaturase activities of heart mitochondria from control or atenolol treated Wistar rats.

DESATURASES		Control	Atenolol
Δ9 (n-7)	**C16:1/C16:0**	0.046±0.001	0.048±0.002
Δ9 (n-9)	**C18:1/C18:0**	0.561±0.010	0.604±0.013*
Δ8 (n-6)	**C20:3/C20:2**	0.828±0.044	0.846±0.025
Δ5 (n-6)	**C20:4/C20:3**	15.387±0.795	12.123±0.493**
n-6*	**C22:5/C18:2**	0.099±0.009	0.058±0.004**
n-3*	**C22:6/C18:3**	14.289±1.494	9.141±0.566**

* Integrated desaturase/elongase activities for the n-6 and n-3 series.

Table 6.- Estimation of elongase activities of heart mitochondria from control or atenolol treated Wistar rats.

ELONGASES		Control	Atenolol
ELOVL 3 (n-9)	**C20:1/C18:1**	0.019±0.003	0.015±0.001
ELOVL 6	**C18:0/C16:0**	1.033±0.029	0.991±0.023
ELOVL 1/3	**C20:0/C18:0**	0.007±0.001	0.007±0.001
ELOVL 1/3	**C22:0/C20:0**	1.371±0.048	1.018±0.051***
ELOVL 1/3	**C24:0/C22:0**	1.392±0.096	1.077±0.036**
ELOVL 5 (n-6)	**C20:2/C18:2**	0.086±0.004	0.065±0.003**
ELOVL 2/5 (n-6)	**C22:4/C20:4**	0.028±0.001	0.023±0.001*
ELOVL 2/5 (n-3)	**C22:5/C20:5**	7.508±0.418	6.071±0.443*

4. DISCUSSION

In the present investigation the effect of the selective β-1 blocker atenolol on oxidative stress related parameters was studied in the Wistar rat heart for the first time. This drug, which has been used in humans for decades without known important side effects, and with beneficial effects like reduced mortality and morbidity reported in many clinical studies (37), decreased the fatty acid unsaturation degree, protein lipoxidation levels and desaturase activities after two weeks of treatment. The reported decrease in fatty acid unsaturation and oxidative stress after interruption of β-adrenergic receptor signalling induced by atenolol could be one of the mechanisms, among other, responsible for the longevity increase observed in 129/SvJ-C57BL/6 AC5KO mice (1). Atenolol decreased the heart fatty acid unsaturation degree of atenolol-treated C57BL/6 mice, reducing DBI, PI and total PUFA. Atenolol treatment was able to lower protein oxidation and lipoxidation and to increase p-ERK levels in those mice too (38). In the present investigation we wanted to clarify if atenolol has the same effects in genetically

heterogeneous animals like the Wistar rat, and to ascertain whether β-adrenergic blockade, which increases longevity (1), decreases oxidative stress.

Atenolol treatment did not modify body weight, heart weight or animal food intake discarding the possibility that the observed changes could be secondary effects of caloric restriction. In our study, atenolol treatment did not change either complex I or III mitROS generation rate, with their specific substrates glutamate/malate and pyruvate/malate and the inhibitors rotenone and antimicyn A. These results agree with previous studies (38), and are in contrast to dietary, protein and methionine restriction models in which mitROS generation decreases at complex I (10). Many studies from different laboratories have shown that dietary restriction lowers mitROS generation (39). After testing restriction of different diet components, we concluded that only protein restriction decreased mitROS production (8) and methionine was the aminoacid responsible for it (9). Dietary, protein and methionine restriction also increased maximum longevity. Thus β-adrenergic blockade does not seem to follow the same pattern as these three types of restriction and probably the increase in longevity observed in AC5 KO mice would not be related to a reduced mitROS generation rate. In relation to the lack of effect of atenolol treatment on mitROS generation, the level of 8-oxodG in mtDNA (which indicates the balance between mtDNA oxidative damage and repair) did not change in the heart of atenolol treated rats. Both parameters, mitROS generation and 8-oxodG levels in mtDNA, change together and in similar direction in different models of dietary restriction studied and both are lower in long-lived compared to short-lived animal species (40).

Since atenolol did not modify mitROS generation or mtDNA oxidative damage, we focused on the other oxidative stress longevity-related parameter: the fatty acid unsaturation degree. Membrane phospholipids are susceptible to oxidative alterations due to physico-chemical properties of the membrane bilayer, in which oxygen and free radicals are more soluble than in the aqueous medium . For this reason membrane lipids are highly sensitive to oxidative damage. On the other hand, PUFA residues of phospholipids are extremely sensitive to oxidation, and this sensitivity increases exponentially as a function of the number of double bonds per fatty acid molecule (15). It has been observed in many different animal species (5) that the total number of double bonds (DBI) and the peroxidizability index (PI) from membrane fatty acids are lower in long-lived than in short-lived animals. A low membrane fatty acid unsaturation degree is also present in extraordinarily long-lived animals like birds (41,42), naked mole-rats (43), echidna (44) and queen honeybees (45). This also occurs in long-lived wild-derived strains of mice compared to genetically heterogeneous laboratory mice (46). In our study, atenolol treatment significantly decreased the PI (15.20% total decrease) and tended to decrease the DBI (6.49% total decrease) in the rat heart.

It is known that the low DBI observed in long-lived species is due to changes in the type of unsaturated fatty acids in the membrane composition. There is a systematic redistribution between the type of PUFAs present from the highly unsaturated docosahexaenoic (22:6n-3) and sometimes arachidonic (20:4n-6) acids in short-lived species to the less unsaturated linoleic acid (18:2n-6) and, in some cases, linolenic acid (18:3n-3) in the long-lived ones at mitochondrial and tissue levels (16). Among these, the fatty acid contributing most to decrease the global fatty acid unsaturation degree in long-lived animals is 22:6n-3. With the purpose of checking if similar changes occur in our model, we measured the full fatty acid composition of heart mitochondria membranes. We found that atenolol treatment in the Wistar rat heart leads to variations in several fatty acids, but among them, the most important, quantitatively, was the decrease in the highly unsaturated 22:6n-3, which was responsible to a great extent for the decrease in the PI. This fatty acid is present in tissue cellular membranes at lower levels in long-lived than in short-lived animals, including the long-lived naked mole-rats (47) and it also decreases after atenolol treatment in C57BL/6 mice (38). In the present study, moreover, we found other atenolol-induced decreases in fatty acids which were involved in the decrease of fatty acid unsaturation (lower PI): decreases in 20:4n-6, 22:4n-6, 22:5n-6, 22:5n-3 and 22:6n-3, whereas 18:2n-6 increased. It is also interesting that in the senescence-accelerated mouse (SAM) strain, the SAM-prone mice had greater levels of the highly polyunsaturated peroxidation-prone fatty acids 22:6 n-3 and 20:4n-6 and lower levels of the less peroxidation-prone 18:2n-6 PUFA in their membranes, and consequently they had a greater PI than the SAM-resistant mice (48,49). SAM-prone mice also showed greater degree of lipid peroxides in their tissues than SAM-resistant mice (50).

What are the consequences of this decrease in PI? A low PI (sensitivity to peroxidation) and DBI (double bound index) confer higher resistance of membranes to lipid peroxidation and lower lipoxidation-dependent damage to macromolecules. Previous studies have shown that the heart of long-lived animals has lower levels of MDAL, a specific marker of lipoxidation-dependent damage to proteins, compared to short-lived species (26). In the present study the fatty acid unsaturation decrease (lower PI) after atenolol treatment correlated with a remarkable decrease in MDAL (49% lower in the atenolol group). Lipid peroxidation generates products like MDAL or hydroxynonenal, but it also produces secondary free radicals. The decreased fatty acid unsaturation degree in the atenolol group could thus be responsible for a lower lipid-derived secondary free radical formation, decreased specific lipoxidation markers like MDAL and damage to other macromolecules (51).

On the other hand, there were no changes in the protein oxidation markers measured (GSA and AASA), but in contrast, protein glycoxidation, quantified as the

level of CEL and CML**,** was increased in the heart after atenolol treatment. These results are in agreement with previous experiments in mice where increases in CEL and CML were also observed (38). Several studies have reported dissociation between markers of lipoxidation and those of glycoxidation or pure oxidation. Thus, increasing fatty acid unsaturation in the rat heart by dietary manipulation strongly elevated MDAL, whereas CML (which can be formed by lipoxidation and glycoxidation) was only slightly elevated (52). On the other hand, MDAL negatively correlated with longevity in the heart of mammals (26), but no correlation between longevity and heart CML or CEL levels was observed in the same investigation. Our results do not clarify why CML and CEL were increased. The formation of those protein adducts could involve chemical reaction with oxidized fragments coming from carbohydrates like glucose, but they may also be formed at a high rate from glycolytic intermediates (40). It is possible that the atenolol treatment increased the concentration of glycolytic intermediates, and that could explain why the glycoxidation markers were increased in our experiment.

Polyunsaturated fatty acids are generally synthesized by modification of saturated fatty acid precursors that are products of fatty acid synthase. This process is catalized by two kind of specific enzymes: desaturases and elongases. The enzymatic steps of microsomal fatty acid elongation involve the addition of two-carbon units to a fatty acyl-CoA employing malonyl-CoA as the donor and NADPH as the reducing agent. To date, seven ELOVL proteins (elongase enzymes referred to as Elongation of very-long-chain fatty acids) have been identified, with ELOVL1, 3, 6 and 7 preferring saturated and monounsaturated fatty acids as substrate and ELOVL2, 4 and 5 being selective for polyunsaturated fatty acids (PUFAs). All ELOVL proteins contain several stretches of amino acids that are fully conserved in mice, rats and humans (53). The desaturase enzymes, which are also highly conserved, insert double bonds at specific carbon atoms (the Δ number indicates the position at which the double bond is introduced) in the fatty acid chain and the fatty acid elongation system elongates the precursors in two-carbon increments (54). The fatty acid desaturation pathway and the deacylation-reacylation cycle are the main mechanisms responsible for the fatty acid composition of cell membranes.

In the present investigation we estimated the desaturase and elongase activities. The Δ5 (n-6) activity was lower in the atenolol group , as it has also been reported in long-lived species, which show several fold lower Δ5 and Δ6 desaturase activities than short-lived ones (16,55). This can explain why 22:6n-3 and 20:4n-6 decrease, and 18:2n-6 and 18:3n-3 increase from short to long-lived animals, since desaturases are the rate-limiting enzymes of the n-3 and n-6 pathways synthesizing the highly unsaturated PUFAs 20:4n-6 and 22:6n-3 from their dietary precursors, 18:2n-6 and 18:3n-3, respectively. In our study the global

n-6 and n-3 desaturases were also significantly lower in the atenolol treated animals. This fact can explain the fatty acid unsaturation decrease in the atenolol group. Desaturation pathways would make available in situ the n-6 and n-3 fatty acids to phospholipid acyltransferases in order to remodel the phospholipid acyl groups. The fact that acyltransferase/n-6 desaturase activity ratio is about 10:1 in tissues (56) reinforces the idea that regulation of desaturases can be the main limiting factor responsible for the observed fatty acid unsaturation-longevity relationship (40), as well as for the fatty acid changes observed in the present study. On the other hand, in this study we found a decrease in the following elongase activities in the atenolol group: ELOVL 1/3, which catalyzes the formation of saturated fatty acids containing as many as 26-carbons; ELOVL 5 (n-6), that catalyzes the initial and rate-limiting desaturation of C18:2n-6 and C18:3n-3 for the production of longer-chain PUFAs; and ELOVL 2/5 (n-6) and (n-3) which are involved in PUFA elongation from C20:3n-6 and C20:4n-3. The lower elongase activities in atenolol treated animals may be responsible for the decrease in the acyl chain length in this group.

An intact respiratory chain is needed to get the maximum capacity available for mitochondrial energy production. Besides, studies in rodents show that the amount of respiratory complexes in the respiratory chain can change in experimental modifications that extend life-span. Therefore, we measured the amounts of the four respiratory complexes in our study. There were no differences in the amount of any of the complexes except in the NDUFA9 complex I subunit, which was lower in the atenolol group. In previous studies of methionine restriction (57) and caloric restriction (58) it has been observed that the amount of complex I was decreased. It is well known that both complex I and complex III produce ROS in isolated mitochondria (23,59). However, the difference in ROS production between species with different longevities and between caloric restricted and *ad libitum*-fed animals seems to come exclusively from complex I (23). Then, the slight decrease observed in the complex I amount in our study can be responsible for the non-significant trend to decrease mitochondrial ROS production with complex I linked substrates (glutamate/malate), and probably the adaptive response of the MnSOD content. This decrease would not be as remarkable as in the case of caloric or methionine restriction, in which the time of treatment was longer (7 weeks) in contrast to the 15 days of atenolol treatment implemented here.

AIF is a mitochondrial flavoprotein involved in the assembly/manteinance of complex I, and besides its role in apoptosis, it is also required for mitochondrial oxidative phosphorylation (22). Similarly to what was previously reported in atenolol treated mice (38), in the present investigation AIF did not change after atenolol treatment, suggesting that the decrease in apoptosis that was observed in

the long-lived AC5 KO mice was probably AIF independent. On the other hand the atenolol treatment did not modify SIRT3 values, which would agree with the absence of changes in mitROS production rate observed in our study. SIRT3 has been reported to increase the activity of complex I through direct interaction and deacetylation of several of its subunits (27). Finally, the unchanged content observed for SIRT5 also agree with the absence of changes in oxidative metabolism (oxygen consumption) observed in the present work.

In conclusion, the results of the present investigation, together with previous reports in mice (1, 38) suggest that β-adrenergic receptor signaling blockade can be a useful model looking for pharmacologically-induced decreases in oxidative stress and possible increases in lifespan. Atenolol treatment improved parameters related to oxidative stress and longevity such as membrane fatty acid unsaturation degree, the peroxidizability index and protein lipoxidation in a time period as short as fifteen days. It would be very interesting to further investigate the effect of atenolol in different organs (other than the heart), species and times of action**,** and to clarify the cellular signaling mechanisms by which this β-blocker decreases fatty acid unsaturation. The interruption of the beta-adrenergic receptor signaling pathway in AC5 KO mice resulted in delaying bone and heart aging and increasing mean and maximum longevity. Those beneficial changes seemed to be under the stimulation of the Raf/MEK/ERK signalling pathway. Previous studies also observed that atenolol-treated mice had higher levels of p-ERK (38). That could suggest that the decrease in fatty acid unsaturation and oxidative stress induced by atenolol could be due to changes in gene expression activated by increases in ERK-dependent signalling. Finally, lowering oxidative stress with atenolol can be easier to implement in humans than caloric restriction. Atenolol seems to be a rather well tolerated drug, which has been used for decades in large human populations without, apparently, important side effects.

5. ACKNOWLEDGMENTS

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