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Renin–angiotensin system inhibitors protect against age-related changes in rat liver mitochondrial DNA content and gene expression

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Abstract

Chronic renin–angiotensin system inhibition protects against liver fibrosis, ameliorates age-associated mitochondrial dysfunction and increases rodent lifespan. We hypothesized that life-long angiotensin-II-mediated stimulation of oxidant generation might participate in mitochondrial DNA “common deletion” formation, and the resulting impairment of bioenergetic capacity. Enalapril (10 mg/kg/d) or losartan (30 mg/kg/d) administered during 16.5 months were unable to prevent the age-dependent accumulation of rat liver mitochondrial DNA “common deletion”, but attenuated the decrease of mitochondrial DNA content. This evidence – together with the enhancement of NRF-1 and PGC-1 mRNA contents – seems to explain why enalapril and losartan improved mitochondrial functioning and lowered oxidant production, since both the absolute number of mtDNA molecules and increased NRF-1 and PGC-1 transcription are positively related to mitochondrial respiratory capacity, and PGC-1 protects against increases in ROS production and damage. Oxidative stress evoked by abnormal respiratory function contributes to the pathophysiology of mitochondrial disease and human aging. If the present mitochondrial actions of renin–angiotensin system inhibitors are confirmed in humans they may modify the therapeutic significance of that strategy.

Keywords

Aging; Mitochondria DNA; Reactive oxygen species; Gene expression; Anti-aging; Antioxidant

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1. Introduction

Free radical-induced oxidative damage to mitochondria has been proposed as a cause of reduced mitochondrial function, and may provide a mechanism to explain aging. This concept is known as the mitochondrial free radical theory of aging (Miquel, 1998), and is supported by abundant evidence indicating an age-related decline of mitochondrial function (Krishnan et al., 2007). In particular, oxidative damage to mitochondrial DNA (mtDNA) is likely involved in the decay of mitochondrial energy production, since mtDNA encodes for 13 polypeptide components of the mitochondrial electron transport chain and oxidative phosphorylation. Aging is associated with the accumulation of rat mtDNA “common deletion” (mtDNA⁴⁸³⁴) that arises from the loss of a 4834 bp fragment in rodents (Yowe and Ames, 1998), and is homologous to human mitochondrial DNA “common deletion” (mtDNA⁴⁹⁷⁷) (Baumer et al., 1994). The deleted DNA fragment encodes polypeptides from respiratory chain complexes I, IV and V, and 5 transfer RNAs (tRNA). In the skeletal muscle of individuals with increasing age, a direct correlation was found between mtDNA⁴⁹⁷⁷ levels and bioenergetic deficiencies due to the reduction of respiratory chain enzyme activities (Lezza et al., 1994). Experimental evidence suggests that mtDNA⁴⁸³⁴ formation results from the accumulation of oxidized nucleotide bases, which by stimulating DNA strand separation, favours the recombination of mtDNA segments in the presence of direct repeats (Ozawa, 1995; Phadnis et al., 2005). Also, a correlation was found between the accumulation of 8-oxodG, an oxidized DNA base, and the incidence of mtDNA⁴⁹⁷⁷ in several human tissues (Ozawa, 1999). This suggests a direct relationship between oxidative damage and the formation of mtDNA deletions. Mutations in the mitochondrial Twinkle helicase (Zeviani and Carelli, 2005) and inefficient repair of double strand breaks (Srivastava and Moraes, 2005) also seem to contribute to the accumulation large-scale deletions in mtDNA.

Angiotensin II (Ang II) stimulates oxidant release by activating NAD(P)H oxidase (Li and Shah, 2003), uncoupling endothelial NOS (eNOS) (Mollnau et al., 2002), and directly or indirectly affecting mitochondrial metabolism (Larkin et al., 2004). In this regard, renin-angiotensin system (RAS) inhibitors were proposed to act as magic bullets against oxidative stress (Munzel and Keaney, 2001). Furthermore, RAS inhibition protects against fibrosis in the liver (Toblli et al., 2002), oxidative stress (Munzel and Keaney, 2001) and mitochondrial dysfunction (de Cavanagh et al., 2003); however, its effects on mtDNA age-related changes have not been investigated.

We have previously shown that during rodent aging, chronic administration of RAS-inhibitors protects hepatic, renal, and cardiac tissues from functional and structural decay, and prevents the age-dependent decrease of mitochondrial number (Ferder et al., 1993, 2002). Also, RAS inhibition, either with an angiotensin converting enzyme (ACE) inhibitor (enalapril) or an Ang II type 1-receptor (AT1) blocker (losartan), prevents the deterioration of mitochondrial structure and function in aged rats (de Cavanagh et al., 2003). Based on these results, we hypothesized that life-long Ang II-mediated stimulation of oxidant generation, might be involved in the formation of mtDNA⁴⁸³⁴ and the resulting impairment of bioenergetic capacity. Thus, the objective of the present study was to investigate whether chronic enalapril- or losartan-mediated RAS inhibition might prevent or attenuate the age-

related accumulation of mtDNA⁴⁸³⁴ in rat liver. We also examined mtDNA copy number, and the expression of nuclear genes that encode proteins involved in mtDNA replication and transcription (mitochondrial transcription factor A (Tfam), mitochondrial single stranded binding protein (mtSSB)), in the transcription of nuclear genes for mitochondrial respiratory chain proteins (nuclear respiratory factor-1 (NRF1), nuclear respiratory factor-2 (NRF2)), in protein import from the cytosol to the mitochondrial matrix (translocase of the outer mitochondrial membrane 20 (Tomm20)), and in coactivating several transcription factors and thereby coordinating the expression of the mitochondrial and nuclear genomes into a program of mitochondrial biogenesis (peroxisome proliferator activator receptor gamma coactivator-1 α (PGC1- α)).

2. Materials and methods

2.1. Animals and treatments

The experiments, approved by the Animal Care and Ethics Committee from the Institute for Cardiovascular Research (Buenos Aires, Argentina), were conducted according to the NIH Animal Care and Use Policy Manual (National Institutes of Health publication No. 3040-2, revised 2002).

Twenty-four inbred male Wistar rats were divided into 3 groups of 8 animals each that received drinking water containing either: (1) enalapril (10 mg/kg/d) (Old + Enal); (2) losartan (30 mg/kg/d) (Old + Los), or (3) no additions (Old), starting at 6 weeks up to 18 months (mo) of age. Four-month-old rats that received water with no additions were used as young controls (Young). Rats had free access to water and standard rat chow (Cargill, Buenos Aires, Argentina), and were housed in metabolic cages (21 ± 2 °C; 12 h light/darkness cycle) to allow for food consumption determination. Water intake was determined twice weekly in order to adjust drug doses. Systolic blood pressure (SBP) was determined every two weeks by tail plethysmography. At the end of the 16.5-mo treatment period, rats were anesthetized with pentobarbital (40 mg/kg, IP), blood was drawn from the thoracic aorta, and a systemic perfusion was performed with 0.9% (w/v) NaCl before excision of the liver. A liver section from the left lateral lobe was used for mitochondrial isolation, and another was fixed in phosphate-buffered 10% (v/v) formaldehyde (pH 7.2), and embedded in paraffin. Three-micron sections were cut and stained with hematoxylin–eosin, and Masson's trichrome. Body weight, plasma glucose, plasma cholesterol and triglyceride contents, and hepatic enzyme activities were determined at the end of the study. Unless otherwise stated, all reagents were from Sigma Chemical Co. (St. Louis, MO).

2.2. Real-time quantitative PCR (qPCR)

Total liver DNA was isolated using the Trizol (Invitrogen, Carlsbad, CA) method, and fluorescence based qPCR was conducted on an (iCycler, BioRad, Hercules, CA) using the iQ SYBR Green Supermix (BioRad) to determine (a) the proportion of total mtDNA genomes carrying the mtDNA⁴⁸³⁴, and (b) the level of mtDNA genomes relative to the level of nuclear DNA (nDNA) (i.e., mtDNA copy number or mtDNA/nDNA). Primers for nDNA, represented by the housekeeping gene for GAPDH, mtDNA (D-loop region) and mtDNA⁴⁸³⁴ (flanking the deletion and representing a fusion sequence that is detectable

only in deleted mtDNA), were from IDT (Coralville, IA). The level of mtDNA⁴⁸³⁴ was determined with respect to total mtDNA (mtDNA⁴⁸³⁴/mtDNA ratio), where the total mtDNA was represented by a PCR product derived from the mitochondrial D-loop region. mtDNA/nDNA was calculated by using mtDNA primers for the D-loop region and primers for a single-copy nuclear gene (i.e., GAPDH), that is assumed to be present in two copies per diploid genome. Ten nanograms of template DNA were used for qPCR performed under the following conditions: initial denaturation at 94.0 °C for 4 min, followed by 50 cycles of 30 s at 94 °C, 30 s at the optimal annealing temperature for each primer set, and 40 s at 72 °C. The efficiency of each real-time PCR and standard regression analyses were conducted in each run by using different amounts of a standard DNA. Melting curve analysis was conducted to ensure reaction specificity and accurate quantification. The sequences of the primers used are shown in Table 1.

2.3. Reverse transcription qPCR

For the evaluation of liver mRNA levels, total RNA was extracted by the Trizol method followed by treatment with DNase (DNA free RNA kit, Zymo Research, Orange, CA), and then reversed transcribed using iScript cDNA synthesis kit (BioRad). A qPCR was conducted as described above to measure the abundance of PGC-1 α , NRF-1, NRF-2, Tfam, mtSSB, Tomm20, AT1 and Ang II type 2-receptor (AT2) mRNAs. Samples for mRNA were run in triplicate, and expression was normalized for GAPDH expression. Individual standard curves for each gene were run in parallel.

Two-fold serial dilutions from liver total DNA or RNA were analyzed for each target gene in order to construct linear standard curves from which the concentrations of the test samples was calculated. The Ct values in each run were used to determine starting DNA or RNA levels by standard regression analysis if the correlation coefficient was greater than 0.98, and efficiencies were between 94% and 104%. For the DNA studies the results were normalized to GAPDH contents to correct for variations in DNA input. For the mRNA studies, the results were normalized to GAPDH expression levels to correct for variations in RNA input and differences in reverse transcription efficiencies, and then multiplied by total RNA content per milligram of wet weight, to further correct for variations in total RNA present in the tissue sections used for RNA extraction (Garnier et al., 2003). The latter correction is relevant because gene expression expressed per unit of cDNA used in qPCR does not account for changes in total tissue RNA content, leading to misinterpretation of the data.

2.4. Mitochondrial isolation

Livers were homogenized at 0–4 °C in a solution containing 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulphonylfluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 10 mM Tris-HCl, pH 7.4. After centrifugation of the homogenates at 600g for 10 min, the pellets were discarded and the supernatants centrifuged at 7000g for 10 min. The pelleted mitochondria were washed once and then resuspended in the same buffered solution. This protocol was modified from Lanni et al. (1996). Aliquots of resuspended mitochondria were used to determine respiratory rates, or maintained at –80 °C until used for the determination of hydrogen peroxide production, Mn-SOD activity, and protein content. For the determination of nitric oxide synthase (NOS) activity, mitochondria

were further purified in a self-forming Percoll gradient. Pelleted mitochondria were suspended in 30% (v/v) Percoll in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, 0.1% (w/v) BSA, pH 7.4, and centrifuged at 95,000g for 30 min. Purified mitochondria were washed twice with 150 mM KCl, followed by two washes with homogenization solution. Potential contamination of mitochondrial fractions with other subcellular components was assessed by the negligible presence of non-mitochondrial enzymes. Electron microscopy examination of the purified mitochondrial fraction confirmed the absence of contaminating organelles or organelle fragments. Protein content was assayed according to Bradford, using BSA as standard (Bradford, 1976).

2.5. Mitochondrial respiratory rates

Mitochondrial O₂ uptake was evaluated with a 2-channel respirometer (Oroboros Oxygraph, Par KG, Graz, Austria). Mitochondrial state 3 rates of O₂ uptake (active respiration) were determined in a solution containing 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, 4 mM MgCl₂, 5 mM potassium phosphate, 0.1% (w/v) BSA, 6 mM malate and 6 mM glutamate, 20 mM Tris-HCl (pH 7.4), at 37 °C, after the addition of 1 mM ADP. State 4 respiratory rates (resting respiration) were recorded after the phosphorylation of added ADP was completed. Respiratory control ratio was calculated as rate of O₂ uptake in state 3/rate of O₂ uptake in state 4. The ADP/O ratio was calculated as nanomoles of ADP added to the reaction medium/nanogram atoms of oxygen used during state 3 respiration (Estabrook, 1967).

2.6. Hydrogen peroxide production

Mitochondrial hydrogen peroxide (H₂O₂) production was determined by following the decrease in fluorescence intensity at 365–450 nm (excitation–emission) in a medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), 0.8 µM horse-radish peroxidase, 1 µM scopoletin, 0.3 µM SOD, 30 µM azide, 6 mM malate, 6 mM glutamate, 3 µM antimycin, and 0.033 mg mitochondrial protein/ml. H₂O₂ (0.05–0.35 µM) was used as standard (Jones and Hancock, 1994).

2.7. Mitochondrial NOS activity

Mitochondrial NOS activity was determined by conversion of ¹⁴C-Arg to ¹⁴C-citrulline in 50 mM potassium phosphate, pH 7.2, 1 µM flavin adenine dinucleotide, 1 µM flavin adenine mononucleotide, 10 µM tetrahydrobiopterine, 100 µM NADPH, 0.1 µM calmoduline, 300 µM CaCl₂, 60 mM valine, 50 µM L-arginine, and 0.025 µCi ¹⁴C-L-arginine and 0.15 mg mitochondrial protein. The assay mixtures were incubated at 37 °C for 5 min. The reaction was stopped by addition of 3 volumes of a solution containing 2 mM EDTA and 20 mM HEPES, pH 5.5, followed by 6 volumes of a Dowex exchange resin (Bio-Rad, Hercules, CA). An aliquot of the supernatant was used for scintillation counting (Knowles and Salter, 1998).

2.8. Mn-superoxide dismutase (Mn-SOD) activity

Mn-SOD activity was determined after the inhibition of cytochrome *c* reduction by superoxide anion at 550 nm, in the presence of 2 mM NaCN. One unit of SOD was defined

as the amount of enzyme necessary to cause a 50% inhibition of the reduction of cytochrome (Flohe and Otting, 1984).

2.9. Immunolabelling and optical microscopy

Liver immunolabelling was carried out with a modified avidin–biotin–peroxidase complex technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, CA). To identify monocytes and macrophages on liver sections, a mouse anti-rat ED1 monoclonal antibody (Serotec Ltd., Oxford, UK) at a dilution 1:300 in PBS (pH 7.2–7.6) was used. Collagen fibers were identified with Masson's trichrome staining.

2.10. Morphological analysis

Two liver sections were studied for each animal using a Nikon E400 (Nikon Instrument Group, Melville, NY) light microscope. Morphological analysis was assessed by an Image ProPlus® 4.1 (Media Cybernetic, Silver Spring, MD) image analyzer, on ten microscopic fields per section examined at a magnification of 100×, with the observer blind to the animal group. Resulting data was averaged. Lesion scores related to (1) unspecific mononuclear inflammatory cell infiltrates; (2) positive ED1 cells (monocytes/macrophages); and (3) fibrosis (collagen deposition), were graded according to the following scale: 0, absent; 1, mild (involving 10% per microscopic field); 2, moderate (>10% and 25%); 3, severe (>25% and 50%); 4, very severe (>50%).

2.11. Statistical methods

Values in text, tables and figure are means $\pm$ SEM. Statistical analyses were performed by one-way ANOVA and *F*-test (Statview SE + Graphics version 1.03, Abacus Concepts, Berkeley, CA) to establish the significance of differences among groups. *P*-values < 0.05 were considered significant.

3. Results

3.1. Animal outcomes

End-of-study body weight, SBP, plasma cholesterol, plasma triglycerides, serum glutamic oxalacetate transaminase (SGOT), glutamic pyruvate transaminase (SGPT), and alkaline phosphatase (AP) activities are shown in Table 2.

In untreated 18-mo-old rats (Old), and in rats treated with enalapril (Old + Enal) or losartan (Old + Los) during 16.5 mo, body weight showed no statistically significant differences. As expected, in untreated 4-mo-old rats (Young), body weight was significantly lower than in the other groups. When expressed relative to body weight, food intake showed no differences among groups throughout the study (7.2 ± 0.3 g/100 g body wt/d). Water intake was 30% higher ($P < 0.05$) in the Old + Enal group (13 ± 2 ml/100 g body wt/d) than in the Old + Enal, Old + Los, and Young groups. Growth rate, evaluated by measuring the tail length, showed no differences among groups. SBP was significantly higher in the Old group compared with the Old + Enal, Old + Los, and Young groups. In the Old group, plasma cholesterol and triglyceride contents, and SGOT, SGPT, and AP activities were significantly

higher than in the Young group. In the Old + Enal and Old + Los plasma lipids and serum hepatic enzymes activities were similar to those found in the Young group.

3.2. Effects of enalapril and losartan treatments on mtDNA⁴⁸³⁴ levels and mtDNA copy number in the liver

The liver was chosen as a target organ based on data showing that mtDNA⁴⁸³⁴ is at its highest level in that tissue when compared with other rat tissues (Yowe and Ames, 1998). Also, the accumulation of mtDNA⁴⁸³⁴ in the liver can be reversed by some interventions, such as caloric restriction (Cassano et al., 2004). The levels of isolated total hepatic DNA showed no significant differences among the groups studied (Old = 2.58 ± 0.57 ; Old + Enal = 2.41 ± 0.41 ; Old + Los = 2.71 ± 0.38 ; Young = 3.12 ± 0.35 mg DNA/g liver).

The mtDNA⁴⁸³⁴/mtDNA ratios were compared to the ratios found in the Old group, which were taken as equal to 1 (Table 3). The absolute value for the mtDNA⁴⁸³⁴/mtDNA ratio in the Young group, i.e., the frequency of mtDNA⁴⁸³⁴, indicated that the “common deletion” averaged 0.00015 ($0.015 \pm 0.002\%$) of the total mitochondrial genomes, which represents 1/10 of the value found in the Old group. There were no differences in the mtDNA⁴⁸³⁴ content among the Old, Old + Enal and Old + Los groups (Table 3). Thus, contrasting with our hypothesis, chronic enalapril- or losartan-mediated RAS inhibition did not prevent or attenuate the age-related accumulation of mtDNA⁴⁸³⁴ in rat liver.

However, chronic enalapril or losartan treatment did influence mtDNA copy number, i.e., the number of copies of mtDNA per diploid genome. The results showed that in the Young group the D-loop region was present in ≈ 456 more copies than the GAPDH sequence, i.e., 912 copies per diploid nuclear genome. mtDNA/nDNA ratios were compared to the ratio of mtDNA to nuclear DNA found in the Old group, which was set as equal to 1 (Table 3). In the Young group, the mtDNA/nDNA ratio was ≈ 3 -times higher than in the Old group and ≈ 2 -times higher than in the Old + Enal and Old + Los groups ($P < 0.05$) (Table 3). In the Old + Enal and Old + Los groups, the mtDNA/nDNA ratios were 40% higher than in the Old group ($P < 0.05$), indicating that chronic enalapril and losartan treatments attenuated the age-related decrease of mtDNA copy number.

3.3. Effects of enalapril and losartan treatments on liver mRNA levels for nuclear genes that influence mitochondrial biogenesis and energetics

The level of GAPDH mRNA was similar among the groups studied, indicating that GAPDH was suitable to be used as an endogenous reference gene. However, the recovery of total DNA-free RNA, expressed as $\mu\text{g RNA/mg wet tissue}$, showed differences among the groups studied. In the Old, Old + Los and Old + Enal groups, total RNA content per unit tissue weight was significantly higher than in the Young group, and in the Old + Los and Old + Enal groups total RNA recovery was higher than in the Old group, although only in the Old + Enal group it reached statistical significance (Old = $1.62 \pm 0.15^*$, Old + Enal = $2.51 \pm 0.46^{*\dagger}$, Old + Los = $2.29 \pm 0.12^{*\ddagger}$, Young = 0.712 ± 0.16 $\mu\text{g RNA/mg wet tissue}$; $*P < 0.05$ vs. Young, $^\dagger P < 0.05$ vs Old, $^\ddagger P = 0.067$ vs Old). To correct for variations in total liver RNA content among the experimental groups, the levels of mRNA determined by real-time

quantitative PCR (qPCR) were multiplied by the micrograms of total RNA per milligram of wet weight, so that the results represent the total mRNA content per milligram of liver.

The transcript levels of the genes studied displayed a complex pattern of responses to enalapril and losartan treatments (Fig. 1). In the Old group, liver NRF-1 and PGC-1 α transcripts were 2-times (not significant) and 4-times ($P < 0.05$) higher than in the Young group, respectively. In the Old + Enal and Old + Los groups, liver NRF-1 mRNA content was significantly higher than in the Old (2.2-times and 1.8-times, respectively) and Young (5.5- and 4.5-times, respectively) groups (Fig. 1 A). In the Old + Enal and Old + Los groups, liver PGC-1 α mRNA contents were 1.7-times and 7-times higher than in the Old and Young groups, respectively ($P < 0.05$) (Fig. 1B). In the Old group, NRF-2 (Fig. 1C) and Tomm20 (Fig. 1D) transcripts were 2.3-times and 3.7-times higher than in the Young group ($P < 0.05$), and approximately 2-times higher than in the Old + Enal and Old + Los groups ($P < 0.05$).

In the Old, Old + Enal, and Old + Los groups, Tfam mRNA contents were between 3- and 4-times higher than in the Young group ($P < 0.05$) (Fig. 1E). In the Old + Enal group, mtSSB transcript level was approximately 3-times higher than in the Old and Old + Los groups ($P < 0.05$), and 13-times higher than in the Young group ($P < 0.05$). mtSSB mRNA contents were approximately 4 times higher in the Old and Old + Los groups compared with those in the Young group ($P < 0.05$) (Fig. 1F). In the Old, Old + Enal, and Old + Los groups, the expression levels of the Ang II type 1 subtype A-receptor (AT1 $_A$) gene were between 3.5- and 5-times higher than in the Young group ($P < 0.05$) (Fig. 1G). In the Old + Enal and Old + Los groups, AT1 $_A$ receptor mRNA contents were approximately 30% lower than in the Old group ($P < 0.05$). In the Old + Enal group, AT2 mRNA contents were approximately 3-times and 9-times higher than in the Old and Young groups, respectively ($P < 0.05$) (Fig. 1H).

3.4. Effects of enalapril and losartan treatments on liver mitochondria function

Table 4 shows liver mitochondrial respiratory control, ADP/O ratios, H₂O₂ production rate, NOS activity, and Mn-SOD activity data. Respiratory control ratios were used to estimate mitochondrial integrity and ADP/O ratios as indicators of the capacity for energy production. In liver mitochondria from the Old group, respiratory control and ADP/O ratios were significantly lower than in mitochondria from the Old + Enal, Old + Los, and Young groups. In the Old group, liver mitochondria H₂O₂ production rate was three times higher than in the Young group ($P < 0.05$). Old + Enal and Old + Los mitochondrial H₂O₂ production rate was approximately two times higher than in the Young group ($P < 0.05$). Mitochondrial NOS activity was 32% lower ($P < 0.05$) in the Old group relative to the Young group, and showed no significant differences among the Old + Enal, Old + Los, and Young groups. Mitochondrial Mn-SOD activity in the Old group was between 80% and 105% higher ($P < 0.05$) relative to the Old + Enal, Old + Los, and Young groups.

3.5. Effects of enalapril and losartan treatments on liver histology

Unspecific mononuclear inflammatory cell infiltration as well as macrophage/monocyte infiltration (ED1 positive cells), and extracellular collagen fiber deposition scores in the liver

were significantly higher in the Old relative to the Old + Enal, Old + Los and Young groups (Table 5). In the Young group, unspecific inflammatory cell infiltrate, ED1 and collagen fiber scores were significantly lower than in the Old + Enal, and Old + Los groups.

4. Discussion

Notwithstanding that compared with other organs the liver ages reasonably well, hepatic structure and function undergo age-dependent modifications (Serste and Bourgeois, 2006). In aged rodents and humans, the liver displays mild fibrosis (Huang et al., 2005), accumulates oxidized DNA bases -especially in mtDNA (Serste and Bourgeois, 2006), experiences defects of the mitochondrial respiratory chain (Navarro and Boveris, 2007), and displays an impaired capacity to compensate for the experimental inhibition of respiratory enzymes (Ramanujan and Herman, 2007). Here we report that enalapril and losartan, two agents that inhibit the RAS by different mechanisms attenuate the the age-dependent decrease of mtDNA copy number and improve mitochondrial function, but are unable to prevent the accumulation of rat liver mtDNA⁴⁸³⁴. Also, – as evaluated by measuring serum hepatic enzymes activities – enalapril and losartan treatments protected the integrity of the hepatobiliary system, and significantly reduced the extent of fibrosis and inflammatory cell infiltrate in old rat livers.

In enalapril- and losartan-treated rats, protection of liver mitochondrial function from age-related decline was revealed by the capacity of both drugs to prevent the decay of energy production and to attenuate the enhancement of mitochondrial oxidant production. In the Old group mitochondria, state 4 respiration was higher than in the other groups studied. Oxygen consumption during state 4 (resting state), is mainly due to the leakage of protons from the mitochondrial intermembrane space to the matrix, which by-passes ATP synthase, and is associated with both structural disruption of the inner mitochondrial membrane and higher oxidant generation. Enhanced state 4 respiration can also result from increased uncoupling protein activity, which is associated with lower oxidant generation. The increase of state 4 respiration observed in the Old group was accompanied with a higher H₂O₂ production rate, suggesting that the inner mitochondrial membrane had lost its integrity. An age-related increase in mitochondrial Mn-SOD activity that was attenuated by enalapril and losartan treatments was also found. This suggests that Mn-SOD activity in the Old group was upregulated to neutralize an augmentation in superoxide generation, as indicated by the observed increase in H₂O₂ production. Also, both enalapril and losartan thwarted the decrease in NOS activity observed in Old rat mitochondria. It is important to note that aging is associated with decreased NO production/availability (Brandes et al., 2005). Although the identity of mtNOS is still controversial (Ghafourifar and Cadenas, 2005), rat liver mtNOS was identified as a myristoylated alpha isoform of nNOS localized to the inner mitochondrial membrane (Elfering et al., 2002). The present experiments do not allow to univocally ascribe the activity of NOS in mitochondria to the recently described mtNOS (Brookes, 2004). However, regardless of the location of NO production, an increase in NOS activity is expected to result in increased cellular NO steady-state level, affecting mitochondria, as well as other organelles. Although the interrelationship between Ang II and NO is very complex, the general consensus is that endothelial Ang II signaling negatively regulates NO bioavailability (Nakashima et al., 2006). Accordingly, ACE inhibitors and AT1

receptor blockers increase endothelial NO availability through several routes (Fernandez-Alfonso and Gonzalez, 1999). Of note, a linkage between NO metabolism and mitochondria was recently indicated by work showing that elevated levels of NO can stimulate mitochondrial biogenesis by upregulating the expression of PGC-1 α (Nisoli et al., 2003). Repression of PGC-1 α expression leads to a reduction of mitochondrial number and enzyme content (Czubryt et al., 2003), and disruption of the endothelial nitric oxide synthase gene *in vivo* leads to a reduction of the mitochondrial mass (Nisoli et al., 2004). Consequently, prevention of the age-related decay of mitochondrial energy output by Ang II blockade may be – at least partly – dependent on increased NO availability. In fact, in previous work we showed that inhibition of mitochondrial NOS activity prevented some of the mitochondrial changes associated with 2-week enalapril treatment, emphasizing the role of mitochondrial NO in those effects (Piotrkowski et al., 2007).

The protective actions displayed by RAS inhibitors against aging-associated mitochondrial changes reinforce our previous observations in rat kidney (de Cavanagh et al., 2003). Here we went further to investigate whether enalapril's and losartan's mitochondrial actions might be related to the attenuation of the age-related accumulation of mtDNA⁴⁸³⁴ in rat liver. Mitochondrial function decay may be, at least partly, the consequence of mtDNA⁴⁸³⁴ accumulation, since common deletion formation entails the loss of a 4834-bp fragment that encodes four subunits of NADH dehydrogenase (ND3, ND4, ND4L, and part of ND5), one subunit each of cytochrome *c* oxidase (COIII) and ATPase (ATPase6), and 5 tRNA (Lys, Ser, His, Arg, and Gly). In addition, the deterioration of mitochondrial function that accompanied aging may have resulted from the decrease of mtDNA copy number.

The regulation of the mtDNA/nDNA ratio is essential to satisfy the mitochondrial ATP needs of the cell, and the absolute amount of wild-type mtDNA – in addition to its proportion versus mutant mtDNA – plays a key role in determining mitochondrial respiratory capacity (Bentlage and Attardi, 1996). In cell lines obtained by fusing mtDNA-less cells with enucleated fibroblasts carrying varying amounts of a mitochondrial mutation at the tRNA_{Leu} gene, the coexistence of 2–5% wild type mtDNA with a predominance of mutant mtDNA molecules, provided the transformants with a respiratory capacity of 50% or more, possibly as a result of mitochondrial complementation (Bentlage and Attardi, 1996). In mice and humans, strong evidence supports the existence of an ample and continuous exchange of mtDNA and transcripts between mitochondria that compensates for the derangement/lack of transcription from mutated mtDNA molecules, and limits mitochondrial dysfunction caused by mtDNA damage (Nakada et al., 2001) (Nakada et al., 2002). Accordingly, in patients with late-onset mtDNA depletion, despite strikingly low mtDNA contents, at least two mtDNA transcripts displayed normal transcription levels that revealed the existence of a compensatory mechanism for mtDNA depletion (Barthelemy et al., 2001). Considering that in the livers of enalapril – and losartan-treated old rats the frequency of mtDNA⁴⁸³⁴ did not differ from that found in untreated old rats, but mtDNA copy number was 40% higher than in the latter, it follows that in enalapril – and losartan-treated old rat livers the number of deleted mtDNA genomes was 40% higher, but – importantly – also the number of undeleted mtDNA genomes was 40% higher than in non-treated old rats. In this setting, it is feasible that – similar to the observations in tRNA_{Leu} transformants and late-onset mtDNA depletion patients – in rats treated with enalapril or

losartan the 40% increase in the absolute number of undeleted mtDNA molecules provided a number of transcripts that was sufficiently high to successfully complement the mutant mtDNA-derived transcript deficiencies, with the consequent enhancement of mitochondrial bioenergetic capacity. This proposal is consistent with the observed improvement of mitochondrial function in enalapril- and losartan-treated rats, and is supported by evidence showing that mtDNA expression increases in parallel with increased mtDNA copy number (Moraes, 2001). The observed age-related decrease of liver mtDNA copy number is in agreement with work by other authors (Barazzoni et al., 2000), who also reported the association between mtDNA copy number diminution and the reduction of mitochondrial respiratory capacity.

The 0.015% value reported here for the frequency of liver mtDNA⁴⁸³⁴ in the Young group is close to that found by Kang et al. (1998) in 6-mo-old rat liver (0.05%), and the 10-fold increase in “common deletion” frequency that was associated with the 18 mo study period is similar to the 6-fold increase found by that group in a period spanning from 6–24 mo of age. The values reported by other researchers vary across a wide range that probably results from the different methodologies used.

The mtDNA copy number found in this study (≈ 912 copies per diploid nuclear genome) is low compared with the 13,000 mtDNA molecules per liver cell, calculated by Nicklas et al. (2004) on the basis of previous stereological studies. However, the value is close to a copy number of ≈ 860 calculated from a report by Gadaleta et al. (1992). As pointed out in (Nicklas et al., 2004), the disagreement between mtDNA copy numbers calculated from morphological examinations and those that originate from molecular biology studies, may result from the existence of unreported GAPDH pseudogenes, and/or the presence of both binucleate cells and liver cells with different levels of ploidy, which are known to accumulate with aging. In fact, ploidy class distribution determined by measuring the diameters of hepatocyte nuclei, as described in (Enesco and Samborsky, 1983), showed that in the three 18-mo-old groups studied diploid cells represented $\approx 5\%$ and polyploid (tetraploid plus octoploid) cells represented $\approx 95\%$ of hepatocytes. This value corresponds to $\approx 60\%$ polyploid cells when considering all cells in the liver. In 4-mo-old rats, diploid cells represented $\approx 13\%$ and polyploid cells $\approx 87\%$ of hepatocytes, confirming that ploidy levels increase with age in the liver.

We also investigated possible changes in the mRNA levels of nuclear-coded proteins engaged in mtDNA maintenance, mitochondrial biogenesis and function. The increase of total RNA content found in the livers of Old rats relative to Young rats, is in agreement with observations in senescent cultured cells (Halle et al., 1997). mtDNA replication relies on mtDNA transcription, since an RNA primer is necessary to start DNA synthesis. In this regard, evidence for Tfam as a direct regulator of mtDNA copy number in mammals has been provided (Ekstrand et al., 2004). Tfam generates mRNAs coding 13 subunits of the oxidative phosphorylation system, 2 ribosomal and 22 transfer RNAs. The rest of the oxidative phosphorylation system subunits, and other mitochondrial proteins and factors engaged in mtDNA maintenance are coded in nuclear DNA (nDNA). Thus, the control of mitochondrial biogenesis and function requires the coordinated expression of hundreds of genes, both nuclear and mitochondrial, in which PGC-1 α seems to play a key role.

Replication by mtDNA polymerase γ is assisted by mtSSB (Moraes, 2001). NRF-1 and NRF-2 are transcriptional regulators for genes coding for subunits of the oxidative phosphorylation system, as well as for many other genes involved in mtDNA replication, including Tfam and the mitochondrial RNA processing complex (Scarpulla, 1997). Tomm20, is crucially involved in protein import into the mitochondria, and is considered an indicator of mitochondrial biogenesis. In the present study, a general increase in the mRNA levels of the genes studied was observed in the Old group when compared with the Young group. In enalapril and losartan-treated rats, NRF-1 and PGC-1 α gene transcription increased above the levels observed in the Old group. NRF-1 plays a key role in activating the expression of genes involved in energy production (Scarpulla, 1997), and PGC-1 α expression not only activates energy pathways that increase ATP generation (Finck and Kelly, 2006) but also protects against increases in ROS production and damage (Spiegelman, 2007). Thus, the responses of NRF-1 and PGC-1 α transcription to enalapril and losartan treatments are in agreement with the observed improvement of liver mitochondrial function and the decrease of mitochondrial H₂O₂ generation. In addition, the latter observations are in accordance with evidence showing that infusion of Ang II lowers the expression of cardiac mitochondria electron transport chain and Krebs's cycle genes (Larkin et al., 2004), and increases mitochondrial H₂O₂ in endothelial cells (Doughan et al., 2008). In enalapril- and losartan-treated rats, the expressions of NRF-2 and Tomm20 genes remained similar to those found in the Young group, and were lower than in the Old group. This suggests that the increases of NRF-2 and Tomm20 expression found in Old rats corresponds to an unsuccessful effort to compensate for both the failure to attain adequate NRF1 and PGC-1 transcription levels and Ang II's negative actions on mitochondrial function. In addition, in enalapril- and losartan-treated rats Tfam mRNA levels were similar to those found in the Old group and higher than in the Young group, whereas mtSSBP gene expression was significantly higher in the Old + Enal group than in the other groups studied. The latter changes in Tfam and mtSSBP gene transcription are difficult to interpret.

Two AT1 receptor subtypes have been described in the rat (AT1_A and AT1_B), and the liver only expresses the AT1_A receptor (Llorens-Cortes et al., 1994) which predominates over AT2 receptors (Speth and Kim, 1990). Here, we report age-dependent increases in liver AT1_A receptor mRNA levels. This is in agreement with a study in aged rat hearts, where augmentations in both AT1_A receptor mRNA and protein were found (Heymes et al., 1998). In that report, AT2 receptor gene expression and density were also higher in old than in young rat hearts. That observation is consistent with the age-related 2-fold (not significant) increase in liver AT2 receptor mRNA levels found in the present study. Considering that Ang II-mediated activation of AT1 receptors plays a key role in the regulation of liver fibrosis development (Yoshiji et al., 2007), liver cell death, and inflammatory response (Chan et al., 2007), and that RAS inhibition protects against liver fibrosis (Toblli et al., 2002; Yoshiji et al., 2007), it is feasible that the attenuation of age-related increases in liver AT1 receptor gene expression associated with enalapril and losartan treatments in the present study, may have protected hepatic tissues from the effects of aging, provided that the increase in AT1 receptor mRNA was accompanied with increased AT1 receptor density. Blockade of the above Ang II actions, may account for the attenuation of age-related increases in plasma liver enzymes activities by enalapril and losartan treatments. In agreement with the present

data, studies in other tissues have reported reductions in AT1 receptor mRNA levels and protein contents in response to angiotensin converting enzyme (ACE) inhibition and Ang II receptor blockade (Kitami et al., 1992; Sechi et al., 1996).

In addition, the beneficial mitochondrial effects displayed by enalapril and losartan treatments may derive from blockade of Ang II actions on mitochondria. Ang II was shown to promote the depression of mitochondria energy metabolism (Kimura et al., 2005; Sorescu and Griendling, 2002) and the decrease of mitochondrial electron transport chain and Kreb's cycle gene expression (Larkin et al., 2004).

Regarding the relation between aging and the RAS, plasma renin and Ang II levels decrease with aging in the rat (Heymes et al., 1994; Vincent et al., 1976), and in humans (Belmin et al., 1994), whereas tissue expression of RAS components (Belmin et al., 1994; Heymes et al., 1994; Thompson et al., 2000) and responsiveness to Ang II stimulation have been shown to increase with aging (Thompson et al., 2000). The age-related enhancement of Ang II-dependent tissular oxidant production may favour the decay of mitochondrial function and the consequent impairment of organ structure and function, which would provide an explanation for the beneficial actions associated with the administration of compounds that decrease Ang II production or block Ang II binding to its receptors during aging.

Based on the present experiments we cannot completely rule out that the lipid lowering actions displayed by enalapril and losartan influenced mitochondrial function, independently of direct effects related to AngII inhibition. However, previously we showed that in normotensive healthy rats mitochondrial function is modified by 14 day RAS inhibition (enalapril) (Piotrkowski et al., 2007), a treatment period that does not significantly modify the lipid profile. The beneficial actions displayed by AT1 blockers and ACE inhibitors on lipid metabolism have been previously documented (Ernsberger and Koletsky, 2007). The lipid lowering actions of AT1 blockers seem to be mediated by activation of the peroxisome proliferator-activated receptor-gamma (PPAR γ) (Janke et al., 2006), whereas, for both AT1 blockers and ACE inhibitors, blockade of Ang II actions on local adipose tissue RAS may improve adipocyte lipid-storage capacity (Strazzullo and Galletti, 2004).

In summary, the present results suggest that life-long Ang II-mediated stimulation of oxidant generation is not involved in the formation of mtDNA "common deletion", but contributes to the age-dependent declines of liver mtDNA copy number and mitochondrial function in the rat. Chronic enalapril- or losartan-mediated RAS inhibition attenuates the decrease of hepatic mtDNA content, and this – together with the enhancement of NRF-1 and PGC-1 mRNA contents – would participate in the maintenance of adequate mitochondrial function in aging.

Integration of the present results with previous evidence (Belmin et al., 1994; Brandes et al., 2005; Casademont and Miro, 2002; Heymes et al., 1994; Larkin et al., 2004; Li and Shah, 2003; Mollnau et al., 2002; Nakashima et al., 2006; Sanbe et al., 1995; Sorescu and Griendling, 2002; Thompson et al., 2000) suggests a mechanistic framework that connects aging with RAS overstimulation, mitochondrial derangement, and alterations of tissue function and architecture.

RAS inhibitors were proposed as candidates for a new strategy for antifibrosis liver therapy based on their inhibitory actions on hepatic stellate cells proliferation, transforming growth factor- β expression and tissue inhibitor of metalloproteinases expression (Yoshiji et al., 2007). In addition, oxidative stress evoked by abnormal respiratory function and defective antioxidant defenses seems to contribute substantially to the pathophysiology of mitochondrial disease and human aging (Wei et al., 2001). Thus, if the beneficial mitochondrial actions displayed by RAS inhibitors in the present study are confirmed in humans they would afford a new facet to the significance that RAS-blockade can have as a therapeutic approach.

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Fig. 1.

Effects of enalapril and losartan treatments on liver mRNA levels for nuclear genes that influence mitochondrial biogenesis and energetics. Reverse transcription quantitative PCR was used to measure the abundance of mRNAs for nuclear-coded proteins engaged in mtDNA maintenance, mitochondrial biogenesis and function: (A) NRF-1, (B) PGC-1α, (C) NRF-2, (D) Tomm20, (E) Tfam, (F) mtSSB, (G) AT1A and (H) AT2 mRNAs. mRNA expression was normalized for GAPDH expression and then multiplied by total RNA per milligram of wet weight to correct for variations in total RNA present in the tissue sections

used for RNA extraction. Values are means $\pm$ SEM of 8 animals. * $P < 0.05$ vs. Young, $^{\dagger}P < 0.05$ vs. Old, $^{\ddagger}P < 0.05$ vs. Old + Los, $^{\S}P < 0.05$ vs. Old + Enal.

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Table 1

Primer sequences used in qPCR and reverse transcription qPCR

Gene	Accession No.	Primer sequence	Product size (bp)
D-loop	X14848	Left: 5'-TGGTAAAATTCCCGACACA-3' Right: 5'-ATAAGGCCAGGACCAAACCT-3'	175
mtDNA ⁴⁸³⁴	X14848	Left: 5'-TCAGCAACCGACTACACTCA-3' Right: 5'-CGAAGTAGATGATCCGTATG-3'	
when deletion is present:			159
when deletion is absent:			4993
PGC-1 α	NM_031347	Left: 5'-AGGAAATCCGAGCTGAGCTGAACA-3' Right: 5'-GCAAGAAGGCGACACATCGAACA-3'	254
Tfam	NM_031326	Left: 5'-GTGCGGGTTTGTGAAGTTCT-3' Right: 5'-AAAGCCCGGAAGTTCTTAG-3'	122
mtSSB	NM_183328	Left: 5'-ATTTGTGGAAGGCAAAGTGG-3' Right primer: 5'-TCCGTTCAATGGCTTTTCTC-3'	130
NRF-1	NM_010938	Left: 5'-TCGGGCATTTATCCAGAGATGCT-3' Right: 5'-TACGAGATGGGCTATGCTGTGTGT-3'	164
NRF-2	AF037350	Left: 5'-CCTTCCTCTGCTGCCATTAG-3' Right: 5'-GTTCAAGTAAATGCCGGAG-3'	150
Tomm20	NM_152935	Left: 5'-TGTGGACCACCTGACAAATG-3' Right: 5'-GGTTGGAAGCTTGGTCAGAA-3'	118
GAPDH	X02231	Left: 5'-AGACAGCCGCATCTTCTTGT-3' Right: 5'-TGATGGCAACAATGTCCACT-3'	142
AT1RA	BC078810	Left: 5'-CGGCCTTCGGATAACATGA-3' Right: 5'-CCTGTCACTCCACCTCAAAACA-3	67
AT2R	NM_012494	Left: 5'-GTCTGTCCTCATTGCCAACA-3' Right: 5'-CCAGCAGACCACTGAGCATA-3	115

Table 2

Biological outcome of 18-months-old rats untreated or treated with enalapril or losartan from 6-weeks of age, and 4-months-old untreated rats

	Old	Old + Enal	Old + Los	Young
Body weight (g)	702 ± 4 *	670 ± 4 *	756 ± 5 *	402 ± 1
SBP (mmHg)	142 ± 4	107 ± 3 †	109 ± 5 †	108 ± 2 †
Plasma cholesterol (mg/dl)	215 ± 4	84 ± 1 †	89 ± 1 †	87 ± 2 †
Plasma triglycerides (mg/dl)	280 ± 2	77 ± 1 †	94 ± 2 †	103 ± 4 †
SGOT (U/L)	186 ± 3	65 ± 2 †	68 ± 4 †	61 ± 3 †
SGPT (U/L)	119 ± 3	66 ± 1 †	58 ± 3 †	28 ± 1 †
AP (U/L)	231 ± 4	169 ± 3 †	144 ± 6 †	131 ± 5 †

Values are means ± SEM of 8 animals.

SBP, systolic blood pressure; SGOT, serum glutamic oxalacetate transaminase; SGPT, serum glutamic pyruvate transaminase; AP, alkaline phosphatase.

* $P < 0.05$ vs. Young, Old + Los

† $P < 0.05$ vs. Old.

Table 3

Quantification of the 4834-bp mtDNA deletion (mtDNA⁴⁸³⁴) and mtDNA copy number in 18-months-old rats untreated or treated with enalapril or losartan from 6-weeks of age, and 4-months-old untreated rats

Group	ng mtDNA ⁴⁸³⁴ /ng mtDNA compared vs. Old	ng mtDNA/ng nDNA Compared vs. Old
Old	1.00 ± 0.07	1.00 ± 0.25
Old + Enalapril	0.98 ± 0.06	1.42 ± 0.19 [*]
Old + Losartan	1.05 ± 0.09	1.41 ± 0.29 [*]
Young	0.10 ± 0.02 [†]	3.31 ± 0.19 [†]

Values are means ± SEM of 8 animals.

The mtDNA⁴⁸³⁴/mtDNA and mtDNA/nDNA ratios were compared to the ratios found in the Old group, which were taken as equal to 1.

^{*} $P < 0.05$ vs. Old

[†] $P < 0.05$ vs. all other groups.

Table 4

Respiratory control (state 3/state 4), ADP/O, H₂O₂ production rate, NOS activity and Mn-SOD activity in liver mitochondria from 18-months-old rats untreated or treated with enalapril or losartan from 6-weeks of age, and 4-months-old untreated rats

	Old	Old + Enal	Old + Los	Young
State 3 (ng-atom O/min/mg protein)	61.2 ± 9.4	88.9 ± 2.4 *	81.9 ± 4.3 *	79.1 ± 1.6 *
State 4 (ng-atom O/min/mg protein)	15.1 ± 0.74	13.3 ± 0.60 *	11.8 ± 0.45 *	12.4 ± 0.40 *
State 3/state 4	3.45 ± 0.53	6.29 ± 0.89 *	6.01 ± 0.99 *	5.86 ± 0.72 *
Respiratory control	3.97 ± 0.47	6.77 ± 0.42 *	6.98 ± 0.41 *	6.40 ± 0.23 *
ADP/O	1.75 ± 0.22	2.79 ± 0.41 *	3.23 ± 0.30 *	3.01 ± 0.54 *
H ₂ O ₂ (nmol/min/mg protein)	9.42 ± 0.91	6.76 ± 0.93 *	6.34 ± 0.88 *	2.99 ± 0.58 * [†]
NOS (nmol Citrulline/min/mg protein)	0.69 ± 0.04	1.01 ± 0.07 *	1.21 ± 0.43 *	1.05 ± 0.11 *
Mn-SOD (Units/mg protein)	64.1 ± 0.20	35.4 ± 0.21 *	33.6 ± 0.43 *	30.9 ± 0.27 *

Values are means ± SEM of 8 animals.

* $P < 0.05$ vs. Old

[†] $P < 0.05$ vs. Old + Enal, Old + Los.

Table 5

Morphological and immunohistochemical parameter scores in the livers of 18-months-old rats untreated or treated with enalapril or losartan from 6-weeks of age, and 4-months-old untreated rats

	Old	Old + Enal	Old + Los	Young
Unspecific inflammatory cell infiltrate	2.12 ± 0.21	1.14 ± 0.32 *	1.00 ± 0.22 *	0.12 ± 0.04 **†
ED1 (monocytes/macrophages)	2.31 ± 0.25	1.36 ± 0.36 *	0.86 ± 0.28 *	0.05 ± 0.02 **†
Liver fibrosis (collagen deposition)	2.19 ± 0.28	1.29 ± 0.36 *	1.21 ± 0.36 *	0.05 ± 0.01 **†

Values are means ± SEM of 8 animals.

* $P < 0.05$ vs. Old

† $P < 0.05$ vs. Old + Enal, Old + Los.