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Cardiac mitochondrial function and tissue remodelling are improved by a non-antihypertensive dose of enalapril in spontaneously hypertensive rats

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Abstract

Renal and cardiac benefits of renin-angiotensin system inhibition exceed blood pressure (BP) reduction and seem to involve mitochondrial function. It has been shown that RAS inhibition prevented mitochondrial dysfunction in spontaneously hypertensive rats (SHR) kidneys. Here, it is investigated whether a non-antihypertensive enalapril dose protects cardiac tissue and mitochondria function. Three-month-old SHR received water containing enalapril (10 mg/kg/day, SHR + Enal) or no additions (SHR-C) for 5 months. Wistar-Kyoto rats (WKY) were normotensive controls. At month 5, BP was similar in SHR + Enal and SHR-C. In SHR + Enal and WKY, heart weight and myocardial fibrosis were lower than in SHR-C. Matrix metalloprotease-2 activity was lower in SHR + Enal with respect to SHR-C and WKY. In SHR + Enal and WKY, NADH/cytochrome c oxidoreductase activity, eNOS protein and activity and mtNOS activity were higher and Mn-SOD activity was lower than in SHR-C. In summary, enalapril at a non-antihypertensive dose prevented cardiac hypertrophy and modifies parameters of cardiac mitochondrial dysfunction in SHR.

Keywords: *Oxidation, antioxidant, nitric oxide, angiotensin, cardiovascular disease*

Abbreviations: *ACE, angiotensin converting enzyme; ANG-II, angiotensin II; BP, blood pressure; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; MMP, matrix metalloprotease; Mn-SOD, manganese superoxide dismutase; mtMP, mitochondrial membrane potential; mtNOS, mitochondrial nitric oxide synthase; RAS, renin-angiotensin system; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats*

Introduction

Hypertension is associated with functional and structural derangements in target organs, such as the kidney, brain and heart [1,2]. Renin-angiotensin system (RAS) modulators are a major group of anti-hypertensive drugs that include mainly angiotensin-converting enzyme (ACE) inhibitors and AT1 receptor blockers. Interestingly, both kinds of drugs have renal- and cardio-protective effects apparently not related to their action on blood pressure (BP) [3–5].

Increased oxidant production and mitochondrial dysfunction are frequent findings in hypertension and, in consequence, they have been pointed out as likely underlying causes of hypertension-related tissue damage [6,7]. The contribution of dysfunctional mitochondria to the pathogenesis of cardiovascular injury associated hypertension is also relevant [7,8]. Supporting an association between mitochondrial

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damage and hypertension, in spontaneously hypertensive rats (SHR) mitochondrial dysfunction is indicated by the occurrence of alterations in: (a) ATP production [9]; (b) reactive oxygen species (ROS) production and antioxidant defense levels [10,11]; and (c) cytochrome oxidase activities and decreases in inorganic phosphate translocator [12,13]. Mitochondria are not only the major energy producing organelles but are also involved in other cell functions, e.g. the regulation of tissue oxygen gradients [14], cell signalling [15] and cell apoptosis [16]. All these functions are consistently associated with the balance between the production of ROS and nitric oxide (NO).

It has been proposed that alterations in the RAS could explain some of the associations between hypertension and mitochondrial damage [17,18]. Angiotensin II (ANG-II), the key effector of the RAS, can enhance extra-mitochondrial oxidant release by stimulating the production of both superoxide and NO via activation of both plasma membrane NADPH oxidase [19,20] and NOS [21–24] in the vascular smooth muscle cells. Moreover, ANG-II stimulates mitochondrial ROS production in vascular smooth muscle cells and in rat aorta *in vivo* [18,25].

In previous work, we have shown that BP lowering doses of ACE inhibitors and AT1 receptor blockers protect rat kidney mitochondria from the functional and structural changes associated with the ageing process [26]. Later, in an effort to dissociate the mitochondrial effects of RAS inhibitors from their action on BP, we compared the response of SHR kidney mitochondria to administration of AT1 receptor blockers (losartan) or a calcium channel blocker (amlodipine). Although losartan and amlodipine reduced BP to similar extents, only losartan improved mitochondrial function in SHR, suggesting that the effects of this AT1 receptor blocker on mitochondria are (at least partly) unrelated to its anti-hypertensive actions [17]. Taken together, these evidences suggest that mitochondria contribute to the beneficial effects displayed by RAS inhibitors.

To further clarify how RAS inhibitors contribute to mitochondrial and cardiac protection independently of their hemodynamic actions, we investigated in SHR the effects of enalapril (an ACE inhibitor) administered at a dose that did not modify hypertension.

Materials and methods

Chemicals

Antimycin, aprotinin, bovine serum albumin (BSA), calmodulin, EDTA, flavin adenine dinucleotide, flavin adenine mononucleotide, glutamate, H_2O_2, hippuric acid (HA), hippuril-hystidil-leucine (HHL), horseradish peroxidase, leupeptin, malate, Percoll, Ponceau S, sucrose, rhodamine 123 (Rh123), scopoletin, tetrahydrobiopterine, xanthine and xanthine oxidase were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other reagents were of the highest available purity.

Animals and treatments

Sixteen 3 month-old SHR (Instituto de Investigaciones Medicas Alfredo Lanari, University of Buenos Aires, Argentina) were randomly divided in two groups of eight animals each. During 5 months, the rats received either water containing enalapril at a dose of 10 mg/kg/day (SHR + ENAL) or water with no additions (SHR-C). Three month-old Wistar Kyoto rats (WKY), that received water with no additions, were used as normotensive controls to obtain reference values for the parameters studied. The rats had free access to standard rat chow (Cargill, Buenos Aires, Argentina) and were housed in individual cages kept under 12-h light–dark cycles. Systolic BP, evaluated by tail plethysmography (Narco Bio-Systems, Austin, TX), and body weight were determined at the end of the study. Rats were anaesthetized with sodium pentobarbital (40 mg/kg body wt, IP), blood was drawn from the inferior cava vein and a systemic perfusion was performed with NaCl 0.9% (wt/vol) immediately before excision of the heart.

Histological analysis of myocardial tissue

Cardiac tissue was fixed in 10% (vol/vol) formaldehide in 0.1 M phosphate (pH 7.2) buffer and embedded in paraffin. The tissue was cut into 5-micron sections and stained with Hematoxilin-Eosin, Masson's Trichrome and Syrius Red, an anionic dye for collagen fibres [27]. The histological samples were semi-quantitatively evaluated for myocardial fibrosis using an arbitrary score as follows: 0 = normal cardiac structure; 1 = a few tiny collagen fibres radiating from perivascular areas; 2 = many collagen fibres with a mononuclear cells infiltrate localized in one myocardial area; 3 = collagen deposition with inflammatory infiltrate in two or more histological areas in one cardiac section or a single extensive area of scarring.

Isolation of membrane fraction and mitochondria

Immediately after the excision of the heart, the tissue was minced and suspended in a digestion medium containing 0.3 M sucrose, 1 mM $CaCl_2$, 5 mM MOPS, 0.1% (wt/vol) BSA, collagenase 70 μg/ml and 5 mM KH_2PO_4 (pH 7.4). The mixture was incubated at 0°C for 40 min. To stop the incubation, EGTA at a final concentration of 2 mM was added. The digestion medium was discarded and replaced with fresh medium containing 0.3 M sucrose, 1 mM

EGTA, 5 mM MOPS, 0.1% (wt/vol) BSA, 5 mM KH_2PO_4 (pH 7.4); and was homogenized at 4°C [28]. After centrifugation of the homogenates at 1500 *g* for 10 min, the supernatant was centrifuged at 10 000 *g* for 10 min. The ensuing crude mitochondrial fraction was washed once and then resuspended in 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA in 10 mM Tris-HCl (pH 7.4). This fraction was used for determining mitochondrial functional parameters. The supernatant obtained from the 10 000 *g* centrifugation was further centrifuged at 100 000 *g* for 60 min to obtain a fraction containing plasma and microsomal membranes; hereafter referred to as membrane fraction [29], and was used for endothelial nitric oxide synthase (eNOS) protein expression and activity, matrix metalloprotease (MMP) and ACE activity determinations. To evaluate the activity of mitochondrial NOS (mtNOS), the mitochondrial fraction was further purified by suspension in 30% (vol/vol) Percoll in 0.25 M sucrose, 1 mM EDTA, 0.1% (wt/vol) BSA in 10 mM Tris-HCl (pH 7.4) and centrifugation at 95 000 *g* for 30 min. Purified mitochondria were washed twice with 150 mM KCl, followed by two washes with 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA in 10 mM Tris-HCl (pH 7.4) [30]. Protein content was assayed according to Bradford [31], using BSA as standard.

Matrix metalloproteases activity

MMP activity was determined in the membrane fraction. The method was modified from Shimokawa et al. [32]. Briefly, the samples were mixed with non-reducing SDS gel sample buffer and applied to a ready 10% polyacrylamide zymogram gel with 0.1% (wt/vol) gelatin (Bio-Rad, Hercules, CA). After electrophoresis, the gels were washed in 2.5% (vol/vol) Triton X-100 for 30 min and incubated at 37°C for 20 h in 50 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% (vol/vol) Igepal. Proteins were stained with Coomassie brilliant blue and gelatinolytic activity was visualized as clear areas of lysis in the gel. To confirm that gelatinolytic activity was due to metalloproteases, the enzymes were inhibited by incubating samples at a final concentration of 10 mM EDTA for 1 h.

Angiotensin converting enzyme activity

ACE activity was determined in the membrane fraction. Briefly, 100 ml of the fraction containing the enzyme was added to 7 mM of HHL in 300 mM NaCl, 50 mM Tris-HCl (pH 8.3) and incubated 1 h at 37°C. The reaction was stopped by the addition of 100 ml of 12% (vol/vol) phosphoric acid. The HA was separated and quantified by HPLC with UV detection [33]. The chromatographic system consisted of a Supelcosil LC-18-DB column (15 cm × 4.6 mm × 5 μm) using a mobile phase composed of 5 mM phosphoric acid:acetonitrile (80:20 vol/vol) (pH 2.5) (low rate, 1 ml/min; retention time, 3.65 min). The UV detection was carried out at 228 nm (Jasco UV 1575, Intelligent UV/VIS Detector, Japan Spectroscopic Co. Ltd.). Commercial HA was used as standard. ACE activity was expressed as μmol HA/min.mg protein.

Mitochondrial enzyme activities

The activities of NADH/cytochrome c oxidoreductase, cytochrome oxidase and manganese superoxide dismutase (Mn-SOD) were determined in the mitochondrial fraction. NADH/cytochrome c oxidoreductase activity was determined by following the reduction of 25 μM cytochrome c at 550 nm in the presence of 0.2 mM NADH and 0.5 mM KCN, in 100 mM potassium phosphate (pH 7.2) [34]. Cytochrome oxidase activity was measured by following the oxidation of 50 μM cytochrome c at 550 nm in 50 mM potassium phosphate (pH 7.0) [35]. Mn-SOD activity was determined by following the inhibition of cytochrome c reduction by superoxide anion at 550 nm, in the presence of 2 mM NaCN, 10 mM EDTA in 50 mM potassium phosphate (pH 7.8) [36]. One unit of SOD was defined as the amount of enzyme necessary to cause a 50% inhibition of the reduction of cytochrome c.

Mitochondrial membrane potential

Mitochondrial membrane potential (mtMP) was evaluated by determining the accumulation of Rh123 in the mitochondrial fraction. Rh123 accumulation is driven by mtMP and can thus be taken as an mtMP index. The method used here takes advantage of the red shift in Rh123 absorption and emission fluorescence spectra that occurs when the dye accumulates in mitochondria. The maximum difference in the excitation spectra between coupled and uncoupled mitochondria occurs at 497 and 520 nm. The above-mentioned wavelength shift results in a much greater change in the magnitude of the 520/497 excitation fluorescence ratio than the change in the intensity measured at each wavelength. The reaction took place in a medium containing 150 mM sucrose, 5 mM $MgCl_2$, 5 mM potassium phosphate, 20 mM K-HEPES (pH 7.4), in the presence of 0.2 mg mitochondrial protein, 0.24 μM Rh123, 10 mM glutamate and 5 mM malate at 28°C. To calibrate the fluorescence ratio for estimation of the mtMP index, a calibration curve was constructed by plotting 520/490 excitation fluorescence ratios (obtained in the presence of mitochondria and 2,4-dinitrophenol [0, 1, 2, 4, 8 and 16 μM] to attain discrete levels of mtMP) and the corresponding mtMP index values obtained by using the Nernst equation: mtMP index = 59 log $([Rh123]_{in}/[Rh123]_{out})$, where $[Rh123]_{in}$ and $[Rh123]_{out}$ were calculated as described elsewhere

[37]. Rh123 binds to mitochondria, causing deviations of mtMP-dependent accumulation of Rh123 from that predicted by the Nernst equation. To correct these deviations, binding partition coefficients were obtained by incubating mitochondria in media containing 0.5 mM KCl, 20 mM valinomycin, 1 μg/ml rotenone and varying initial Rh123 concentrations, as described in Scaduto and Grotyohann [37].

Mitochondrial hydrogen peroxide production

H_2O_2 production was determined in the mitochondrial fraction by following the decrease of scopoletin fluorescence (excitation, 350 nm; emission 460 nm). The reaction was performed in a 3 ml-fluorescence cuvette in a medium containing 20 mM Tris-HCl (pH 7.4), 0.23 M mannitol, 0.07 M sucrose, 0.8 μM horseradish peroxidase, 1 μM scopoletin, 0.3 μM SOD, 6 mM malate, 6 mM glutamate, 3 μM antimycin, 30 μM sodium azide and 0.05 mg mitochondrial protein/ml. H_2O_2 (0.05–0.35 μM) was used as standard [38].

Nitric oxide synthase activity

Mitochondrial NOS (mtNOS) and endothelial nitric oxide synthase (eNOS) activity were determined by conversion of [^{14}C]-arginine to [^{14}C]-citrulline in a solution containing 50 mM potassium phosphate (pH 5.8 or pH 7.2, respectively), 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide, 10 μM tetrahydrobiopterine, 0.1 μM calmodulin, 300 μM $CaCl_2$, 100 μM NADPH, 60 mM valine, 50 μM arginine, 0.025 μCi [^{14}C]-arginine and 0.15 mg mitochondrial protein or 0.1 mg protein of membrane fraction. The assay mixture was incubated at 37°C for 5 min. The reaction was stopped by addition of 3 vol of a solution containing 2 mM EDTA, 20 mM HEPES (pH 5.5), followed by 6 vol of a 50% (wt/vol) Dowex exchange resin (Bio-Rad, Hercules, CA) solution. The samples were centrifuged at 14 000 *g* during 30 s and an aliquot of the supernatant was used for scintillation counting [39].

Western blot analysis

eNOS protein expression was determined in membrane fractions. Proteins were separated on SDS 10% polyacrylamide gels and then transferred to PVDF membranes, according to Laemmli [40]. Membranes were incubated overnight at 4°C with monoclonal eNOS antibody (1:1500) (Biosystems S.A., Argentina) diluted in 2% (wt/vol) dry low-fat milk. Peroxidase-conjugated secondary antibody was incubated for 90 min at room temperature. To reveal bound secondary antibody an enhanced chemiluminiscence kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used. Western blots were digitalized and analysed with Scion Image software (Scion Corporation, Frederick, MD). To normalize protein loading, membranes were stained with 0.5% (wt/vol) Ponceau S in 1% acetic acid (vol/vol).

Statistical analysis

Values in text, tables and figures are mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) and *F*-test to establish the significance of between-group differences. p-values < 0.05 were considered significant (Statview 5.0, SAS Institute Inc., Cary, NC).

Results

Systolic BP, body weight and heart weight and ACE activity

Table I shows systolic BP, body and heart weight and ACE activity in SHR treated with enalapril during 5 months. At the end of the study, systolic BP was significantly higher in SHR-C and SHR + Enal than in WKY (73% and 83%, respectively). Moreover, in SHR + Enal and SHR-C, systolic BP was not significantly different, indicating that the dose of enalapril used did not affect BP (Table I). Within each experimental group, systolic BP showed no significant differences between values at the beginning and the end of the study.

At the end of the study, body weight showed no significant differences among the groups. Heart weight was significantly higher in SHR-C than in SHR + Enal and WKY (33% and 110%, respectively). In SHR-C, heart weight to body weight ratio was significantly higher than in SHR + Enal and WKY (43% and 130%, respectively) (Table I). ACE activity in SHR + Enal was 38% lower than in SHR-C ($p < 0.05$) but similar to the activity in WKY (Table I).

Histological analysis of myocardial tissue

In SHR-C, severe myocardial sclerosis was evident by the increased deposition of extracellular matrix (ECM), which included an extensive accumulation of interfibrillar and perivascular collagen fibres and was accompanied by mononuclear cell infiltrates. In contrast, in SHR + Enal myocardial sclerosis cell infiltrates were absent and tissue structure was similar to that observed in WKY (Figure 1A–C). According to the semi-quantitative analysis, tissue damage was as follows: SHR-C = 2; SHR + Enal = 0; WKY = 0.

Effect of enalapril treatment on MMP

In-gel MMP activity was ascribed to MMP-2 (Mw 72 KDa), as determined by comparing its migration rate with that of molecular weight markers. In SHR + Enal, MMP-2 activity was 48% lower than in

Table I. Systolic blood pressure, body weight, heart weight, and ACE activity in SHR treated with enalapril for 5 months, in untreated SHR and in normotensive rats.

	SHR-C	SHR+Enal	WKY
Systolic blood pressure (mm Hg)	208±10*	220±11*	120±2
Body weight (g)	304±14	321±8	332±11
Heart weight (g)	1.6±0.1*	1.2±0.1*#	0.8±0.1
Heart weight/body weight (%)	0.53±0.01*	0.37±0.01*#	0.23±0.03
ACE activity (μmol HA/min.mg protein)	6.2±0.4	4.5±0.4#	3.9±0.4#

Values are expressed as means± SEM. *$p < 0.001$ vs. WKY; #$p < 0.01$ vs. SHR-C.

SHR-C ($p < 0.05$) (Figure 1D and E) and similar to the activity found in WKY.

Effect of enalapril treatment on mitochondrial function parameters

In SHR-C, NADH/cytochrome c oxidoreductase activity was 50% lower than in both, SHR + Enal and WKY (Figure 2A). Cytochrome c oxidase activity showed no differences among the groups studied (Figure 2B). In SHR+Enal, mtMP, used as an indicator of the energetic state of the mitochondria [41], was similar to that observed in SHR-C, but significantly lower than in WKY (13%) (Figure 2C). In SHR+Enal, mtNOS activity was three times and 47% higher than in SHR-C and WKY, respectively (Figure 2D). In SHR-C, Mn-SOD activity was 72% and 94% higher than in SHR+Enal and WKY, respectively (Figure 2E). H_2O_2 production rate showed no differences among the groups studied (Figure 2F).

Effect of enalapril treatment on eNOS activity and protein expression

In SHR+Enal, eNOS protein expression was similar to that found in WKY and three-times higher than that observed in SHR-C (Figure 3A). Accordingly, eNOS activity in SHR+Enal was similar to that found in WKY and significantly higher than in SHR-C (52%) (Figure 3B).

Discussion

This study shows that in SHR, long-term enalapril administration at a dose that is unable to counteract hypertension protects cardiac tissue from damage associated with mitochondrial ROS and NO metabolism. The benefits of enalapril treatment on mitochondria were evidenced by the effects on NADH/cytochrome c oxidoreductase, mtNOS and Mn-SOD. After 5 months of treatment, systolic BP in SHR-C and SHR+Enal were not significantly different, confirming that the dose of enalapril used did not correct hypertension, regardless of its ability to inhibit ACE activity. The latter finding suggests that the effects of enalapril on heart structure and mitochondrial function are not related to anti-hypertensive actions, but to the prevention of ANG-II trophic effects.

Hypertension leads to cardiac hypertrophy [42], which may conduct to the increase of heart weight [43] and is associated to elevated levels of ANG-II in SHR [44,45]. In SHR treated with enalapril, the prevention of heart weight increase is consistent with a lower ANG-II tissue content that should follow the observed inhibition of ACE.

Cardiac remodelling refers to the changes in cardiac morphology and contractile function that occur in various pathological conditions, eventually leading to cardiac hypertrophy and changes in the quantity and quality of the ECM [43]. The ECM plays a key role in the maintenance of tissue structure and function and the ECM turnover is largely regulated by MMP activities [46]. Cardiac hypertrophy has been associated with increases in MMP activities [47]. Accordingly, the present results show that in SHR-C the increase in heart weight was accompanied with higher MMP-2 activity and enalapril treatment diminished MMP-2 activity to values found in WKY. This is in agreement with the absence of fibrotic tissue in the hearts of SHR+Enal and confirms the beneficial actions of enalapril treatment on hypertension-dependent cardiac remodelling. It should be emphasized that enalapril-induced protection against cardiac hypertrophy and sclerosis occurred in the presence of hypertension. This would be indicating that the mechanical actions of high BP contribute to the cardiac remodelling to a lesser extent than the molecular mechanisms, such as those derived from ANG-II signalling.

Accumulating evidence suggests the existence of a connection between mitochondria and ECM, underscoring the involvement of mitochondria in the maintenance of tissue architecture [48,49]. Furthermore, MMP and mitochondria display reciprocal interactions: oxidative phosphorylation deficiency activates the transcription of MMP as well as other ECM remodelling genes in cultured osteosarcoma cells [50]; and under oxidative stress conditions, MMP-2 impairs cardiac mitochondrial function [51]. Correspondingly, in the present study, enalapril treatment exhibited beneficial effects on both MMP activity and cardiac mitochondria functional parameters.

Heart mitochondria isolated from SHR-C and SHR+ENAL were 'energetically' similar, as suggested by the equivalent mtMP found in these study groups. In SHR-C, lower NADH/cytochrome c oxidoreductase and higher Mn-SOD activities were observed, as compared to SHR+Enal and

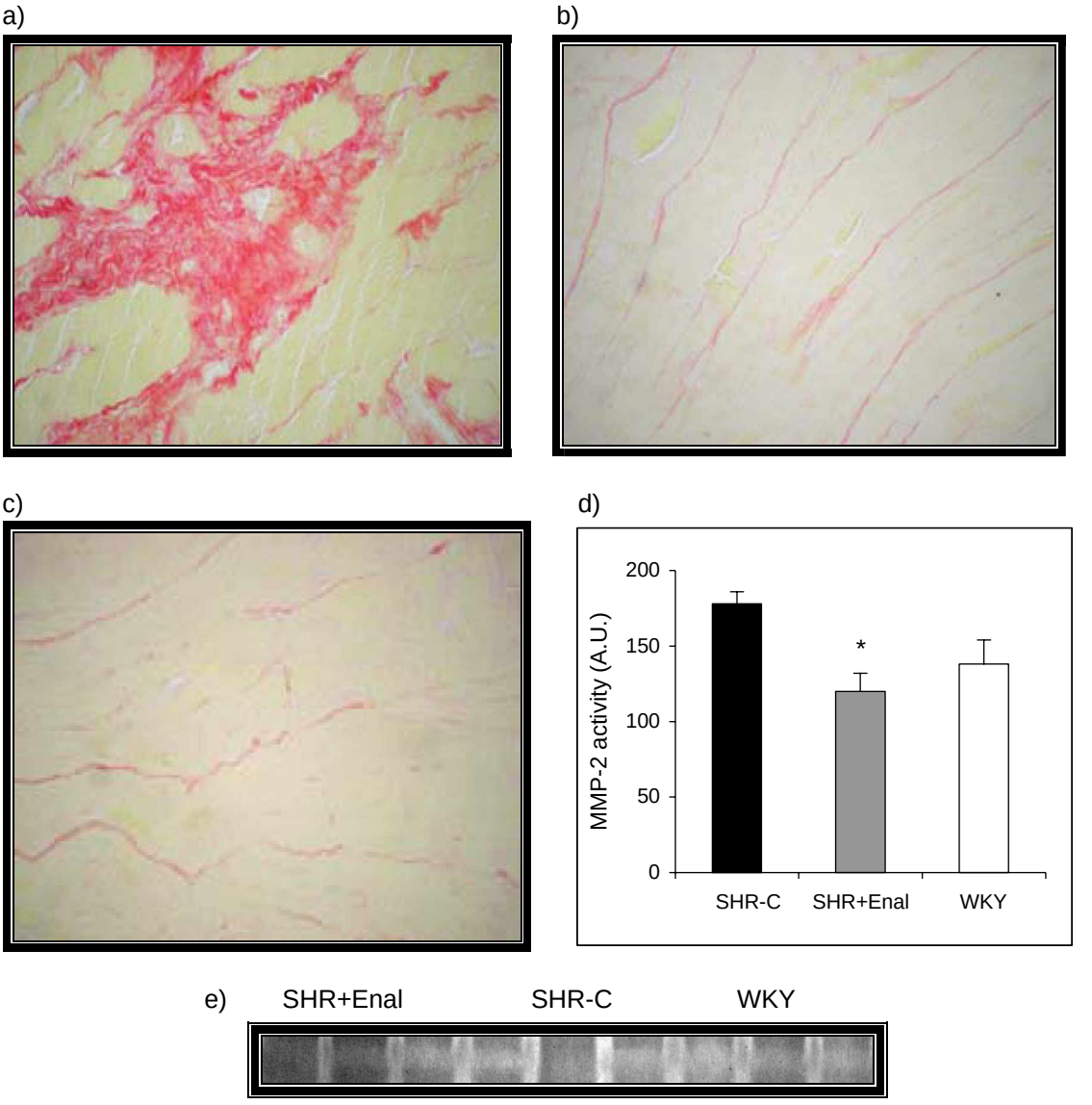

Figure 1. Optical microphotographs of cardiac tissue from untreated SHR [SHR-C] (A), SHR treated with enalapril for 5 months [SHR+Enal] (B) and normotensive control [WKY] (C). MMP-2 activity quantification (D); and a representative zymogram gel electrophoresis (E). Values are expressed as means ± SEM; $^{*}p < 0.05$ vs SHR.

WKY. These findings can be explained considering that mitochondria are not only a major source of, but also are exposed to, self-generated and extra-mitochondrial ROS. Elevated ROS levels eventually play a dual role, they impair mitochondrial function by, for example, inhibiting NADH/cytochrome c oxidoreductase activity [52,53] and they promote the induction of mitochondrial antioxidant enzymes activities, for example Mn-SOD. The latter effect is known to occur in response to long-term oxidative stress in different pathologies, including hypertension [54,55]. Enalapril treatment prevented the changes in NADH/cytochrome c oxidoreductase and Mn-SOD activities observed in SHR-C, indicating a protective role of the treatment with enalapril on mitochondrial oxidation.

The significantly higher cardiac Mn-SOD activity found in SHR-C relative to SHR + Enal suggests that *in vivo* intramitochondrial H_2O_2 production rate was higher in SHR-C than in SHR + Enal. In this context, increased Mn-SOD activity can be associated with higher MMP, as was reported to occur for MMP activity [56] and expression [57] by enhancing mitochondrial H_2O_2 production. However, when determined *in vitro*, mitochondrial H_2O_2 production rate in SHR-C showed no significant differences with that found in SHR + Enal. These contrasting evidences can be explained by considering that H_2O_2 measured under the assay conditions used, i.e. in the presence of externally added SOD, does not totally reflect *in vivo* conditions. It is actually the sum of H_2O_2 derived from Mn-SOD-related conversion of superoxide in the matrix, plus H_2O_2 derived from conversion of superoxide released from mitochondria [58].

Another possible explanation for the lower MMP activity in SHR-Enal relative to SHR-C involves the increases in cardiac eNOS expression and activity

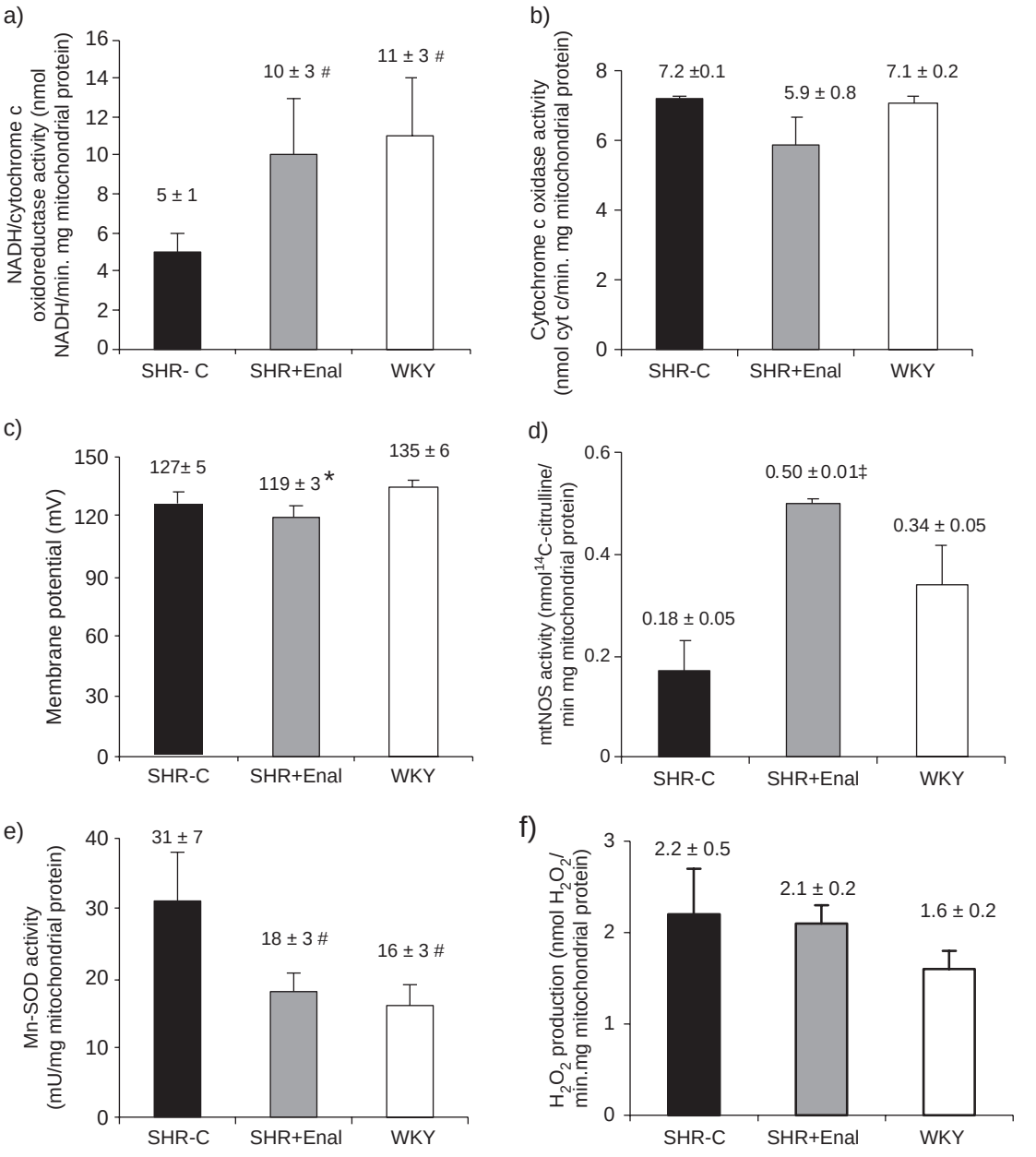

Figure 2. Mitochondrial function parameters and enzymes activities from untreated SHR (SHR-C), SHR treated with enalapril for 5 months (SHR+Enal) and normotensive controls (WKY). Membrane potencial are mtMP index calculated as described in Materials and methods. Values are expressed as means ± SEM; *$p < 0.05$ vs WKY; #$p < 0.05$ vs SHR-C; ‡$p < 0.05$ vs SHR-C and WKY.

observed in enalapril-treated SHR, which is in agreement with a report showing that NO and MMP levels are inversely related [59]. A number of reports point to increased NO production as the mechanism underlying the beneficial tissue actions of ACE inhibitors [56,60]. ACE inhibitors stimulate NO production by preventing the degradation of bradykinin, a peptide that stimulates NOS activity [61]. It is worth noting that, despite enalapril being administered in a non-BP lowering dose, the increases in cardiac eNOS activity and expression that were found in the SHR+Enal group suggest that enalapril succeeded in blocking local heart RAS.

Nitric oxide plays a relevant role in regulating mitochondrial function and oxidant production and evokes cell-protective or cell-damaging actions, essentially depending on the levels of superoxide available to form peroxynitrite [62]. It is likely that the observed increase in mtNOS activity led to a higher steady-state level of mitochondrial NO. Therefore, based on the observed effects of enalapril on Mn-SOD and NADH/cytochrome c oxidoreductase activities and cardiac structure it can be proposed that the higher levels of mitochondrial NO in enalapril-treated SHR as compared with untreated SHR are, at least in part, responsible for the observed positive effects.

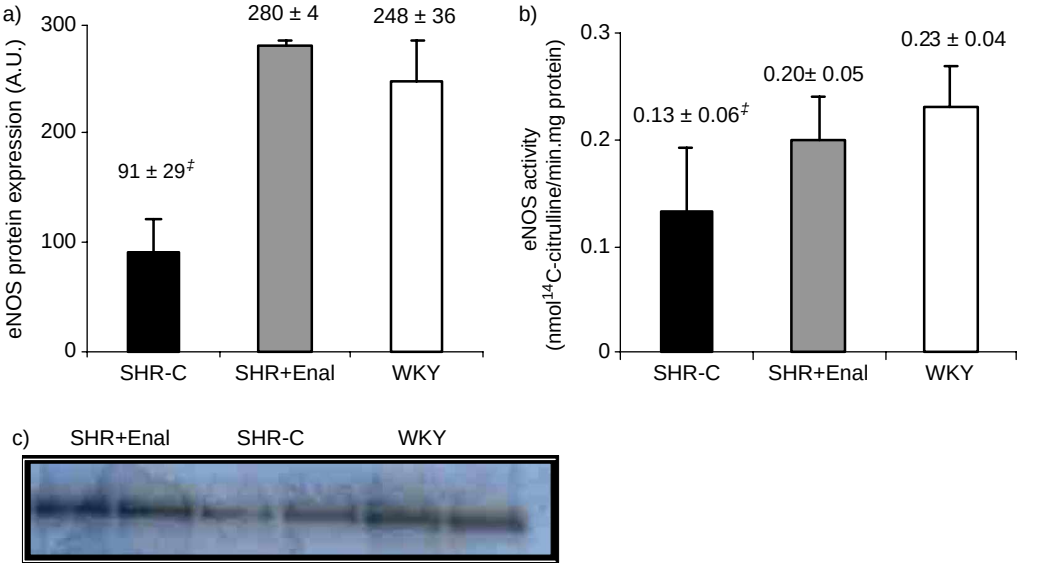

Figure 3. eNOS protein expression (A), eNOS activity (B) and a representative SDS-page electrophoresis in the heart of untreated SHR (SHR-C), SHR treated with enalapril for 5 months (SHR+Enal) in and normotensive controls (WKY). Values are expressed as means ± SEM; $^{\ddagger}p < 0.05$ vs SHR+Enal and WKY.

Concerning the extramitochondrial sources of NO, eNOS activity and protein expression were lower in SHR-C than in WKY. This is in agreement with previous studies that reported hypertension-related decreases in eNOS expression [63] and NO production [64]. The decrease of cardiac eNOS activity observed in the SHR-C group may be the consequence of: (a) ANG-II-induced eNOS uncoupling, i.e. eNOS switches from NO to superoxide production [65,66]; and/or (b) ACE-mediated degradation of bradykinin, a peptide that stimulates NOS activity [61]. Enalapril maintained a high NOS activity in SHR, which is consistent with a number of reports that point to increased NO production as the mechanism underlying the beneficial tissue actions of ACE inhibitors [56,60]. Although the association between hypertension and excessive superoxide production results mainly from increased NAD(P)H oxidase activity in the vasculature [67], the regulation of NO production also seems to play a role.

In summary, RAS inhibition with a dose of enalapril that was unable to correct hypertension protected cardiac tissue structure and mitochondria from hypertension-related damage. The present observations acquire special significance when considering that enalapril prevented cardiac hypertrophy even in the presence of hypertension. These results provide (a) new evidence on BP-independent effects of ACE inhibitors; (b) novel data for understanding the relations between the RAS and mitochondrial function; and (c) a possible explanation for the mechanisms involved in the protective effects of ACE inhibition on cardiac tissue.

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