

Mitochondrial function and nitric oxide metabolism are modified by enalapril treatment in rat kidney

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Piotrkowski B, Fraga CG, de Cavanagh EMV. Mitochondrial function and nitric oxide metabolism are modified by enalapril treatment in rat kidney. *Am J Physiol Regul Integr Comp Physiol* 292: R1494–R1501, 2007. First published December 21, 2006; doi:10.1152/ajpregu.00540.2006.—The renal and cardiac benefits of renin-angiotensin system (RAS) inhibition in hypertension exceed those attributable to blood pressure reduction, and seem to involve mitochondrial function changes. To investigate whether mitochondrial changes associated with RAS inhibition are related to changes in nitric oxide (NO) metabolism, four groups of male Wistar rats were treated during 2 wk with a RAS inhibitor, enalapril (10 mg·kg⁻¹·day⁻¹; Enal), or a NO synthase (NOS) inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME) (1 mg·kg⁻¹·day⁻¹), or both (Enal+L-NAME), or were untreated (control). Blood pressure and body weight were lower in Enal than in control. Electron transfer through complexes I to III and cytochrome oxidase activity were significantly lower, and uncoupling protein-2 content was significantly higher in kidney mitochondria isolated from Enal than in those from control. All of these changes were prevented by L-NAME cotreatment and were accompanied by a higher production/bioavailability of kidney NO. L-NAME abolished mitochondrial NOS activity but failed to inhibit extra-mitochondrial kidney NOS, underscoring the relevance of mitochondrial NO in those effects of enalapril that were suppressed by L-NAME cotreatment. In Enal, kidney mitochondria H₂O₂ production rate and MnSOD activity were significantly lower than in control, and these effects were not prevented by L-NAME cotreatment. These findings may clarify the role of NO in the interactions between RAS and mitochondrial metabolism and can help to unravel the mechanisms involved in renal protection by RAS inhibitors.

angiotensin; membrane potential; uncoupling protein; N^ω-nitro-L-arginine methyl ester; reactive oxygen species

THE RENIN-ANGIOTENSIN SYSTEM (RAS) plays a critical role as a regulator of systemic blood pressure and electrolyte balance (29, 41). Inhibition of the RAS, either with angiotensin-converting enzyme (ACE) inhibitors or ANG II type 1 (AT1) receptor blockers, lowers blood pressure, and protects the kidney and the heart (9, 26, 35, 42, 52) from hypertension-related damage. This protection is not always associated with a reduction in blood pressure (52). Therefore, it is relevant to identify the mechanism(s) underlying the alternative benefits of RAS inhibition.

ACE catalyzes the conversion of ANG I into ANG II, which is the main effector of the RAS. At the cellular level, ANG II binds to the AT1 receptor promoting the generation of both superoxide anion by NADPH-oxidase (72) and nitric oxide (NO) by NO synthase (NOS). Recently, it was shown that ANG II can stimulate the generation of superoxide anion by

uncoupling NOS (15, 47). In addition, ACE degrades bradykinin into inactive metabolites, so ACE inhibition not only prevents ANG II production but also promotes bradykinin accumulation. Bradykinin binds to BK1 and BK2 plasma membrane receptors, thereby activating endothelial NOS (eNOS) and stimulating NO release by endothelial cells (34). Consequently, ACE inhibition may increase eNO production, via a bradykinin-dependent pathway (40).

Mitochondria, the organelles responsible for most of the energy produced in the cell, are also involved in the control of cell signaling and cell death (10, 12, 21). Mitochondria are ubiquitous physiological sources of superoxide anion and NO (7). NO can modulate mitochondrial number (51) and function, and the quantitative relationship between superoxide and NO may be determinant of an appropriate mitochondrial function (8).

Mitochondrial dysfunction has been related to different pathological conditions, such as Alzheimer's and Parkinson's diseases (58), diabetes mellitus (70), obesity and the metabolic syndrome (73), and aging (33). Recent evidence indicates that hypertension may be associated with mitochondrial dysfunction through mechanisms involving increased reactive oxygen species (ROS) production and RAS activation (64). We have shown that long-term RAS inhibition with enalapril, an ACE inhibitor, or losartan, an AT1 receptor blocker, attenuates age-associated structural and functional changes in rat kidney mitochondria (17). Also, losartan was found to attenuate mitochondrial function changes in the kidneys of spontaneously hypertensive rats (18, 19). A recent report from Kimura et al. (38) indicates that ANG II stimulates mitochondrial ROS production in vascular smooth muscle cells and in rat aorta in vivo, albeit this increase in ROS production is not involved in the ANG II-mediated elevation of blood pressure. Taken together, these results point to a role for mitochondria in mediating at least part of the beneficial effects displayed by RAS inhibitors.

In the present study, to investigate whether RAS inhibition-associated changes in NO metabolism might be related to changes in mitochondrial function, we used normotensive rats treated during 2 wk with an ACE inhibitor (enalapril) and/or a NOS inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME). We found that several of the mitochondrial function changes prompted by enalapril treatment are associated with alterations in NO production or bioavailability.

METHODS

Chemicals. L-NAME, Percoll, sucrose, BSA, EDTA, malate, glutamate, horseradish peroxidase, scopolin, antimycin, H₂O₂, aproti-

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nin, leupeptin, rhodamine 123 (Rh123), flavin adenine mononucleotide, flavin adenine dinucleotide, Ponceau S, xanthine, xanthine oxidase, tetrahydrobiopterine, and calmodulin were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest commercially available purity.

Animals and treatments. Thirty-two male Wistar rats (3 mo old) were randomly divided into four groups of eight animals each. The experimental groups received tap water (control), enalapril (Enal), L-NAME, or enalapril and L-NAME (Enal+L-NAME) during 14 days. Enalapril and L-NAME were administered in the drinking water, so as to provide daily doses of 10 mg enalapril/kg body wt, and/or 1 mg L-NAME/kg body wt. The dose of L-NAME was chosen after considering that similar L-NAME doses were shown to have no deleterious effects on kidney structure and function (45, 58). The dose of enalapril was chosen on the basis of data from our previous studies (17). The rats had free access to drinking water and food (standard rat chow, Cargill, Buenos Aires, Argentina) and were kept under 12:12-h light-dark cycles. Body weight was determined at the beginning and at the end of the study (*day 15*). Systolic blood pressure (SBP) was evaluated by tail plethysmography (Polygraph Grass Instrumental, Quincy, MA) on *day 15*. For kidney isolation, the rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip); blood was drawn from the inferior cava vein, and a systemic perfusion was performed with 0.9% (wt/vol) NaCl immediately before excision of the kidneys.

Mitochondria isolation. Kidneys were homogenized at 4°C in a medium containing 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, and 10 mM Tris·HCl (pH 7.4), and centrifuged at 600 *g* for 10 min, to eliminate nuclei and cellular debris. The supernatant was further centrifuged at 9,000 *g* for 10 min. The ensuing pellet was washed once, then it was resuspended in the same medium used to homogenize the tissue (2) and centrifuged at 9,000 *g* for 10 min. The pellet obtained—referred to as the “mitochondrial fraction”—consists of intact coupled mitochondria able to carry out oxidative phosphorylation (65). The supernatant was further centrifuged at 100,000 *g* for 60 min to obtain the “membrane fraction” (25).

To evaluate the activity of mitochondrial NOS (mtNOS), the mitochondrial fraction was suspended 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 centrifuged 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, and 10 mM Tris·HCl (pH 7.4) (17). The purity of the mitochondrial preparation was established by the negligible content of potential contaminants, as evaluated by determining the activities of marker enzymes for microsomes, plasma membrane, lysosomes, and peroxisomes (glucose-6-phosphatase, 0.13%; 5'-nucleotidase, 0.10%; acid phosphatase 2.9%; and catalase, 0.30%, respectively). Protein content was assayed according to Lowry et al. (44) using BSA as a standard.

NO metabolites. NO metabolites content (NO_x) was determined by use of the Griess colorimetric reaction, after enzymatic reduction of nitrates to nitrites. Aliquots containing 2 mg of membrane fraction protein were filtered through 30 kDa cut-off membranes (UltraFuge Ultrafiltration Centrifuge Filters, Micron Separations, Westboro, MA) to remove proteins that interfere with the Griess reagent. The filtrates were incubated at room temperature in a medium containing 50 μM NADPH, 25 mM glucose-6-phosphate, 8,000 U/L glucose-6-phosphate dehydrogenase, 4,000 U/L nitrate reductase and sodium phosphate 0.7 M (pH 7.4). After 2 h, 1% (wt/vol) sulphanilamide and 1% (wt/vol) naphthylethylenediamine were added, and the mixtures were incubated for 10 min. The absorbance of the samples was determined at 540 nm, and sodium nitrate was used as a standard (67).

Mitochondrial enzyme activities. Mitochondrial fractions were used to determine the activities of NADH/cytochrome *c* oxidoreductase, cytochrome oxidase, and manganese superoxide dismutase (Mn-SOD). 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) (32). 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) (13). 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, 50 mM potassium phosphate, and 10 mM EDTA (pH 7.8) (24). 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 (MMP) was evaluated by determining the accumulation of Rh123 in isolated mitochondria. Rh123 accumulation is driven by MMP and can thus be taken as a MMP index (MMPI) (56). 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 MgCl₂, 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 MMP, 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 MMP), and the corresponding MMPI values obtained by using the Nernst equation: $MMPI = 59 \log ([Rh123]_{in}/[Rh123]_{out})$, where [Rh123]_{in} and [Rh123]_{out} were calculated as described elsewhere (56). Rh123 binds to mitochondria causing deviations of MMP-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 (56).

Mitochondrial hydrogen peroxide production. H₂O₂ production was determined in the mitochondrial fraction by following the decrease of scopoletin fluorescence (excitation, 350 nm; emission 460 nm) (37). 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 0.1 mg mitochondrial protein/ml. H₂O₂ (0.05–0.35 μM) was used as a standard.

Mitochondrial NOS activity. The activity of mitochondrial NOS (mtNOS) was evaluated in purified mitochondria by determining the conversion of [¹⁴C]arginine to [¹⁴C]citrulline, in a solution containing 50 mM potassium phosphate (pH 5.8), 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide, 10 μM tetrahydrobiopterine, 0.1 μM calmodulin, 300 μM CaCl₂, 100 μM NADPH, 60 mM valine, 50 μM arginine, 0.025 μCi [¹⁴C]arginine, and 0.15 mg of mitochondrial protein. The assay mixture was incubated at 37°C for 5 min and the reaction stopped by addition of 3 vol of a solution containing 20 mM HEPES (pH 5.5), 2 mM EDTA, and 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 min, and an aliquot of the supernatant was used for scintillation counting (39).

Western blot analysis. Uncoupling protein 2 (UCP-2) and eNOS protein contents were determined in the mitochondrial and membrane fractions, respectively. Proteins were separated on SDS-12.5% polyacrylamide gels and then transferred to polyvinylidene difluoride membranes, as previously described (17). Membranes were incubated overnight at 4°C with either polyclonal UCP-2 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA), or monoclonal eNOS (1:1,500) antibodies (Biosystems, Buenos Aires, Argentina) diluted in 2%

(wt/vol) low-fat milk. Peroxidase-conjugated secondary antibodies were incubated for 90 min at room temperature. To reveal bound secondary antibodies, both diaminobenzidine tablets (Sigma, St. Louis, MO) and an enhanced chemiluminescence kit (Santa Cruz Biotechnology) were used. Western blots were photographed using a digital camera and analyzed with Scion Image software (Scion, Frederick, MD). To normalize samples for protein loading, membranes were stained with 0.5% (wt/vol) Ponceau S in 1% (vol/vol) acetic acid.

Statistical analysis. Values in the text, Table 1, and figures are expressed as means \pm SE. Statistical analyses were performed by one-way ANOVA and *F* test (Statview 5.0, SAS Institute, Cary, NC) to establish the significance of between-group differences. *P* values < 0.05 were considered significant.

RESULTS

Body weight, kidney weight, and systolic blood pressure. Table 1 shows body weight, kidney weight, and SBP data in rats treated during 14 days with enalapril, L-NAME, or enalapril and L-NAME. At the beginning of the study, body weight showed no differences among groups. At the end of the experimental period, body weight in the Enal group was 14% lower than in the control group (*P* = 0.01). In the L-NAME group, body weight was higher than in the other groups studied. However, in the Enal+L-NAME group, body weight was similar to that in the control group, suggesting a potential counterbalance between the effects of enalapril and L-NAME. In the L-NAME group, two-kidney weight was significantly higher than in the Enal and Enal+L-NAME groups (Table 1). However, when two-kidney weight was expressed relative to body weight, values were similar in all groups studied. At the end of the study period, SBP in the Enal group was 15 and 22% lower than in the control (*P* < 0.02) and L-NAME (*P* < 0.002) groups, respectively. In the Enal+L-NAME group, SBP was similar to that found in the control group (*P* < 0.05) (Table 1). Notwithstanding the blood pressure-lowering effects of enalapril, in the Enal group, SBP still remained within the normal physiological range (4, 71).

Effects of enalapril and L-NAME on nitric oxide metabolites and eNOS protein contents. Nitrates and nitrites are stable end-products of NO metabolism; therefore, the sum of their contents (NO_x) is used as an index of tissue NO production (67). In the Enal and Enal+L-NAME groups, kidney NO_x (10.5 \pm 3.3 and 10.9 \pm 1.7 μ g NO₃⁻/mg protein, respectively) were significantly higher than in the L-NAME (4.9 \pm 0.4 μ g NO₃⁻/mg protein) and control groups (4.6 \pm 1.1 μ g NO₃⁻/mg protein) (Fig. 1A). In the Enal group, kidney eNOS protein

content was three to four times higher than in the other groups studied (Fig. 1B).

Effects of enalapril and L-NAME on mitochondrial function parameters. NADH/cytochrome *c* oxidoreductase activity was used to examine the capacity for electron transfer through mitochondrial complexes I to III (32). In the control group, NADH/cytochrome *c* oxidoreductase activity (30 \pm 9 nmol NADH·min⁻¹·mg protein⁻¹) was significantly higher compared with the other groups studied (Fig. 2A). In the Enal group NADH/cytochrome *c* oxidoreductase activity was significantly lower than in the Enal+L-NAME group (2.7 \pm 0.3 vs. 15.6 \pm 3.0 nmol NADH·min⁻¹·mg protein⁻¹). Cytochrome *c* oxidase, the final electron acceptor in the respiratory chain, was determined as another index of mitochondrial respiratory chain capacity. Cytochrome *c* oxidase activity in the Enal group (5.1 \pm 1.1 nmol cytochrome *c*·min⁻¹·mg protein⁻¹) was significantly lower than in the control (13 \pm 1 nmol cytochrome *c*·min⁻¹·mg protein⁻¹), Enal+L-NAME (8.9 \pm 0.9 nmol cytochrome *c*·min⁻¹·mg protein⁻¹), and L-NAME (9.8 \pm 1.4 nmol cytochrome *c*·min⁻¹·mg protein⁻¹) groups (Fig. 2B). In the Enal+L-NAME group, cytochrome *c* oxidase activity was significantly lower than in the control group. NADH/cytochrome *c* oxidoreductase and cytochrome *c* oxidase activities showed a positive correlation (*r* = 0.42; *P* < 0.03).

MMP is an indicator of the energy state of the mitochondria (46) that elevated as MMPI was similar among the groups studied (Fig. 3A). In the control group, mitochondrial H₂O₂ production (0.93 \pm 0.22 nmol H₂O₂·min⁻¹·mg protein⁻¹) was higher than in the Enal (0.47 \pm 0.08 nmol H₂O₂·min⁻¹·mg protein⁻¹), L-NAME (0.34 \pm 0.04 nmol H₂O₂·min⁻¹·mg protein⁻¹), and Enal+L-NAME (0.41 \pm 0.08 nmol H₂O₂·min⁻¹·mg protein⁻¹) groups (Fig. 3B). Mn-SOD is the antioxidant enzyme responsible for the dismutation of superoxide anion, yielding H₂O₂. Mn-SOD activity in the Enal (63 \pm 9 mU/mg protein) and Enal+L-NAME (72 \pm 7 mU/mg protein) groups was significantly lower than in the control (129 \pm 23 mU/mg protein) and L-NAME (132 \pm 21 mU/mg protein) groups (Fig. 3C). In the Enal group, mtNOS activity was significantly higher than in the control group (1.47 \pm 0.27 and 0.69 \pm 0.25 nmol ¹⁴C-citrulline·min⁻¹·mg protein⁻¹, respectively), whereas enzyme activity was undetectable in the L-NAME and Enal +L-NAME groups (Fig. 3D). UCP-2 is a widely expressed mitochondrial protein that dissipates the proton electrochemical gradient, thereby uncoupling electron transport and ATP synthesis and decreasing oxidant generation (53). UCP-2 protein content was higher in kidney mitochondria from the Enal group (122.6 \pm 4.6 AU) relative to the control (101.2 \pm 2.1 AU), Enal +L-NAME (85.5 \pm 0.5 AU), and L-NAME (88.8 \pm 3.7 AU) groups (Fig. 3E).

Table 1. Body weight, kidney weight, and systolic blood pressure in rats treated with enalapril, L-NAME, or enalapril plus L-NAME, for 14 days

	Control	Enal	L-NAME	Enal + L-NAME
Initial body weight, g	186.7 \pm 8.6	182.6 \pm 6.7	180.7 \pm 4.8	185.7 \pm 8.4
Final body weight, g	213.4 \pm 5.1	176.4 \pm 7.7*	238.2 \pm 7.8*	193.1 \pm 7.9
SBP, mm Hg	113.0 \pm 1.4	96.5 \pm 5.3*†	121.9 \pm 6.8	110.7 \pm 5.3
Kidney weight, g	1.65 \pm 0.11	1.55 \pm 0.06†	1.91 \pm 0.11	1.42 \pm 0.11†
Kidney weight/body weight, %	0.82 \pm 0.06	0.89 \pm 0.04	0.79 \pm 0.03	0.80 \pm 0.02

Values are expressed as means \pm SE. **P* < 0.05 vs. control; †*P* < 0.05 vs. L-NAME. SBP, systolic blood pressure.

DISCUSSION

This study shows that short-term RAS inhibition with enalapril modulates NO production, and this modulation is associated with changes in kidney mitochondrial function.

Enalapril treatment lowered SBP relative to control rats, and L-NAME, a competitive NOS inhibitor, prevented the reduction. These results confirm previous observations showing a null effect of the combined administration of enalapril and L-NAME on SBP (20, 54), and reinforce the participation of

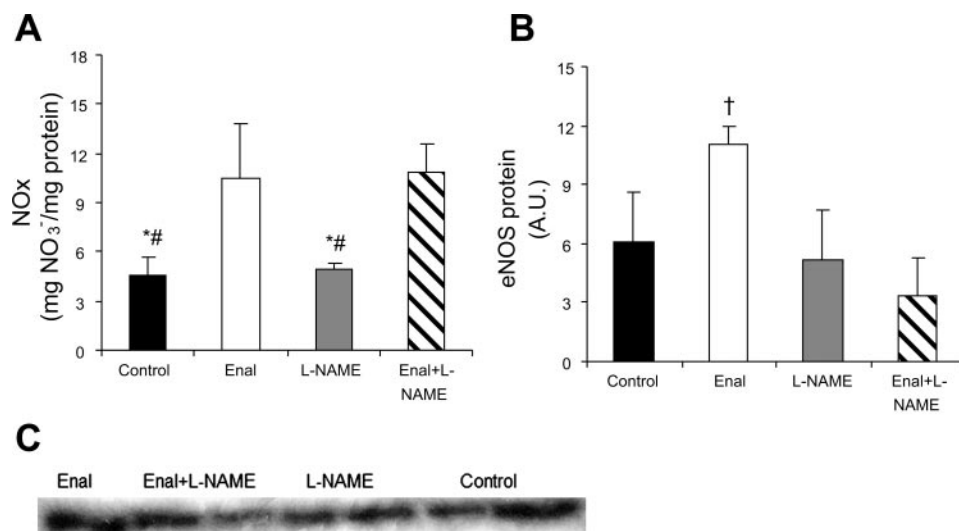


Fig. 1. Kidney nitric oxide (NO) metabolites content (NO_x) (A) endothelial NO synthase (eNOS) protein contents (B), and a representative SDS-PAGE (C), in rats treated with enalapril (Enal), N^G -nitro-L-arginine methyl ester (L-NAME), or Enal+L-NAME for 14 days, and controls. Values are expressed as means \pm SE; * P < 0.05 vs. control; # P < 0.05 vs. L-NAME; † P < 0.05 vs. control, L-NAME, and Enal+L-NAME.

NO in the blood pressure-lowering effect of enalapril. In agreement with other reports (31, 62), the administration of enalapril resulted in weight loss and that of L-NAME led to weight gain. These changes were not observed when enalapril and L-NAME were administered together. The inverse relation between NO production and body weight is supported by previous observations showing that in eNOS knockout mice body weight increases. This finding was accompanied with the reductions of mitochondrial biogenesis and energy expenditure (5, 51). The experimental design used here, addressing the effects of ACE inhibition on mitochondrial function in the kidney, does not allow us to draw conclusions on the regulation of body weight by enalapril. Rather, it seems reasonable to believe that the effect of enalapril on animal weight is related to the effects of ACE inhibition on adipose tissue RAS and lipid metabolism. In fact, a link between the RAS and lipid metabolism was suggested by studies showing that ANG II can increase both adipose tissue lipid storage and the activity of two key lipogenic enzymes (fatty acid synthase and glycerol-3-phosphate dehydrogenase) and that these effects are blocked by RAS inhibitors (36, 60). This evidence suggests that adipose tissue RAS may play a role in body fat accumulation.

Enalapril treatment was associated with a higher production or bioavailability of NO. Accordingly, kidney NO_x was higher in both groups of rats treated with enalapril (Enal and Enal+L-NAME groups) compared with the control and L-NAME groups. These results are in agreement with previous studies

indicating an increase in plasma NO_x in enalapril-treated rats (16, 20, 74). In the Enal group, the increase in kidney NO_x was accompanied by an increase in kidney eNOS protein content, suggesting that enalapril treatment increased NO production by stimulating eNOS protein expression. In contrast, in the Enal+L-NAME group kidney eNOS protein level was lower and NO_x was higher than in the Enal group. The seemingly paradoxical effect of L-NAME, being unable to block the ability of enalapril to increase kidney NO_x content but being effective in preventing the enhancement of eNOS expression, may be explained considering that 1) inducible NOS (iNOS) is a relevant source of NO in the kidney compared with eNOS and neuronal NOS (nNOS; 1, 27, 66); and 2) L-NAME is 10- to 30-fold selective for inhibiting n-NOS and eNOS relative to iNOS (6). This suggests that kidney iNOS is the isoform responsible for increased NO_x in Enal+L-NAME rats. This interpretation is also supported by evidence showing that both renal iNOS expression and NO production are negatively modulated by TGF- β in vivo (68), and enalapril downregulates TGF- β levels in the kidney (52, 61).

Interestingly, mtNOS activity was increased in the Enal group compared with control, L-NAME, and Enal+L-NAME groups, pointing to mtNOS as a relevant contributor to enalapril-mediated NO production. Regarding the contrasting effect of L-NAME on renal NO_x and mtNOS activity, it can be proposed that, as indicated above, the extent of inhibition depends on the selectivity of L-NAME for the different NOS isoforms. Even when there is no data on the L-NAME selec-

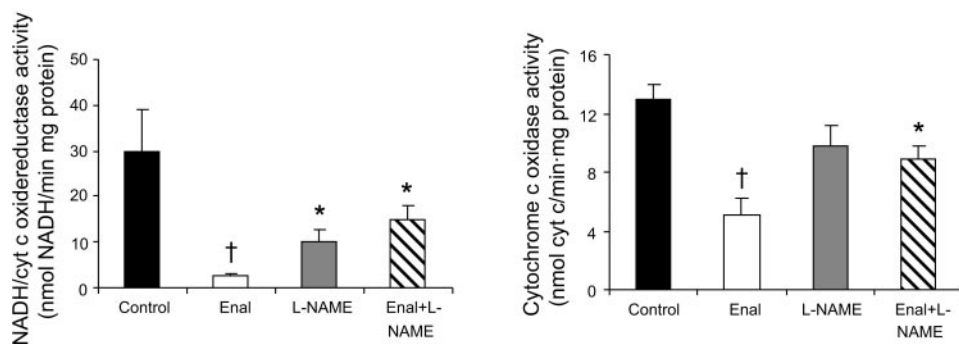


Fig. 2. Mitochondrial respiratory chain enzymes activities in rats treated with Enal, L-NAME, or Enal+L-NAME for 14 days and controls. Values are expressed as means \pm SE. * P < 0.05 vs. control; † P < 0.05 vs. control, L-NAME and Enal+L-NAME.

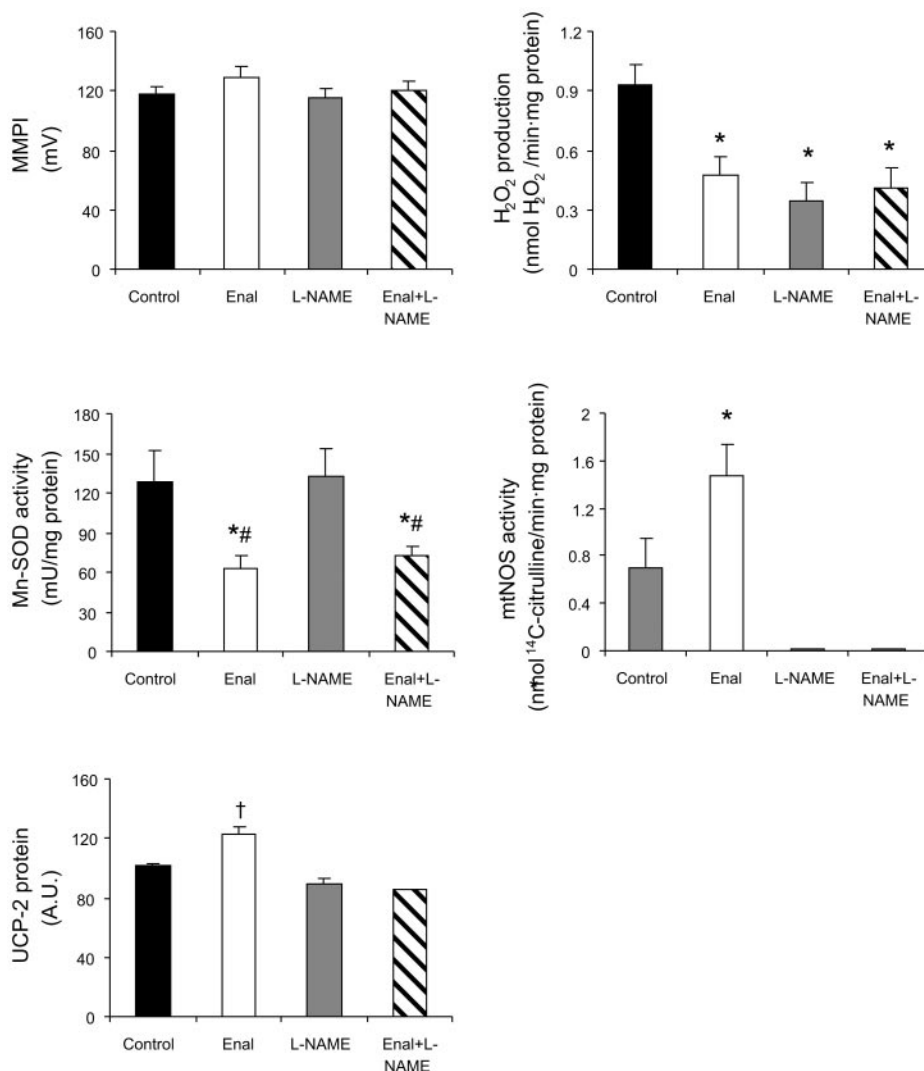


Fig. 3. Mitochondrial function parameters and enzymes activities in rats treated with Enal, L-NAME, or Enal+L-NAME for 14 days, and controls. Values are expressed as means \pm SE. * $P < 0.05$ vs. control; # $P < 0.05$ vs. L-NAME; † $P < 0.05$ vs. control, L-NAME and Enal+L-NAME. Mitochondrial NOS (mtNOS) activity in L-NAME and Enal+L-NAME groups was below the detection limit. MMPI, mitochondrial membrane potential index; UCP-2, uncoupling protein 2.

tivity for mtNOS, being that mtNOS is a strong candidate for a subtype of nNOS (11, 30), it is expected to be highly susceptible to inhibition by L-NAME. In summary, enalapril upregulated renal eNOS, iNOS, and mtNOS, and cotreatment with L-NAME prevented eNOS and mtNOS upregulation. This suggests that among the mitochondrial effects prompted by enalapril, those that were prevented by cotreatment with L-NAME may be attributed to either eNOS or mtNOS, or both.

A wide body of evidence shows that, not only enalapril, but ACE inhibitors in general, as well as AT1 receptor blockers, increase endothelial NO availability via one or more of the following events: 1) attenuation of ANG II-dependent inhibition of eNOS; 2) bradykinin-mediated induction eNOS; and 3) increase in NO steady-state level as a consequence of the attenuation of superoxide production. In addition, NO was found to negatively modulate the RAS through both inhibition of ACE activity and downregulation of AT1 receptors (23). These mechanisms point to a mutual regulation of the RAS and NO-generating enzymes, including mtNOS (40, 63). The relationships between NO and the RAS are further complicated by ANG II being able to stimulate NO generation, as it was reported in endothelial cells, as well as in the kidney (23). The ANG II-mediated release of NO from the endothelium was

proposed to act as a modulator of the vasoconstrictor action of ANG II on smooth muscle cells. It was also suggested that ANG II may lead to contrasting effects, depending on the cell type with which it interacts. Regarding the effects of ANG II on renal NO production, the results are conflicting. Some studies showed that ANG II can stimulate NO release, whereas others reported a decrease in renal NO after ANG II infusion (3, 59). Recently, it was shown that in response to ANG II, endothelial NADPH oxidase-derived superoxide anion causes a deficiency of tetrahydrobiopterine, a cofactor of eNOS, leading to a switch from NO to superoxide production by eNOS (15). Taken together, this evidence indicates that the interrelationship between ANG II and NO is very complex. However, the general consensus is that endothelial ANG II signaling negatively regulates NO bioavailability (48). The effects of enalapril treatment on NO metabolism observed in the present study, that is, increases in renal NO_x, eNOS protein, and mtNOS activity, favor the concept that, a decrease in ANG II formation favors NO bioavailability.

At the mitochondrial level, in the Enal group, electron transfer through complexes I to III (NADH/cytochrome *c* oxidoreductase activity) was lower than in the control group, and this effect was prevented by the coadministration of

L-NAME. The observed response of electron transfer to enalapril treatment is in agreement with a study showing that incubation of isolated kidney cortex mitochondria with enalapril maleate leads to the inhibition of both, respiratory rate (state 3), and respiratory control (46), thus supporting a direct effect of enalapril on NADH/cytochrome *c* oxidoreductase. Also, it is worth mentioning that NADH/cytochrome *c* oxidoreductase activity can be inhibited by high concentrations of NO (12, 57).

At physiological concentrations, NO competes with oxygen for the active site of cytochrome oxidase. The binding of NO to cytochrome oxidase may lead to the: 1) modulation of tissue oxygen gradients, 2) regulation of mitochondrial respiration (5) and superoxide production (55), and 3) inhibition of apoptosis by preventing cytochrome *c* release (10). In the Enal group, the observed decrease in cytochrome oxidase activity may also be ascribed to the relatively higher levels of kidney NO found in this group. However, in the Enal+L-NAME group, mtNOS was completely inhibited by L-NAME, suggesting a role for mitochondrial NO production in the decline of cytochrome oxidase activity observed in the Enal group.

It is interesting to note that the existence of a mtNOS has been reported in different tissues. In rat liver, mtNOS was identified as a myristoylated α -isoform of nNOS localized to the inner mitochondrial membrane (22). However, in human endothelial cells, it appears to be the eNOS isoform docked to the outer mitochondrial membrane (28). Recently, evidence was presented in support of the existence of an inducible mtNOS that is likely coded by the same gene as iNOS, in the diaphragm of septic mice (43). Thus, because no unique nuclear gene has been identified for mtNOS, it is assumed to be a variant of one of the other NOS isoforms (11).

In accordance with previous results in long-term enalapril-treated rats (17), in this study, we observed that enalapril treatment led to a decrease in Mn-SOD activity. This decrease may be explained by 1) enalapril contributing to the lowering of RAS-dependent superoxide generation and the ensuing prevention of superoxide-induced Mn-SOD synthesis; or 2) the enalapril-dependent increase in NO production, leading to the inactivation of the enzyme. In this setting, it was observed that nitration of a critical residue in the active site of the enzyme leads to Mn-SOD inactivation (50).

In this study, the higher level of UCP-2 protein observed in the Enal group relative to the other groups studied, was associated both with higher NO and lower H₂O₂ productions but was unrelated to changes in MMP. These results are in line with previously published evidence indicating that NO (12) and H₂O₂ (49) are involved in UCP-2 regulation and that increased UCP-2 levels result in a lower production of cell oxidants (14, 49). Also, in brain mitochondria subjected to malate/glutamate sustained respiration, oxidant production by complex I was shown to be independent of mitochondrial membrane potential (69), and in rat myoblasts, UCP-3 overexpression decreased mitochondrial oxidant production but did not affect mitochondrial membrane potential (45).

In summary, enalapril treatment modified several parameters of kidney mitochondrial function, that is, respiratory chain activity (electron transfer from complex I to complex III, and complex IV activity), Mn-SOD activity, and UCP-2 content. These effects may provide a mechanism mediating the positive health effects of RAS-inhibition, involving mitochondria as a

key organelle in such actions. The question still remains as to whether enalapril can reach the mitochondria and exert direct effects on mitochondrial components, or whether enalapril acts only at the plasma membrane level, for example, inhibiting ACE activity, leading to an increase in the cellular levels of NO, or other signaling molecules, which would be the species that eventually reach the mitochondria, and improve mitochondrial function. More research in this direction is necessary to assess the physiological relationships between RAS modulation and mitochondrial function.

The results shown in this paper indicate that in healthy rats, inhibition of the RAS with enalapril modifies mitochondrial function and that several of these changes are associated with changes in NO metabolism. These findings provide new insights regarding the relationship between the RAS and mitochondrial metabolism and can help unravel the mechanisms involved in the renal protective effects afforded by RAS inhibition.

GRANTS

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