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Enalapril Increases Mitochondrial Nitric Oxide Synthase Activity in Heart and Liver

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ABSTRACT

Heart and liver mitochondria isolated from rats treated with enalapril, 3–30 mg/kg/day in the drinking water for 7–120 days, showed a time- and dose-dependent increased nitric oxide (NO) production in the range of 14–250%. Heart and liver mitochondria from control rats produced 0.69 and 0.50 nmol of NO/min/mg of protein, respectively, as determined by dual wavelength spectrophotometry (577–591 nm) following hemoglobin oxidation to methemoglobin. The response to enalapril treatment, attributed to a gene-mediated up-regulation of mitochondrial nitric oxide synthase (mtNOS) activity, was half-maximal at 5–6 days and was maintained up to 120 days. Enalapril-treated animals showed an increased mtNOS functional activity in heart mitochondria that inhibited state 3 O_2 uptake (from 22% in control rats to 43%) and increased state 4 hydrogen peroxide (H_2O_2) production (from 30% in control rats to 52%). Calculated heart intramitochondrial NO and H_2O_2 steady-state concentrations were increased 66% and 20%, respectively, by enalapril treatment. Signaling pathways dependent on mitochondrial NO and H_2O_2 may account for the beneficial effects of enalapril in aging mammals. *Antioxid. Redox Signal.* 5, 691–697.

INTRODUCTION

CHRONIC ADMINISTRATION of the angiotensin-converting enzyme inhibitor enalapril to aging mice extended their median life span and prevented myocardial fibrosis (24). It was suggested that the protective effect was due to an increased level of antioxidant enzymes, to an increase in the number of mitochondria, and to a shift in intracellular redox balance (19, 24). Increased superoxide dismutase (SOD) and catalase activities were associated with increased longevity in lower organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (34, 40), but there are no other reports of a similar effect on mammals, except for the mentioned study (24).

Enalapril effects on mice, beyond its action as an antihypertensive drug, opened a new area of research that may be called the ROS-RAS link in aging (ROS: reactive oxidative species; RAS: renin–angiotensin system) (2), by which angiotensin II, through specific receptors, modifies cellular oxidative metabolism, redox levels, and physiological functions in heart, kidney, liver, and other organs (19, 20). Long-term treatment of rats with enalapril or losartan, the latter an inhibitor of AT_1 angiotensin II receptor, failed to show an increase in antioxidant enzymes by either of the two drugs, but instead recognized an increased nitric oxide (NO) production by heart mitochondria (17).

The production of NO by heart mitochondrial nitric oxide synthase (mtNOS) has been recently reported in a few studies (17, 27, 32). The activity of mtNOS, a specialized isoform of nitric oxide synthase (NOS), has also been determined in other tissues, such as liver (28, 30), brain (33, 37), thymus (12, 13), kidney (8), and skeletal muscle (6). Recently, Giulivi and co-workers (23) reported the sequence of the 1,429 amino acids of liver mtNOS, identified as nNOSα (neuronal NOS splice variant α) myristoylated and phosphorylated in posttranslational processes. This important achievement establishes mtNOS as a constitutive protein of the inner mitochondrial membrane. The identity of heart mtNOS as neuronal NOS had been advanced in studies with knockout mice by Kanai *et al.* (32).

This study describes the time and dose relationships of heart mtNOS activity response to enalapril treatment. Liver

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mtNOS activity was determined for comparative purposes. The effects of the increased activity of mtNOS on mitochondrial oxygen uptake and hydrogen peroxide (H_2O_2) production in the physiological mitochondrial metabolic states 3 and 4, respectively, were determined. Intramitochondrial steady-state concentrations of NO and H_2O_2 were estimated as an approach to the intracellular signaling by these molecules.

MATERIALS AND METHODS

Experimental design

Male Wistar rats (200–250 g) were administered either tap water (controls) or water containing enalapril at doses of 3–30 mg/kg/day for periods of up to 4 months.

Mitochondrial isolation

Rats were killed under pentobarbital anesthesia, and heart and liver were immediately excised. Tissues were homogenized in a medium consisting of 0.23 *M* mannitol, 0.07 *M* sucrose, 10 m*M* Tris-HCl, and 1 m*M* EDTA (pH 7.4). Rat heart and liver homogenates were centrifuged at 700 *g* for 10 min to discard nuclei and cell debris, and the pellet was washed to enrich the supernatant that was centrifuged at 8,000 *g* for 10 min. The operations were carried out at 0–2°C (4). The obtained pellet, washed and resuspended in the same buffer, consisted of intact mitochondria that carried out oxidative phosphorylation with respiratory control ratios higher than 5 with malate-glutamate as substrate. Submitochondrial membranes were obtained by twice freezing and thawing mitochondrial preparations and by homogenizing them by passage through a 29 G hypodermic needle (7). Protein was assayed with the Folin reagent using bovine serum albumin (BSA) as standard.

NO production

NO production was measured in heart and liver submitochondrial membranes by following spectrophotometrically the oxidation of oxyhemoglobin (HbO_2) to methemoglobin (metHb) at 37°C, in a reaction medium containing 50 m*M* phosphate (pH 7.4), 1 m*M* $CaCl_2$, 0.1 m*M* L-arginine, 0.1 m*M* NADPH, 10 μ*M* dithiothreitol, 4 μ*M* SOD, 0.1 μ*M* catalase, submitochondrial membranes (0.60–1.50 mg of protein/ml), and 25 μ*M* HbO_2 (expressed per heme group) (7). The kinetics were followed at 577–591 nm in a PerkinElmer 356 double-beam double-wavelength spectrophotometer; the absorbance changes due to HbO_2 oxidation ($\epsilon = 11\ mM^{-1}\ cm^{-1}$) that were sensitive to the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) were expressed as nanomoles of NO per minute per milligram of protein. Preincubation with 1–2 m*M* L-NNA inhibited by 87–94% the rate of HbO_2 oxidation (7).

Oxygen uptake

Respiratory rates were determined in coupled heart and liver mitochondria suspended in 0.23 *M* mannitol, 0.07 *M* sucrose, 20 m*M* KCl, 1 m*M* EDTA, 5 m*M* phosphate buffer, 4 m*M* $MgCl_2$, 6 m*M* malate, 6 m*M* glutamate, 0.2% BSA, and 20 m*M* Tris-HCl (pH 7.4) at 37°C. To set state 3 active respiration, 0.1 m*M* ADP was added (15). Respiratory rates were measured with a Clark-type oxygen electrode.

H_2O_2 production

H_2O_2 generation was determined in heart and liver mitochondria (0.1–0.3 mg of protein/ml) in metabolic state 4 (3, 15) by the scopoletin–horseradish peroxidase (HRP) method, following the decrease in fluorescence intensity at 365–450 nm ($\lambda_{excitation} - \lambda_{emission}$) at 37°C (3). The reaction medium consisted of 0.23 *M* mannitol, 0.07 *M* sucrose, 6 m*M* malate, 6 m*M* glutamate, 20 m*M* Tris-HCl (pH 7.4), 0.8 μ*M* HRP, 1 μ*M* scopoletin, and 0.3 μ*M* SOD.

Manganese superoxide dismutase (Mn-SOD) activity

Mn-SOD activity was determined in heart frozen and thawed mitochondria by the inhibition of adrenochrome formation in a reaction medium containing 1 m*M* epinephrine and 50 m*M* glycine-NaOH (pH 8.5). One picomole of Mn-SOD was equal to 0.87 Misra and Fridovich units (35).

Drugs and chemicals

Enalapril maleate was a gift from Roemmers Laboratories (Buenos Aires, Argentina). All reagents, enzymes, and enzyme substrates were reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistics

All results are expressed as mean values ± SEM. Student's *t* test was used to analyze differences between mean values.

RESULTS

NO production

The production of NO by heart submitochondrial membranes was 87% increased after 14–28 days of enalapril treatment (10 mg/kg/day), with a half-maximal effect at 5–6 days and maintained up to 120 days (Fig. 1). Enalapril treatment similarly increased NO production by liver submitochondrial membranes, both in quantitative effect and in time dependence (Fig. 1). The effect of different enalapril doses on heart and liver mtNOS activity was assayed after 14 days of treatment; a linear relationship was observed between mtNOS activity and enalapril dose in the range of 3–30 mg/kg/day (inset in Fig. 1), or of 6.4–64 μg of enalapril/kJ if expressed in terms of rat basal metabolic rate. The lower limit is comparable to the human daily dose (10–20 mg) that corresponds to 1.4–2.8 μg of enalapril/kJ considering human basal metabolic rate. Extrapolation of the rat data to humans would indicate that 20 mg/day enalapril would increase mtNOS activity by 7% in human heart.

The characteristics and the specificity of the spectrophotometric assay utilized to determine heart mtNOS activity are illustrated in Fig. 2. The addition of the substrates NADPH and arginine immediately started a consumption of HbO_2 due to NO production ($HbO_2 + NO \rightarrow metHb + products$). Trace

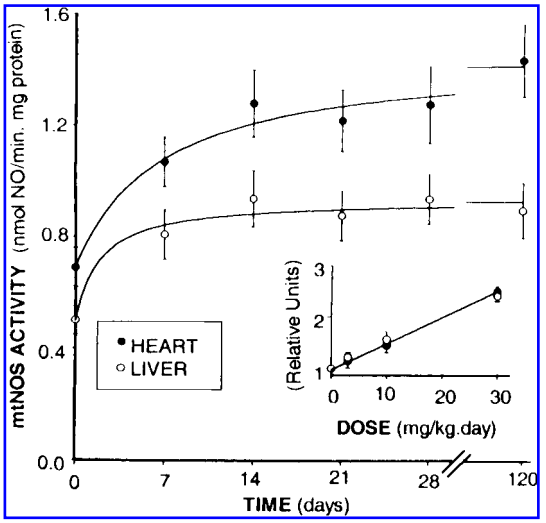

FIG. 1. Time and dose dependency of the effect of enalapril treatment on the mtNOS activity of rat heart (●) and liver (○) mitochondria. Time-effect relationship at the enalapril dose of 10 mg/kg/day. **Inset:** Dose-effect relationship at 14 days of treatment.

(a) corresponds to an enalapril-treated rat and trace (b) to a control untreated animal. Preincubation of the submitochondrial membranes isolated from an enalapril-treated rat with the NOS inhibitor L-NNA, trace (c), decreased the reaction rate by ~90%.

mtNOS functional activity in the regulation of O_2 uptake

The effect of mtNOS activity on mitochondrial O_2 uptake is easily assayed by the determination of state 3 respiration, which is the maximal physiological rate of O_2 uptake and ATP synthesis, i.e., active respiration, in the presence of excess respiratory substrate and ADP (4, 15); respiratory rates are determined in two limit conditions of intramitochondrial NO: at its maximal and minimal levels. The first condition is achieved by supplementation with arginine and SOD, and the second by addition of a NOS inhibitor and of HbO_2 (7, 29). The difference between the state 3 respiratory rates (a) with arginine and SOD and (b) with L-NNA and HbO_2 indicates mtNOS functional activity on the regulation of respiration (Table 1). Enalapril treatment increased mtNOS functional activity in inhibiting state 3 respiration from 22% to 43% in heart mitochondria and from 20% to 53% in liver mitochondria (Table 1).

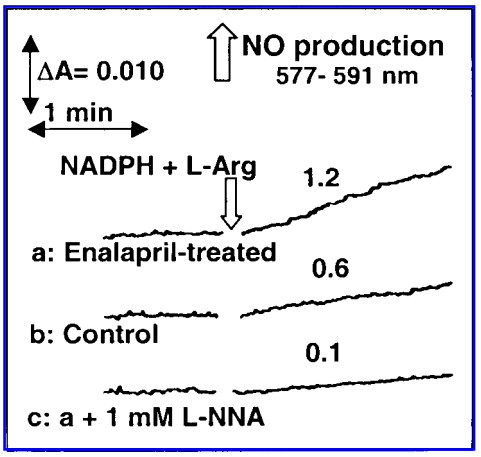

FIG. 2. Spectrophotometric determination of NO production by rat heart submitochondrial membranes. Reaction medium and other conditions are described in Materials and Methods. Mitochondrial protein, 0.60 mg/ml; L-NNA, 1 m*M*.

mtNOS functional activity in the regulation of H_2O_2 production

The regulatory activity of mtNOS on mitochondrial H_2O_2 production was assayed in mitochondrial state 4, the resting and nonphosphorylating mitochondrial state, *i.e.,* controlled respiration, in the presence of excess respiratory substrate and in the absence of ADP (15). Production of H_2O_2 is physiologically maximal in mitochondrial state 4 (4). Addition of arginine and SOD increases H_2O_2 production, whereas the supplementation of the same preparations with a NOS inhibitor and HbO_2 decreases H_2O_2 production (7, 39). The difference in H_2O_2 production rate between the conditions of maximal and minimal NO levels, *i.e., the functional activity of mtNOS* on the regulation H_2O_2 production, increased from 0.22 nmol of H_2O_2/min/mg of protein in control heart mitochondria to 0.43 nmol of H_2O_2/min/mg of protein in heart mitochondria from enalapril-treated rats (30% to 52% of the state 4 rate of H_2O_2 production, respectively). Similarly, enalapril treatment increased the mtNOS-sensitive H_2O_2 production of liver mitochondria from 28% to 49% of the state 4 rate (Table 2). Enalapril treatment did not change mitochondrial H_2O_2 production in the presence of antimycin, *i.e.,* the maximal rate of H_2O_2 generation, in the heart, but increased by 33% H_2O_2 production in the liver (Table 2). These results are at variance with the decrease in antimycin-stimulated heart H_2O_2 production observed after 18 months of enalapril treatment (17), likely by the complex relationships of aging and a long treatment.

Mn-SOD

Enalapril treatment (10 mg/kg/day for 28 days) significantly decreased Mn-SOD content in heart mitochondria from 5.0 ± 0.4 to 3.1 ± 0.3 pmol/mg of protein, whereas liver mitochondrial Mn-SOD was slightly decreased (7.2 ± 0.4 to 6.0 ± 0.4 pmol/mg of protein, respectively).

DISCUSSION

Heart and liver mitochondrial NO production was markedly increased, up to 2.5 times, by enalapril treatment, in a dose-dependent manner and with a half-time for maximal effect of 5–6 days, the latter in agreement with mitochondrial turnover time. The observed effect opens questions about the molecular mechanism of the link between the RAS and the

TABLE 1. EFFECT OF ENALAPRIL TREATMENT ON MTNOS ENZYMATIC ACTIVITY AND ON MTNOS FUNCTIONAL ACTIVITY IN THE INHIBITION OF OXYGEN UPTAKE OF RAT HEART AND LIVER MITOCHONDRIA

ORGAN/condition	*mtNOS activity (nmol of NO/min/mg of protein)*	*Respiratory rate (ng-at O/min/mg of protein)*
HEART/control	0.69 ± 0.04	117 ± 5
[a] + arginine + SOD		101 ± 4
[b] + 1 m*M* L-NNA + HbO_2		127 ± 5
mtNOS functional activity [b] − [a]		26 ± 6
HEART/enalapril-treated	1.28 ± 0.09*	93 ± 5
[a] + arginine + SOD		75 ± 4
[b] + 1 m*M* L-NNA + HbO_2		115 ± 5
mtNOS functional activity [b] − [a]		40 ± 6*
LIVER/control	0.49 ± 0.05	60 ± 3
[a] + arginine + SOD		60 ± 2
[b] + 1 m*M* L-NMMA + HbO_2		72 ± 3
mtNOS functional activity [b] − [a]		12 ± 4
LIVER/enalapril-treated	0.94 ± 0.08*	58 ± 3
[a] + arginine + SOD		48 ± 2
[b] + 1 m*M* L-NMMA + HbO_2		79 ± 3
mtNOS functional activity [b] − [a]		31 ± 4*

Experiments were carried out with malate and glutamate as substrate and mitochondria in metabolic state 3. Enalapril was given at 10 mg/kg/day for 14 days. Values are means ± SEM of three determinations. L-NMMA, N^G-methyl-L-arginine.

*$p < 0.05$.

intracellular redox state in the cardiovascular system and in other organs, especially in relation to aging (2, 25). Enalapril and captopril, both inhibitors of the angiotensin-converting enzyme, have been reported to produce a series of antiaging effects in rodents that seem to exceed their well known antihypertensive effects. Extended median life span (24), decreased cardiac fibrosis and hypertrophy (19, 24, 31), decreased diabetes-induced oxidative stress (21), and increased tissue levels of antioxidant enzymes (18–20) were reported. The protective action in the cardiovascular function and in tissue oxidative stress is likely exerted through a decrease in plasma angiotensin II levels and its binding to its receptor.

Activated angiotensin II receptors initiate a cascade of intracellular signaling that regulates gene activation and ex-

TABLE 2. EFFECT OF ENALAPRIL TREATMENT ON MTNOS ENZYMATIC ACTIVITY AND ON MTNOS FUNCTIONAL ACTIVITY IN THE INCREASE OF H_2O_2 PRODUCTION OF RAT HEART AND LIVER MITOCHONDRIA

	H_2O_2 production (nmol/min/mg of protein)	
ORGAN/condition	*Control*	*Enalapril-treated*
HEART	0.70 ± 0.04	0.84 ± 0.03
[a] + arginine + SOD	0.90 ± 0.03	1.07 ± 0.04
[b] + NOS inhibitor + HbO_2	0.68 ± 0.03	0.64 ± 0.03
[c] + 3 μ*M* antimycin	1.05 ± 0.05	1.08 ± 0.03
mtNOS functional activity [a] − [b]	0.22 ± 0.05	0.43 ± 0.05*
LIVER	0.50 ± 0.04	0.76 ± 0.04
[a] + arginine + SOD	0.61 ± 0.04	0.88 ± 0.04
[b] + NOS inhibitor + HbO_2	0.47 ± 0.03	0.51 ± 0.03
[c] + 3 μ*M* antimycin	0.72 ± 0.05	0.86 ± 0.04
mtNOS functional activity [a] − [b]	0.14 ± 0.05	0.37 ± 0.05*

Experiments were carried out with malate and glutamate as substrate and mitochondria in metabolic state 4. Enalapril was given at 10 mg/kg/day for 14 days. NOS inhibitor was L-NNA or L-NMMA, both at 1 m*M*, for heart or liver mitochondria, respectively. Values are means ± SEM of three determinations.

*$p < 0.05$.

pression. At present, inhibition of the angiotensin II/receptor interaction has been reported to increase antioxidant enzyme activities and mtNOS activity. Concerning antioxidant enzymes, Mn-SOD, Cu,Zn-SOD, catalase, and glutathione peroxidase activities were reported increased in 11-week enalapril- or captopril-treated mice (18–20). In this context, the RAS to ROS link is the angiotensin II/receptor-mediated down-regulation of antioxidant enzyme activities, which in turn leads to oxidative stress. Concerning mtNOS, its activity has been found up-regulated after enalapril administration during short periods (1–17 weeks) in rat heart and liver (this study), in rat kidney (30 mg/kg enalapril, i.p. for 14 days) (8), and after long-term treatments (6 and 18 months) in rat heart (17). Of note, the increase in antioxidant enzymes was not confirmed in rats after long-term administration of enalapril and losartan (17). In this case, the RAS to ROS link includes NO as intermediate and operates through an angiotensin II-mediated down-regulation of mtNOS activity that decreases the intracellular levels of NO. The mtNOS activities of heart, liver, and kidney, and the endothelial NOS of aorta endothelium (31) are increased after enalapril and losartan treatments. NO has been referred to as an effective antioxidant, both in membrane and in low-density lipoprotein lipoperoxidation, due to its capacity to annihilate free radicals; the NO unpaired electron matches the unpaired electrons of alkyl ($R^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals, yielding nonradical adducts that terminate free radical chain reactions (38, 42).

The physiological heart intramitochondrial NO and H_2O_2 concentrations, calculated by a double steady-state approach (36), were 30 n*M* NO and 100 n*M* H_2O_2 (Fig. 3). Concerning NO steady-state concentrations, 30–50 n*M* NO were reported for heart and liver (5, 36) and measured as 29 n*M* NO by electrochemical detection in a single isolated mitochondrion after mtNOS activation by Ca^{2+} (32). Enalapril treatment increased these values, by 66% to 50 n*M* NO and by 20% to 120 n*M* H_2O_2 (Fig. 3). The increase in NO concentration reflects the measured increases in mtNOS activity (Fig. 1 and Table 1), and the increase in H_2O_2 level reflects the increased rate of H_2O_2 generation (Table 2). Higher intramitochondrial NO and H_2O_2 steady-state concentrations provide a higher NO and H_2O_2 diffusion to the cytosol in the heart of enalapril-treated animals (Fig. 3). The current hypotheses to explain the biological effects of angiotensin II inhibitors is that both H_2O_2 and NO constitute a pleiotropic signal that indicates high mitochondrial energy charge, with both molecules acting together on cytosolic sensitive regulatory proteins that modulate the cellular cycle and the apoptosis pathway. The character of pleiotropic signal for the H_2O_2 and NO diffusion from mitochondria to cytosol is supported by the direct relationship of H_2O_2 production to mitochondrial energy charge: the rate is high in the energized mitochondrial metabolic state 4 and is low in state 3 and in the deenergized and uncoupled state 3u (4). Boyd and Cadenas postulated that NO and H_2O_2 activate mitogen-activated protein kinases by *S*-nitrosation in a process that is increased by reduced glutathione depletion (9).

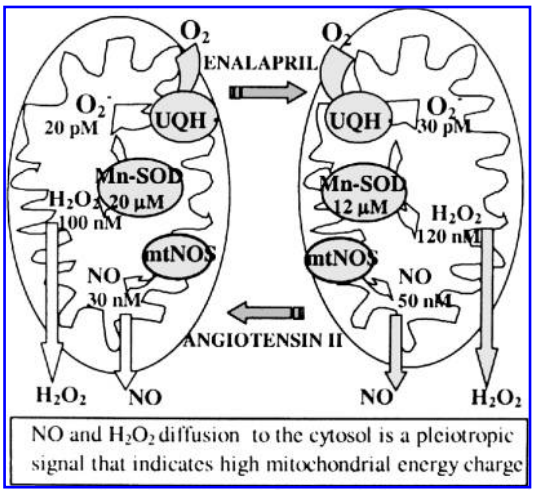

FIG. 3. Effects of enalapril, the inhibitor of the angiotensin-converting enzyme, and of angiotensin II on heart mtNOS biochemical activity, and on NO and H_2O_2 intramitochondrial steady-state concentrations and diffusion to the cytosol. The steady-state concentrations of NO and H_2O_2 reflect their generation rates (Figs. 1 and 2 and Tables 1 and 2); the content of Mn-SOD expresses the measured activities (see text). UQH, ubisemiquinone.

Mitochondrial NO modulates oxygen consumption by a reversible and O_2-competitive inhibition of cytochrome oxidase (5, 11, 16, 41) that slows down electron flow and substrate oxidation and stores chemical energy. In isolated myocytes, an increased mtNOS activity was associated with decreased myocardial contractility (32), in agreement with an inhibitory NO effect in electron transfer and oxidative phosphorylation (10, 41).

The enalapril effect described in Fig. 3, considering enalapril as inhibitor of the angiotensin-converting enzyme, constitutes an inverse description of the physiological effect of angiotensin II in sensitive cells, also indicated in Fig. 3. It can be postulated that angiotensin II is a prooxidant that leads cells to oxidative stress through decreased intracellular NO levels. Moreover, the effect of angiotensin II is similar to the changes in cellular and mitochondrial metabolism associated with aging. Down-regulation of heart mtNOS by angiotensin II resembles the effect of thyroxine in rat liver; hypothyroidism increased 2.6 times mtNOS activity, a change that was reverted by triiodothyronine (14).

Angiotensin II and NO are biological antagonists that appear integrated in homeostatic mechanisms for the regulation of vascular contraction and dilation; NO acts as a regulating factor of the RAS at different levels, whereas angiotensin II has a regulatory function on NO generation (26). The same antagonism is apparent in cellular oxygen uptake and energy metabolism, in intracellular redox state and oxidative stress. Enalapril treatment is associated with a decrease of heart mitochondrial Mn-SOD activity; a similar NO-dependent decrease in Mn-SOD activity has been observed in C6 glial cells (22). The direct effect of a decrease in Mn-SOD activity is an almost quantitatively similar increase in intramitochondrial superoxide anion (O_2^{-}) steady-state concentration (36). Recently, Cadenas and co-workers have recognized that moderate increases in O_2^{-} and H_2O_2 levels signal for cellular proliferation and growth (1, 9). Antunes and Cadenas reported that graded increases in H_2O_2 concentration in the reaction

medium lead fibroblasts, first to proliferation and after that to apoptosis (1).

Increased NO production by mtNOS leading to augmented NO and H_2O_2 intramitochondrial steady-state levels and diffusion to the cytosol may account through signaling cascades and gene activation for the beneficial effects observed in mammals in long-term enalapril treatments.

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ABBREVIATIONS

BSA, bovine serum albumin; HbO_2, oxyhemoglobin; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; metHb, methemoglobin; Mn-SOD, manganese superoxide dismutase; mtNOS, mitochondrial nitric oxide synthase; L-NMMA, N^G-methyl-L-arginine; L-NNA, N^ω-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; RAS, renin–angiotensin system; ROS, reactive oxygen species; SOD, superoxide dismutase.

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