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Kidney Mitochondrial Nitric Oxide Synthase

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ABSTRACT

Nitric oxide synthase activity was recognized in rat renal cortex mitochondria (mtNOS) with nitric oxide (NO) production rates of 0.14–0.78 nmol/min/mg of protein. Rat pretreatment with enalapril (30 mg/kg/day i.p., up to 15 days) increased NO production in kidney, liver, and heart mitochondria. In kidney, mtNOS activity and mtNOS protein, measured by western blot densitometry, were 5 and 2.3 times increased, respectively. Electron paramagnetic resonance analysis with the probe *N*-methyl-D-glucamine dithiocarbamate/$FeSO_4$ detected NO production in mitochondria isolated from enalapril-treated rats, but not in control untreated animals. Polyclonal antibodies anti–iNOS and anti–nNOS detected kidney mtNOS in western blots and inhibited mtNOS biochemical activity. The enzymatic activity of kidney mtNOS generates intramitochondrial NO concentrations that regulate mitochondrial functions: state 3 respiration was decreased by 12–28%, and state 4 hydrogen peroxide production was increased 12–35%.
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INTRODUCTION

THE MITOCHONDRIAL PRODUCTION OF NITRIC OXIDE (NO) by a specialized form of nitric oxide synthase (NOS) localized in the mitochondrial inner membrane was independently and simultaneously reported in rat liver mitochondria by Ghafourifar and Richter (26) and by Giulivi *et al.* (29, 43). Later on, other studies further reported mitochondrial NOS (mtNOS) activity in liver mitochondria and advanced its physiological function (10, 17, 27, 28, 34, 41). Simultaneously, NO production was also reported in brain (7, 34, 36, 40), thymus (15, 16), and heart (20, 25, 31) mitochondria. Recently, Giulivi and co-workers sequenced the 1,429 amino acids of liver mtNOS that was identified as neuronal NOS splice variant α (nNOSα) myristylated and phosphorylated in posttranslational processes (22). This important achievement establishes mtNOS as a constitutive protein of the inner mitochondrial membrane.

Earlier immunochemical studies had recognized a protein, named mtNOS, reacting with anti–endothelial NOS (anti-eNOS) antibodies in liver, brain, heart, kidney, and skeletal muscle mitochondria (3, 4, 32). In more recent studies, in which the determination of enzymatic activity was complemented with western blot analysis, liver mtNOS was reported reactive with anti–inducible NOS (anti-iNOS) antibodies (17, 43) and with anti-eNOS antibodies (34); brain mtNOS was found reactive with both anti-eNOS (34) and anti–neuronal NOS (anti-nNOS) antibodies (8, 40); and thymus mtNOS was found reactive with anti-iNOS antibodies (16). Then, there is an open question concerning the antibody reactivity of the mtNOS of different tissues.

At present, there is no report on the mtNOS of kidney cortex, a tissue characterized by high energy demands and oxidative phosphorylation, except for the cytochemical study mentioned early (3).

Interestingly, mtNOS activity is biologically modulated; it was reported down-regulated by thyroid hormones in liver (17) and susceptible to pharmacological regulation in liver, brain, and heart (8). Enalapril, the well known inhibitor of the angiotensin-converting enzyme, supplied in chronic treat-

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ments, was reported (a) to increase half-life span and the level of tissue antioxidant enzymes in mice (21, 23) and (b) to increase mtNOS activity in the rat heart (20); the two effects constitute a subject that opens a research area (2, 24). Enalapril effects are usually more marked in kidney than in other organs (8).

In this study, we report on the biochemical and functional activities of kidney cortex mtNOS, including the effect of an enalapril *in vivo* treatment. Some measurements were also performed in rat liver and heart mitochondria for comparative purposes.

MATERIALS AND METHODS

Mitochondrial preparations

Rats (200–240 g) fed a conventional laboratory diet and free water were anesthetized (pentobarbital, 50 mg/kg i.p.) and the kidneys rapidly excised. Renal cortex was separated from medulla and papilla and homogenized in 0.23 *M* mannitol, 0.07 *M* sucrose, 1 m*M* EDTA, 10 m*M* Tris-HCl (pH 7.4). The homogenate was centrifuged at 750 *g* for 10 min and the supernatant centrifuged at 7,000 *g* for 10 min; the mitochondrial pellet was resuspended and washed in similar conditions. Kidney cortex mitochondria showing respiratory controls of ~4.2 ± 0.2 with succinate as substrate were used for respiration and hydrogen peroxide (H_2O_2) production measurements (30). Kidney submitochondrial membranes were obtained by twice freezing and thawing mitochondrial preparations and by homogenizing them by passage through a 29G hypodermic needle (8).

Production of NO

The production of NO by submitochondrial membranes was determined in a reaction medium consisting of 150 μ*M* NADPH, 0.2 m*M* arginine (L-arginine), 0.3 m*M* $CaCl_2$, 2 μ*M* Cu,Zn-superoxide dismutase (SOD), 0.3 μ*M* catalase, and 100 m*M* phosphate buffer (pH 7.4). Mitochondrial protein was used in the range of 1.5–3.0 mg/ml (8). The spectrophotometric determination of mtNOS activity was made by measuring the rate of hemoglobin (HbO_2) oxidation to its met derivative and following the reaction at 579–591 nm ($\varepsilon_{579-591}$ = 11 mM^{-1} cm^{-1}), where 579 nm is the active wavelength and 591 nm is the isosbestic point, in a 356 PerkinElmer (PerkinElmer Instruments, Shelton, CT, U.S.A.) double-beam and double-wavelength spectrophotometer at 37°C (14). Detection of NO production by electron paramagnetic resonance (EPR) was performed in the presence of 20 m*M* *N*-methyl-D-glucamine dithiocarbamate (MGD; Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.) and 2 m*M* $FeSO_4$. Submitochondrial membranes were incubated for 10 min at room (25°C) temperature, transferred to Pasteur pipettes, and the EPR spectra recorded in Bruker ECS 106 ESR spectrometer with an ERY 1025T cavity (Bruker BioSpin GmbH, Rheinstetten, Germany). EPR settings were as follows: 20 mW microwave power; 6.175 G modulation amplitude; 164 ms time constant and conversion time; 50 kHz modulation frequency; 2 × 10^4 receiver gain; 150 G sweep; and 9.81 GHz microwave frequency.

Oxygen uptake and H_2O_2 production

Respiratory rates and H_2O_2 production were determined in coupled mitochondria suspended in 0.23 *M* mannitol, 0.07 *M* sucrose, 20 m*M* KCl, 1 m*M* EDTA, 7 m*M* phosphate buffer, 5 m*M* $MgCl_2$, 10 m*M* succinate, and 20 m*M* Tris-HCl (pH 7.3) at 30°C. To set state 3 active respiration, 0.1 m*M* ADP was added. Respiratory rates were measured with a Clark-type oxygen electrode. H_2O_2 production was measured fluorometrically at 365 nm (excitation) and 450 nm (emission) through the coupled oxidation of the hydrogen donor scopoletin (1 μ*M*) in the presence of 2 U/ml horseradish peroxidase (type VI-A, Sigma Chemical, St. Louis, MO, U.S.A.) (7, 44).

Cytochrome content

Cytochrome content was determined in a Beckman Coulter DU 7400 spectrophotometer from the 500 to 650 nm spectra of dithionite reduced minus oxidized submitochondrial membranes with the following wavelengths and extinction coefficients: cytochromes $c + c_1$, 550–540 nm and 19 mM^{-1} cm^{-1}; cytochromes *b*, 562–575 nm and 22 mM^{-1} cm^{-1}; cytochromes a + a_3, 605–630 nm and 21 mM^{-1} cm^{-1} (5).

Enalapril treatment

Enalapril was given intraperitoneally to 200–240 g rats at a dose of 30 mg/kg/day for periods of 7 and 14 days.

Antibodies

The following antibodies were used: polyclonal antibody (Pab) anti-iNOS [carboxy terminus (C-term), amino acids (aa) 1,126–1,144], monoclonal antibody (Mab) anti-iNOS (C-term, clone NOS-IN), Pab anti-nNOS [amino terminus (N-term), aa 251–270], Pab anti-nNOS (C-term, aa 1,409–1,429), Mab anti-nNOS (C-term, clone NOS-B1); Pab anti-eNOS (N-term, aa 596–609), Pab anti-eNOS (C-term, aa 1,185–1,205), and Mab anti-eNOS (C-term, clone NOS-E1) from Sigma Chemical; and Pab anti-iNOS [NOS-2 (C-19): sc-649], Pab anti-nNOS [NOS-1 (R-20): sc-648], and Pab anti-eNOS [NOS-3 (N-20): sc-653] from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

RESULTS

Production of NO by mitochondrial membranes of kidney cortex

Submitochondrial membranes from renal cortex mitochondria produce NO as shown in Fig. 1. In the traces, a stable baseline was recorded before the reaction was initiated by the addition of NADPH and arginine. The upward deflection of the trace [see trace (a)] indicates a decrease at 579 nm with respect to 591 nm, which identifies met-hemoglobin (metHb) formation and NO generation (HbO_2 + NO → metHb + products). It is worth noting that submitochondrial membranes totally expose the M face of the inner mitochondrial membrane, where mtNOS is located, with full availability of the substrates and cofactor (NADPH, arginine, and Ca^{2+}). A 2-min preincubation of the mitochondrial membranes with the NOS

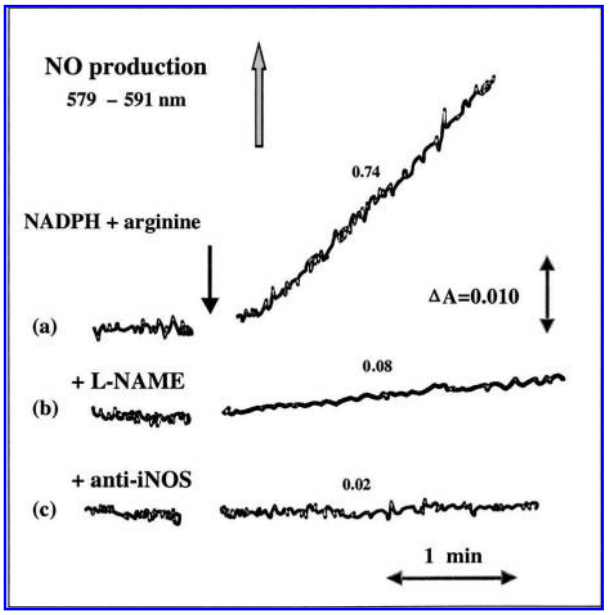

FIG. 1. Spectrophotometric determination of NO production by submitochondrial membranes of rat kidney cortex. Reaction medium and other conditions are described in Materials and Methods. Mitochondrial protein was present at 1.95 mg/ml and L-NAME at 3 m*M*. The antibody was Pab anti-nNOS (C-term, aa 1,409–1,429). The numbers near the traces indicate the rate of metHb formation in nmol/min/mg of mitochondrial protein.

inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) produced an almost complete suppression of NO generation, as shown in trace (b). The rate of NO production is safely calculated from the rate of metHb formation that is suppressed by the NOS inhibitor L-NAME; in other words, the slope of trace (a) minus the slope of trace (b), which in the case of Fig. 1 gives a rate of 0.66 nmol of NO/min/mg of protein and corresponds to a rat treated with enalapril (see below). Trace (c) shows the effect of an anti-nNOS antibody, which was preincubated with the mitochondrial membranes for 5 min, on the enzymatic activity of the mtNOS of kidney mitochondria. The effect of a series of anti-NOS antibodies on mtNOS activity is listed in Table 1. Only two Pabs, anti-iNOS and anti-nNOS, were able to inhibit mtNOS activity effectively.

Up-regulation of kidney, liver, and heart mtNOS activities by enalapril

Treatment with the drug enalapril increased mitochondrial NO production by 75%, 40%, and 50% after 7 days, and by five, four, and four times after 14 days in kidney, liver, and heart mitochondria, respectively (Fig. 2). The estimated $t_{1/2}$ of the response (~8 days) is similar to mitochondrial turnover and suggests an activation by enalapril of cytosolic factors that regulate the transcription of specific genes.

The mitochondrial production of NO by kidney and liver mitochondria was also assayed by EPR in the presence of the probe MGD-Fe (Fig. 3). The NO signal was detected in kidney and liver mitochondria isolated from enalapril-treated rats, barely seen in mitochondria isolated from untreated control rats, and abolished after supplementation of mitochondrial membranes from enalapril-treated animals with the NOS inhibitor L-NAME (Fig. 3).

TABLE 1. EFFECT OF ANTI-NOS ANTIBODIES ON THE mtNOS ACTIVITY OF KIDNEY CORTEX MITOCHONDRIA

	mtNOS activity (nmol of NO/min/mg of protein)	*Inhibition (percentage)*	*Reactivity in western blot*
No addition	0.76	—	—
Pab anti-iNOS (C-term)	0.28	63%	Yes
Mab anti-iNOS (C-term)	0.76	0%	ND
Pab anti-nNOS (N-term)	0.72	5%	ND
Pab anti-nNOS (C-term)	0	100%	Yes
Mab anti-nNOS (C-term)	0.76	0%	ND
Pab anti-eNOS (N-term)	0.76	0%	No
Pab anti-eNOS (C-term)	0.76	0%	ND
Mab anti-eNOS (C-term)	0.76	0%	ND

ND, not determined.

Kidney mtNOS reacted with anti-iNOS antibodies; rats treated for 14 days with enalapril showed a 125% increased amount of mtNOS protein as determined by western blot analysis and densitometry. The reactive protein moved in the gels as an 80-kDa polypeptide (Fig. 4).

Inhibition of respiration by intramitochondrial NO

Supplementation of kidney mitochondria in state 3 (active respiration in the presence of ADP) with the mtNOS substrate arginine and with SOD slightly decreased the respiration rate

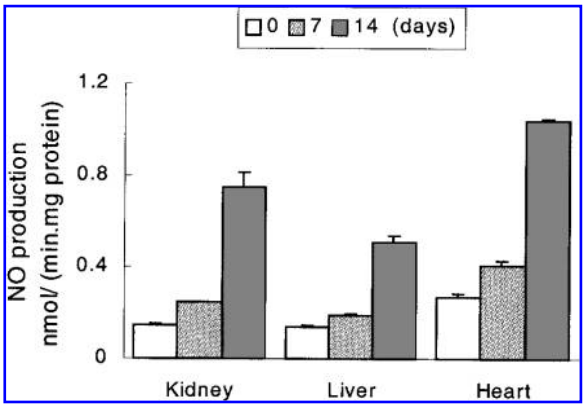

FIG. 2. Effect of enalapril (30 mg/kg/day, 7 and 14 days) on the mtNOS activity of kidney, liver, and heart.

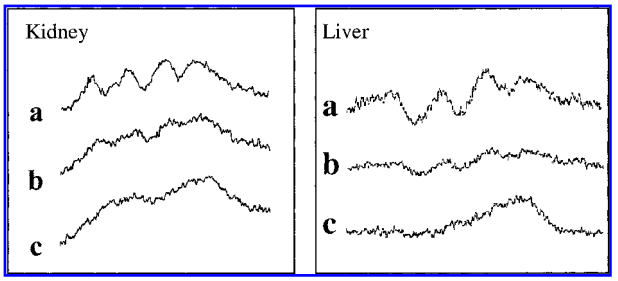

FIG. 3. NO production by kidney and liver mitochondrial membranes as detected by EPR. Kidney and liver: (**a**) 14 days enalapril-treated rat; (**b**) control untreated rat; (**c**) (a) + L-NAME. Reaction medium and other conditions are described in Materials and Methods.

(4%). It is understood that arginine and SOD have synergic effects in maximizing the intramitochondrial steady-state level of NO. Supplementation of the same mitochondrial preparation with the NOS inhibitor L-NAME and HbO_2, a condition that will minimize intramitochondrial NO levels, slightly increased respiration (8%). Altogether, the difference in respiratory rate between the conditions of maximal and minimal intramitochondrial NO steady-state concentrations was 12% [conditions (b) and (a) in Table 2] of the physiological state 3 respiratory rate. This effect is termed the *functional activity of mtNOS* in the regulation of mitochondrial respiration (Table 2) and corresponded to a mtNOS biochemical activity of 0.14 nmol of NO/min/mg of protein. In the case of kidney mitochondria isolated from enalapril-treated animals, the addition of arginine and SOD decreased respiration by 8% and the addition of L-NAME and hemoglobin increased respiration by 20%; the overall effect, *i.e.,* the functional activity of mtNOS, amounted to 28% of the physiological state 3 rate of respiration and corresponded to a mtNOS biochemical activity of 0.72 nmol of NO/min/mg of protein (Table 2).

Increase of H_2O_2 production by intramitochondrial NO

Addition of arginine and SOD to state 4 kidney mitochondria increased H_2O_2 production by ~10%, whereas the supplementation of the same preparations with L-NAME decreased H_2O_2 production by 10%; altogether, the difference in

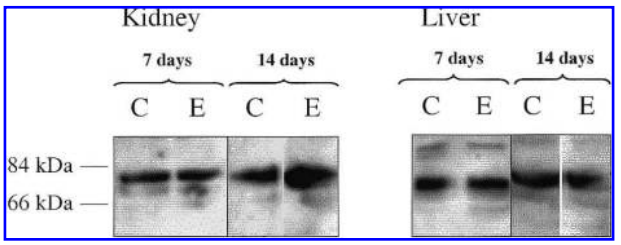

FIG. 4. Western blot analysis: mtNOS of kidney and liver mitochondrial membranes from control (C) and enalapril-treated (E) rats. mtNOS was recognized by an anti-iNOS antibody (Santa Cruz Biotechnology).

TABLE 2. EFFECT OF ENALAPRIL ON mtNOS ACTIVITY AND ON THE REGULATION OF MITOCHONDRIAL RESPIRATION BY NO IN KIDNEY CORTEX MITOCHONDRIA

	mtNOS activity (nmol of NO/min/mg of protein)	*Respiratory rate (ng-at O/min/mg of protein)*
Control rats	0.14 ± 0.02 (100%)	201 ± 7
(a) + L-arginine + SOD		193 ± 5
(b) + L-NAME + HbO_2		219 ± 6
mtNOS functional activity (b) − (a)		26 ± 8*
Enalapril-treated rats†	0.75 ± 0.06 (536%)	184 ± 68
(a) + L-arginine + SOD		158 ± 5
(b) + L-NAME + HbO_2		221 ± 6
mtNOS functional activity (b) − (a)		65 ± 8*

Mitochondria in metabolic state 3, with succinate as substrate, were used for respiration measurements.

*$p < 0.05$ (Student test).

†Enalapril was given for 14 days.

H_2O_2 production rate between the conditions of maximal and minimal NO levels, *i.e.,* the functional activity of mtNOS, 0.08 nmol of NO/min/mg of protein, amounted to 20% of the physiological state 4 rate of H_2O_2 production (Table 3). When the same additions were made to kidney mitochondria isolated from enalapril-treated rats, the effects of arginine plus SOD and of L-NAME on H_2O_2 generation rates were ~20% each, and the combined effect, *i.e.,* the functional activity of mtNOS, 0.28 nmol of NO/min/mg of protein, amounted to 53% of the physiological state 4 rate of H_2O_2 production (Table 3).

TABLE 3. EFFECT OF ENALAPRIL ON mtNOS ACTIVITY AND ON THE REGULATION OF THE MITOCHONDRIAL PRODUCTION OF H_2O_2 BY NO IN KIDNEY CORTEX MITOCHONDRIA

Condition (mtNOS activity)*	*H_2O_2 production (nmol/min/mg of protein)*
Control rat (0.16)	0.44 ± 0.03
(a) + L-arginine + SOD	0.48 ± 0.03
(b) + L-NAME	0.40 ± 0.03
(c) + antimycin	0.70 ± 0.05
mtNOS functional activity (a) − (b)	0.08 ± 0.04†
Enalapril-treated rat‡ (0.78)	0.67 ± 0.04
(a) + L-arginine + SOD	0.80 ± 0.05
(b) + L-NAME	0.52 ± 0.04
(c) + antimycin	0.82 ± 0.06
mtNOS functional activity (a) − (b)	0.28 ± 0.06†

Mitochondria in metabolic state 4, with succinate as substrate, were used for H_2O_2 production measurements.

*In nmol of NO/min/mg of protein.

†$p < 0.05$ (Student test).

‡Enalapril was given for 14 days.

Cytochrome content

Cytochrome content was determined in cortex kidney mitochondria isolated from enalapril-treated and control rats to assay the specificity of the up-regulation of mtNOS by enalapril. Enalapril-treated rats showed ~33% decreased cytochrome content, which indicates that the up-regulation of mtNOS activity is a specific effect (Table 4).

DISCUSSION

The experiments reported in this study add kidney cortex to the list of mammalian organs from which isolated mitochondria have been shown to produce NO. In this way, mtNOS activity has been recognized in liver, brain, heart, skeletal muscle, thymus, and kidney cortex. Liver mtNOS has been found to be identical to nNOSα in its 1,429 amino acids, with two posttranslational modifications, phosphorylation and myristylation (22). Transcripts corresponding to nNOSα were found in liver, brain, heart, kidney, skeletal muscle, lung, testis, and spleen, indicating that these tissues have the capacity to express nNOSα and mtNOS (22). It is then clear that mtNOS is a constitutive protein of the inner mitochondrial membrane of mammalian organs.

The antibody reactivity of mtNOS of different organs is not fully clear at the present time. As stated in the Introduction and shown in Table 1, there is more than one reactivity toward NOS antibodies for the mtNOS of some organs. Phosphorylation and myristylation may contribute to cross reactions and to loss of the immunoreactivity of mtNOS with anti-nNOS antibodies in the immune reaction in the solid phase as pointed out by Elfering *et al.* (22). Of note, two different Pabs, anti-nNOS and anti-iNOS, directed toward the reductase domain at the C-term of the sequence (iNOS aa 1,126–1,144, nNOS aa 1,409–1,429), were able to inhibit kidney mtNOS activity in solution (Table 1).

The important question concerning mtNOS, now that it is established as an inner membrane protein, is which is its physiological role. The continuous intramitochondrial generation of NO by mtNOS, provided the availability of intramitochondrial NADPH, arginine, and Ca^{2+}, generates an intramitochondrial NO steady-state concentration that has been measured (31) and calculated (6, 39) in the range of 30–50 n*M* NO. Concerning the biological significance of mtNOS activity, there are currently two main views. In the pathophysiological view, NO and peroxynitrite ($ONOO^-$) [the product of the reaction of NO with superoxide anion (O_2^-)], in a rapid or slow process, generate mitochondrial dysfunction that in turn leads to apoptosis; the process is considered relevant in aging and neurodegenerative diseases (11, 16, 24). In the regulatory view, NO exerts a regulatory role on mitochondrial O_2 uptake and H_2O_2 production. In this latter conception, mtNOS has a biochemical activity associated with a regulatory functional activity (6, 9, 31, 33). It is clear that the two views are not contradictory but complementary; the regulatory functional role may occur at physiological levels of NO and the pathophysiological pathway may be triggered at increased NO levels, as in inflammation.

TABLE 4. CYTOCHROME CONTENT OF CORTEX KIDNEY MITOCHONDRIA ISOLATED FROM ENALAPRIL-TREATED AND CONTROL RATS

	Enalapril-treated	*Control*
Cytochromes $c + c_1$	0.39 ± 0.01	0.59 ± 0.03
Cytochromes b	0.21 ± 0.02	0.31 ± 0.02
Cytochromes $a + a_3$	0.17 ± 0.01	0.26 ± 0.02

Enalapril was given for 14 days. Cytochrome content is in nmol/mg of protein.

Concerning the functional activity of mtNOS in regulating mitochondrial respiration, it is clear that the phenomenon occurs *in vitro* with mitochondria isolated from liver, brain, and kidney in a significant range. Supplementation of respiring mitochondria with the mtNOS substrate L-arginine decreases the respiration rate by ~10–30%, depending on the organ. The effect is observed with mitochondria in metabolic states 4 and 3, and with malate-glutamate or succinate as substrates (8, 28). Similarly and on the contrary, the addition of mtNOS inhibitors to respiring mitochondria increases the respiratory rate by ~20–50%, depending on the type of organ mitochondria (8, 28). In the different types of mitochondria, a marked effect of L-arginine is usually associated with a low effect of the NOS inhibitor, and vice versa. The functional activity of kidney mtNOS in the two conditions of low and high NO, in regulating mitochondrial state 3 O_2 uptake, was 13% and 35%, respectively, which is remarkable in terms of cell energy supply. The described effects are explained by the continuous production of NO by mtNOS and by the inhibition of cytochrome oxidase by NO; NO binds to the enzyme in its reduced and oxidized forms in a process that is competitive with O_2 (6, 13, 18, 19, 37, 42). Binding and inhibition are removed by washing or by addition of myoglobin or hemoglobin. The inhibition of mitochondrial respiration by NO can be expressed as a function of the ratio $[O_2]/[NO]$; half-maximal inhibition of state 3 respiration is reached at O_2/NO ratios of 150–500 (6, 13, 42). For cortex kidney mitochondria, the intramitochondrial NO level is calculated as 40 n*M,* which at physiological 20 μ*M* O_2 yields an O_2/NO ratio of 500 and a cytochrome oxidase inhibition of 25% (6, 39). The contemporary concept is that mitochondrial and cellular O_2 uptakes are regulated by ADP, O_2, and NO and that the rate of cell energy supply depends on the mitochondrial O_2/NO ratio. However, the quantitative physiological situation is not clear, because tissue myoglobin and blood hemoglobin are effective NO scavengers, decreasing the NO steady-state concentrations in cells and tissues (14).

Concerning the functional activity of mtNOS in regulating mitochondrial H_2O_2 production, it is also clear that the phenomenon occurs *in vitro* with isolated mitochondria. Addition of arginine to state 4 kidney, liver, and brain mitochondria increases H_2O_2 production by ~10–30%, whereas the supplementation of the same preparations with NOS inhibitors decreases H_2O_2 production by 10–40% (Table 3) (41). When the arginine effect is high, the NOS inhibitor effect is low, as described for the similar situation concerning cytochrome oxidase. The effects are again explained by the intramitochondrial NO steady-state concentrations and, in this case, by the NO inhibition of ubiquinol–succinate–cytochrome *c* reduc-

tase (complex III) activity that enhances H_2O_2 production (38) and by the reaction of NO with ubiquinol, yielding ubisemiquinone that by autooxidation produces O_2^- (38).

The observation by Antunes and Cadenas (1) that H_2O_2 is able to exert a tight control of the proliferation–apoptosis transition in fibroblasts, where 0.7 μ*M* H_2O_2 maintains cell proliferation and H_2O_2 levels over 1 μ*M* set up apoptosis, gave the experimental basis to postulate a role of both H_2O_2 and NO in cell intracellular signaling (1, 11). Both molecules acting together on a sensitive molecular transducer may constitute a pleiotropic signal, indicating high mitochondrial energy charge, for the regulatory cascades that modulate cellular cycle and apoptosis. Specific up-regulation of mtNOS, as shown here, and up-regulation of antioxidant enzymes are the two current molecular hypotheses for the beneficial effects of the inhibitors of the renin–angiotensin system upon normal aging (2, 24).

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ABBREVIATIONS

aa, amino acids; C-term, carboxy terminus; EPR, electron paramagnetic resonance; HbO_2, oxyhemoglobin; H_2O_2, hydrogen peroxide; Mab, monoclonal antibody; metHb, met-hemoglobin; MGD, *N*-methyl-D-glucamine dithiocarbamate; mtNOS, mitochondrial nitric oxide synthase; L-name, N^{ω}-nitro-L-arginine methyl ester; nNOS, iNOS, and eNOS, neuronal, inducible, and endothelial nitric oxide synthases, respectively; nNOSα, neuronal nitric oxide synthase splice variant α; NO, nitric oxide; NOS, nitric oxide synthase; N-term, amino terminus; O_2^-, superoxide anion; Pab, polyclonal antibody; SOD, Cu,Zn-superoxide dismutase.

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