Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart

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Laboratory of Oxygen Metabolism, University Hospital, School of Medicine and Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, 1120 Buenos Aires, Argentina; and Department of Molecular Pharmacology and Toxicology, University of Southern California, Los Angeles, California 90033

Poderoso, Juan J., Jorge G. Peralta, Constanza L. Lisdero, Maria Cecilia Carreras, Marcelo Radisic, Francisco Schöpfer, Enrique Cadenas, and Alberto Boveris. Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C112–C119, 1998.—Isolated rat heart perfused with 1.5–7.5 μM NO solutions or bradykinin, which activates endothelial NO synthase, showed a dose-dependent decrease in myocardial O_2 uptake from 3.2 ± 0.3 to 1.6 ± 0.1 (7.5 μM NO, $n = 18$, $P < 0.05$) and to 1.2 ± 0.1 μM $O_2 \cdot min^{-1} \cdot g\ tissue^{-1}$ (10 μM bradykinin, $n = 10$, $P < 0.05$). Perfused NO concentrations correlated with an induced release of hydrogen peroxide (H_2O_2) in the effluent ($r = 0.99$, $P < 0.01$). NO markedly decreased the O_2 uptake of isolated rat heart mitochondria (50% inhibition at 0.4 μM NO, $r = 0.99$, $P < 0.001$). Cytochrome spectra in NO-treated submitochondrial particles showed a double inhibition of electron transfer at cytochrome oxidase and between cytochrome *b* and cytochrome *c*, which accounts for the effects in O_2 uptake and H_2O_2 release. Most NO was bound to myoglobin; this fact is consistent with NO steady-state concentrations of 0.1–0.3 μM, which affect mitochondria. In the intact heart, finely adjusted NO concentrations regulate mitochondrial O_2 uptake and superoxide anion production (reflected by H_2O_2), which in turn contributes to the physiological clearance of NO through peroxynitrite formation.

Langendorff preparation; regulation of myocardial oxidative metabolism; nitrosylmyoglobin; role of oxygen free radicals; peroxynitrite

THE NITROGEN RADICAL nitric oxide (NO) is a physiological vasodilator in arterial vascular beds such as vertebral, pulmonary, and coronary arteries (17). The effects of NO are mediated by its binding to the heme group of guanylyl cyclase to produce guanosine 3′,5′-cyclic monophosphate, which relaxes vascular smooth muscle by lowering cytoplasmic Ca^{2+} levels (12).

The pharmacological use of nitrites, nitrates, and derived compounds to alleviate symptoms of coronary insufficiency is well known by physicians. It is now accepted that the pharmacological actions of nitrates and nitrites are mediated by the release of NO (12). In the last years, solutions of NO or NO donors have been reported to produce coronary vasodilation, to protect ischemic myocardium, and to prevent the deleterious effects of reperfusion in the heart (16).

Recently, interest has been focused on the actions of NO on mitochondrial function and on oxidative metabolism of mammalian cells. NO reversibly inhibits cytochrome oxidase and O_2 uptake in skeletal muscle and heart mitochondria (5, 20) and in heart submitochondrial particles prepared from rat heart (20). Previously, inhibition of mitochondrial enzymes by NO was described as a toxic and nonreversible effect rather than as a physiological and regulatory action (3). Toxic effects are understood to be primarily produced by peroxynitrite ($ONOO^-$), the product of the reaction of NO with superoxide anion (O_2^-; see Ref. 1). The effects of $ONOO^-$ appear to be selectively exerted on matrix mitochondrial enzymes such as aconitase and on membrane-bound enzymes such as succinate dehydrogenase (3).

On the other hand, the overproduction of superoxide radicals and derived reactive O_2 species has been recognized as the initial molecular phenomenon in experimental and clinical myocardial ischemia-reperfusion (30). Mitochondria are the main source of cellular O_2 free radicals; O_2^- and its dismutation product H_2O_2 are by-products of mitochondrial electron transfer from a deviation of electrons that reduce O_2 in collisional noncatalyzed reactions (25). Irreversible inhibition of electron transfer and increase in O_2^- production are obtained in the presence of the nonphysiological drug antimycin. We have reported that 0.5–1 μM NO is able to inhibit mitochondrial electron transfer at the ubiquinol-cytochrome *b*-cytochrome *c* region of the respiratory chain and to produce an increase in O_2^- and H_2O_2 production in submitochondrial particles and in rat heart mitochondria (20). The mitochondrial production of O_2^- seemed to revert NO inhibitory effects on cytochrome oxidase. On this basis, it is interesting to consider the hypothesis that the heart, under physiological conditions, is subjected to a NO regulation of O_2 uptake and mitochondrial electron transfer with an effect in O_2^- production in a much more causal way than previously recognized.

The aim of the present study was to determine whether changes in O_2 uptake and in H_2O_2 release could be observed in the beating heart of the Langendorff preparation after the infusion of a NO gas solution, the NO donor *S*-nitrosoglutathione (GSNO), or autacoids that promote NO release, like bradykinin. The experimental conditions of the Langendorff preparation do not rule out some degree of myocardial ischemia, but they allow approximation of the physiological setting in which O_2 concentration can be consid-

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ered to be at the edge of effectively regulating respiration (8).

The results suggest that NO released by endothelium not only dilates the vessels but also plays a regulatory role increasing the generation of O_2^- and lowering heart O_2 uptake.

MATERIAL AND METHODS

General experimental design. The effects of different doses of NO, GSNO, and bradykinin were tested in different heart preparations. In each experiment, cardiac physiological functions such as mean coronary perfusion pressure, left ventricular developed pressure, heart O_2 uptake, and release of NO and H_2O_2 were assessed simultaneously. To determine NO and H_2O_2 concentrations, 10-ml samples of the effluent perfusate of the isolated rat hearts were collected every 5 min. The samples were taken before NO or bradykinin infusion to set the basal values, during NO and bradykinin infusion, and up to 30 min after the treatments were discontinued. Control hearts perfused with saline solution during a similar period (50 min, $n = 4$) neither exhibited changes in O_2 uptake, aortic coronary pressure, and developed left ventricular pressure nor released detectable NO or H_2O_2 during the experiments. The direct effect of NO on mitochondrial respiration was determined by measuring the O_2 uptake of isolated rat heart mitochondria supplemented with NO. To obtain enough mitochondrial protein concentration and considering the weight of the rat hearts (1–1.3 g), two animals were used in each experiment, and four experiments were done to assess the NO effects on mitochondrial functions.

Animals. Eighty female Sprague-Dawley rats weighing 250–300 g fasted overnight with ad libitum access to water were used. Rats were anesthetized with ether. To remove blood cells, 60 units of sodium heparin were administered by the tail vein before the heart was excised. The protocol followed international ethics guidelines for laboratory animals.

Heart perfusion and pressure determinations. Hearts were perfused by the Langendorff technique (21) at 37°C with a perfusion medium consisting of 128 mM NaCl, 4.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 20.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 5 mM glucose, and 2 mM pyruvic acid (pH 7.4) that was oxygenated with 95% O_2-5% CO_2. Hearts were paced at 5 Hz with a pacemaker. The aortic-coronary flow was kept constant at a rate of 6–7 ml/min with a peristaltic pump (model 7554-20; Cole Parmer, Chicago IL). Mean aortic-coronary perfusion pressure was obtained from the perfusion line. Developed left ventricular pressure (peak systolic left ventricular pressure − end diastolic left ventricular pressure) was determined at a fixed preload (5–7 mmHg) with a balloon-tipped catheter directly inserted in the left ventricle. The lines were connected to a pressure transducer (78342A monitor; Hewlett-Packard, Boeblingen, Germany), and pressure curves were recorded in Dyne programmable multichannel equipment (Dyne, Buenos Aires, Argentina).

To avoid a possible contamination of the perfusion fluid, a cannula was inserted in the pulmonary artery to collect the effluent samples.

Infusion of NO. After 15 min of perfusion and under stable beating and perfusion conditions, NO or GSNO was added in parallel to the perfusion medium with a Harvard pump at a rate of 0.2 ml/min to achieve concentrations of 1–8 μM NO and of 0.1–1.5 mM GSNO in the perfusate. At these slow rates, the degassed NO solution did not diminish P_{O_2} of the perfusion fluid by >2.8%. The electrochemical detection of NO in the effluent during the perfusion with true NO solutions was ~3–10% of the added NO (0.14, 0.17, and 0.27 μM for 2, 5, and 7.5 μM, respectively). The NO concentration in the effluent amounted to ~0.6% of added GSNO (20). The NO levels in the effluent decreased promptly to be undetectable a few minutes after NO infusion was stopped.

Measurement of myocardial O_2 uptake. The O_2 uptake was calculated from the difference between the O_2 content in the input minus the content in the output multiplied by the perfusate flow according to the Fick principle. The difference in O_2 content was determined with an on-line Clark type electrode and recorded continuously.

Detection of NO. The NO concentration in the perfused solutions and in the effluent was measured with a sensitive NO electrode (ISO-NO; World Precision Instruments, Sarasota, FL) connected to a recorder. The electrode was calibrated daily with standard nitrite solutions in acid medium.

Measurement of H_2O_2 in the effluent. The concentration of H_2O_2 in the effluent samples was determined fluorometrically at 315 nm (excitation) and 425 nm (emission) in an F-2000 Hitachi spectrofluorometer (Hitachi, Tokyo, Japan), using an assay medium containing 12 U/ml horseradish peroxidase and 500 μM *p*-hydroxyphenilacetic acid in 2-ml cuvettes at 37°C under soft magnetic stirring (2, 20). Determinations were performed in duplicates with and without 0.1 μM catalase, and the difference between both fluorescence measurements was calculated as H_2O_2 concentrations (11).

Mitochondrial respiration. Rat heart mitochondria were isolated in 0.23 M mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.3; see Ref. 20). Mitochondrial O_2 uptake was determined polarographically with a Clark type O_2 electrode in a reaction medium consisting of 0.23 M mannitol, 70 mM sucrose, 0.2 mM EDTA, 5 mM Na_2HPO_4-KH_2PO_4, and 20 mM Tris·HCl buffer (pH 7.4) at 30°C. O_2 uptake rates were measured with 8 mM succinate as substrate in the presence (state 3 O_2 uptake) or absence (state 4 O_2 uptake) of 0.2 mM ADP as phosphate acceptor. Cytochrome differential spectra of rat heart submitochondrial particles obtained by sonication of rat heart mitochondria as previously described (20) were recorded with a model 356 Perkin-Elmer-Hitachi double beam-double wavelength spectrophotometer. Mitochondria and submitochondrial particles were exposed to different GSNO concentrations by 8 min to provide a known rate of NO release (d[NO]/d*t*) and a known effective NO concentration (20). Cytochrome redox state was controlled by adding succinate and, to achieve a complete cytohrome reduction, with sodium dithionite. The supplementation with NO did not modify the final reduction level produced by sodium dithionite.

Myoglobin spectra in heart slices. Rat hearts were placed in liquid nitrogen immediately after NO infusion, and 6- to 7-μm slices from the left ventricles were obtained with an electronic microtome at −20°C (4). The slices were placed on thin glass covers that were vertically adhered to one of the transparent walls of quartz cuvettes. The wavelength scan spectra were recorded between 560 and 600 nm in a Hitachi U-3000 spectrophotometer. The absorbance difference between 581 and 592 nm was proportional to oxyMb concentrations with an extinction coefficient ($\epsilon_{581-592}$) = 11 mM/cm (2).

Infusion of bradykinin. Bradykinin was infused at 1–10 μM. As reported by Zhang et al. (29) in isolated myocardial slices, the effects of bradykinin were potentiated by inhibition of kininase II with enalapril (10 mg/kg), which was administered to the rats in the tail vein 30 min before the start of the experiments. The NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) was added to the perfusion medium at a concentration of 100 μM. In other experiments, indomethacin

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(100 μM) was added to bradykinin to rule out prostacyclin-dependent effects.

Chemicals and drugs. GSNO was synthesized at 25°C by reacting equimolar concentrations (200 mM) of reduced glutathione in 100 mM phosphate buffer (pH 7.4) with sodium nitrite in 100 mM HCl. NO solutions (1–1.5 mM) were obtained freshly on the day of the experiment or on the day before by bubbling 99% pure NO gas in N_2-degassed water for 30 min at room temperature and afterward were kept at 4°C. Other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Statistics. The statistical analysis included paired Student's *t*-test, one-way analysis of variance, Dunnett's test, and regression studies.

RESULTS

Effects of NO infusion on myocardial O_2 uptake and coronary perfusion. The infusion of either NO solutions or GSNO to the isolated and perfused rat heart clearly induced dose-dependent decreases in myocardial O_2 uptake and coronary perfusion pressure and a slight nonsignificant change in developed left ventricular pressure. A comparison between the percentage of variation with respect to control values induced by 1.5–7.5 μM NO infusion on heart functions is shown in Fig. 1. O_2 uptake decreased progressively from 3.2 ± 0.3 (basal value) up to 1.6 ± 0.1 μmol $O_2 \cdot min^{-1} \cdot g$ tissue^{-1} (at 7.5 μM NO, $P < 0.05$; controls $n = 6$, each NO concentration subgroup $n = 4$, total $n = 18$). Once infusion was stopped, O_2 uptake recovered to the basal condition after 10–15 min of perfusion. In the same conditions, mean coronary perfusion pressure decreased from 80 ± 3 to 50 ± 5 mmHg (total $n = 18$, $P < 0.05$) and returned back to the basal values within 10 min after stopping NO infusion. The left ventricular developed pressure did not significantly change by effects of NO infusion (from 100 ± 10 to 85 ± 2 mmHg).

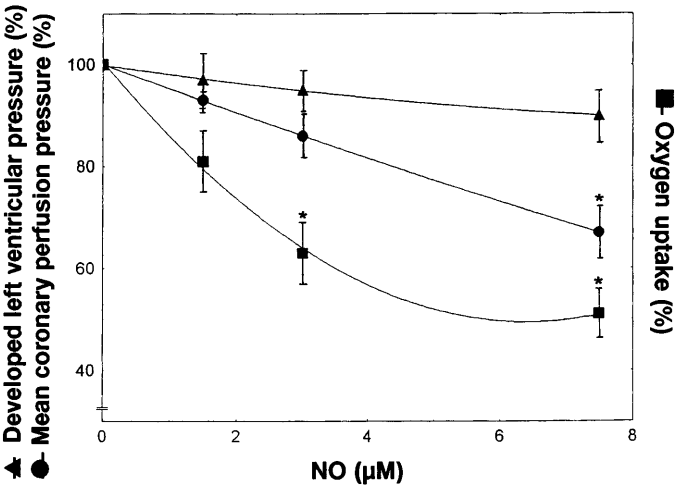

Fig. 1. Comparative effects of 3 different nitric oxide (NO) concentrations on left ventricular developed pressure, mean coronary perfusion pressure, and myocardial O_2 uptake of the perfused beating rat heart. Each point is the mean ± SE of the percentage of variation of 4 separate experiments with respect to 6 controls before and after 10-min NO infusion. * Significant difference with respect to controls by analysis of variance (ANOVA) and Dunnett's test ($P < 0.05$).

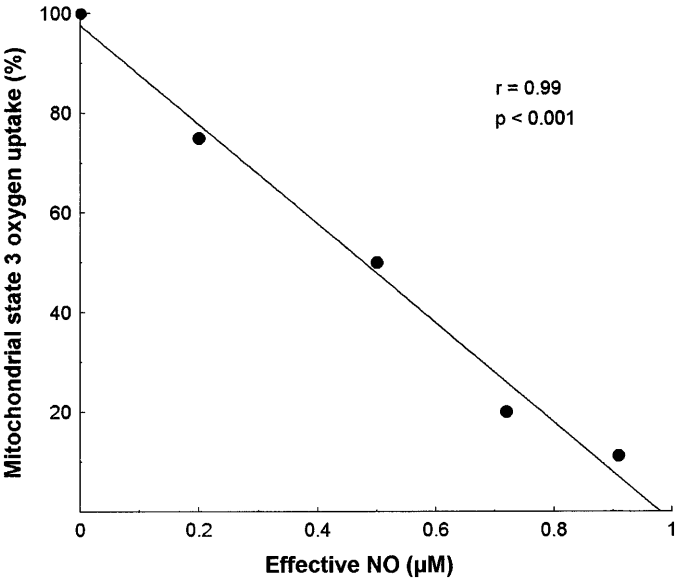

Fig. 2. Inhibition of state 3 O_2 uptake of rat heart mitochondria (1–2 mg protein/ml) in relation to the effective NO concentrations released by the NO donor *S*-nitrosoglutathione (GSNO). O_2 uptake was measured in the presence of 8 mM succinate and 0.2 mM ADP. Data are expressed as percentage of control values. Each point represents the average of 4 determinations in separate experiments.

The hearts did not exhibit any functional impairment up to 60 min after NO infusion was stopped.

Effects of NO on mitochondrial O_2 uptake. The O_2 uptake of isolated rat heart mitochondria was 42 ± 6 ng atoms O_2 $min^{-1} \cdot$ mg protein^{-1} in state 4 (without ADP) and 165 ± 17 ng atoms $O_2 \cdot min^{-1} \cdot$ mg protein^{-1} in state 3 (with ADP), with succinate as substrate. The exposition to NO inhibited 50% state 3 O_2 uptake at ~0.4 μM and reached maximal 80% inhibition at ~0.9 μM NO (33 ± 3 ng atoms O_2 $min^{-1} \cdot$ mg protein^{-1}, total $n = 16$, $P < 0.05$). The negative relationship between NO concentration and mitochondrial state 3 O_2 uptake is shown in Fig. 2.

Kinetics of H_2O_2 release after NO infusion. The infusion of either NO solutions or the NO donor GSNO induced the appearance of H_2O_2 in the effluent. The release of H_2O_2 was detected ~5–10 min after the end of NO perfusion as a transient phenomenon, increasing to maximal levels in ~10–15 min and decreasing afterward (Fig. 3). Both the concentration of H_2O_2 in the perfusate and the amount of H_2O_2 released (determined as the area under the curve of H_2O_2 release as a function of time) depended linearly on the NO concentration in the perfusion fluid between 1.5 and 7.5 μM NO (Fig. 3, *inset*). Similarly, maximal H_2O_2 concentrations in the effluent fluid also correlated with the NO concentrations recovered in the perfusion fluid during GSNO infusion (Fig. 4).

Cytochrome reduction. The cytochrome differential spectra of rat heart submitochondrial particles supplemented with NO showed that addition of 0.8 mM succinate, at relatively low substrate concentrations, produced a complete reduction of cytochrome *c* and cytochrome *a*-a_3 with no further reduction after addition of 8 mM succinate and sodium dithionite; by

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contrast, cytochrome *b* remained only partially reduced (Fig. 5).

Spectra of myoglobin in myocardium slices. The NO-dependent changes in the tissue spectrum in the 560- to 580-nm region were similar to the NO-dependent changes in the spectrum of pure oxymyoglobin solutions (2). The infusion of 2–7 μM NO produced a decrease in the absorption peak of tissue oxymyoglobin at 581 nm that was linearly related to NO concentrations (Fig. 6). The conservation of the absorption at 592 nm, at the isosbestic point, indicates the lack of myoglobin loss and the stoichiometric change of oxymyoglobin to metmyoglobin (Fig. 6).

The action of bradykinin. The infusion of 1–10 μM bradykinin induced a decrease of ~30–40% in coronary perfusion pressure and O_2 uptake. The changes in perfusion pressure and O_2 uptake were associated with an increase in NO concentration in the effluent. Bradykinin infusion also promoted an increase in H_2O_2 concentrations in the effluent in which the time course was similar to that obtained after perfusion with NO (Fig. 7).

The simultaneous administration of L-NMMA at 100 μM inhibited most of the effects of bradykinin and completely abolished H_2O_2 release. In contrast, the administration of enalapril, the inhibitor of the angiotensin-converting enzyme, which potentiates bradykinin action by inhibiting the kininase II (29), increased these effects. Indomethacin had no significant effects on H_2O_2 release (Table 1).

DISCUSSION

Coronary infusion of NO, either as an NO solution or released by GSNO or by bradykinin-activated endothelium, transiently and reversibly decreased aortic perfusion coronary pressure and myocardial O_2 uptake and elicited the release of H_2O_2 in the effluent fluid of the isolated and perfused beating rat heart.

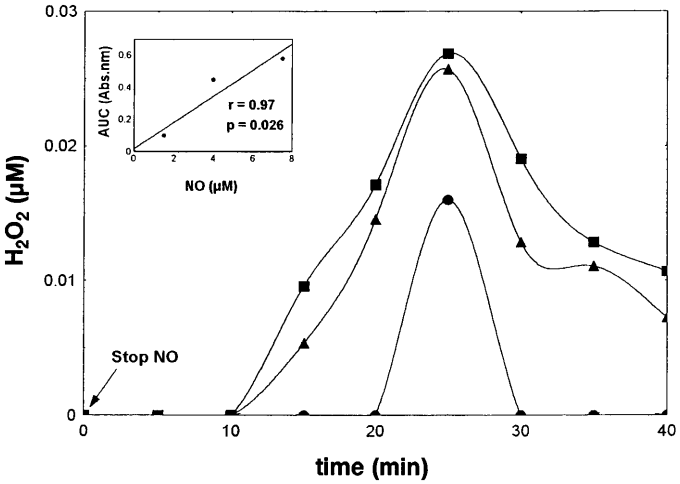

Fig. 3. Release of H_2O_2 in the effluent perfusate in rat heart after 10-min infusion of NO at 1.5 (●), 4 (▲) and 7.5 (■) μM. *Inset:* relationship of the area under the curve (AUC) and NO concentration. Each point is an average of 3 determinations in separate experiments.

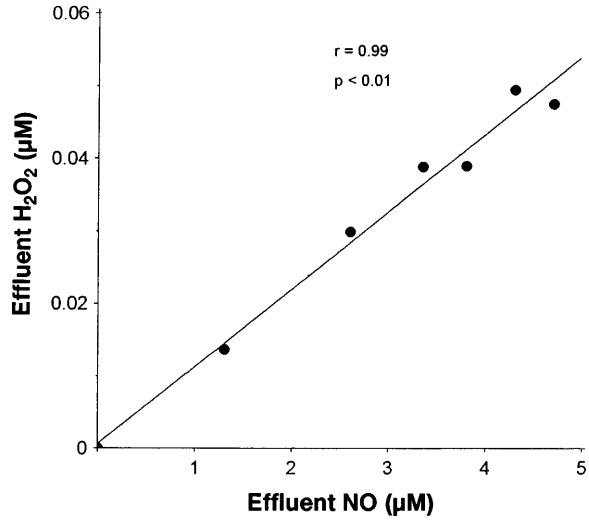

Fig. 4. Relationship between NO and H_2O_2 concentrations in the effluent perfusate after rat heart infusion of 0.1–1.5 mM GSNO; NO was determined at the end of the GSNO infusion, and peak H_2O_2 concentration was obtained 10–15 min after GSNO infusion was stopped. Each point is an average of 4 separate experiments.

The decrease in myocardial O_2 uptake was not the result of a diminished energy demand secondary to a decreased heart performance. Actually, the effects of NO on heart contractility parameters are still controversial, since they probably depend on the doses, i.e., very high doses of NO are required to achieve detrimental effects on the contractility of rat cardiac muscle (28). Hence, the decrease in myocardial O_2 uptake is understood here as derived from a direct NO effect on mitochondrial electron transfer and O_2 uptake, similar to those previously reported in mitochondria isolated from skeletal muscle (5) and heart (20). In this study, half-inhibition of heart mitochondria state 3 respiration was achieved at ~0.4 μM NO. The decrease in mitochondrial O_2 uptake was mostly due to the inhibition of cytochrome oxidase, the final step of the electron transfer chain. In agreement, Cleeter et al. (5) and our group (20) reported that NO half-inhibition of rat heart mitochondrial cytochrome oxidase occurs at even lower levels in the range of 0.05–0.1 μM NO.

In addition, this study shows a significant correlation between NO and the amounts of H_2O_2 released by the heart. Mitochondrial production of O_2^- and H_2O_2 requires an inhibition of mitochondrial electron transfer as is the case for ADP-controlled state 4 respiration or after the addition of nonphysiological inhibitors like antimycin or in the presence of NO. In this way, NO is the unique physiological substance that blocks more than one site in the mitochondrial respiratory chain. In addition to cytochrome oxidase, NO reversibly inhibits the segment cytochrome *b*-cytochrome *c* in which electron transfer capacity is adequately measured by succinate-cytochrome *c* reductase activity (20). In accord, a blockade between cytochrome *b*-cytochrome *c* in the presence of NO and succinate was shown by absorption spectral studies (Fig. 5).

In a previous work, we reported that heart mitochondria exposed to 0.4 μM NO produced ~0.07 nmol

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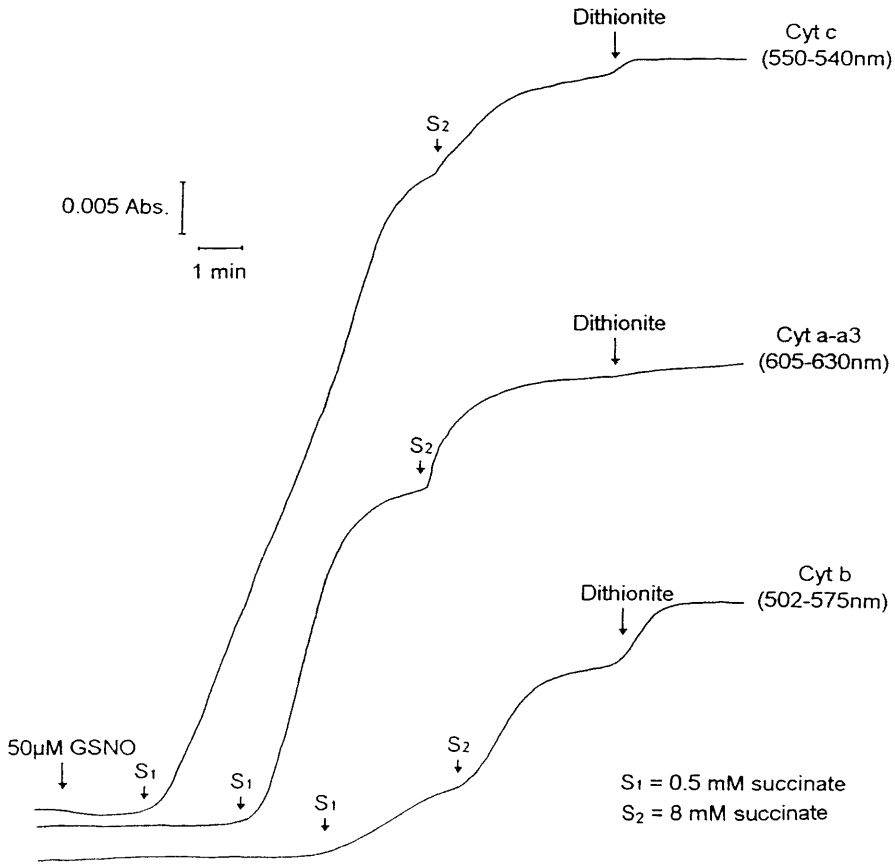

Fig. 5. Cytochrome reduction in rat heart submitochondrial particles (0.4 mg protein/ml) supplemented with NO as released from the donor GSNO and with succinate as substrate. Traces represent the reduced-oxidized difference visible spectra of the cytochromes (Cyt) using a double beam-double wavelength spectrophotometer.

$H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ (20). Thus, considering that 1 g of cardiac tissue provides 54 mg of mitochondrial protein (7), it is apparent that the H_2O_2 production by the whole heart in conditions of 50% inhibition of O_2 uptake should be ~3.8 $nmol \cdot min^{-1} \cdot g\ tissue^{-1}$. The concentration of H_2O_2 detected in the effluent (0.04 μM) accounts for the recovery of 0.28 $nmol \cdot min^{-1} \cdot g\ tissue^{-1}$ and thus ~7.4% of the produced H_2O_2. The relatively low amount of H_2O_2 recovered in the effluent appears to indicate the effective operation of tissue detoxification pathways. Mitochondrial and cytosolic catalase and glutathione peroxidase are certainly using myocardial H_2O_2. Moreover, underestimation of H_2O_2 in the effluent perfusate may be caused by the remaining red blood cells and by the release, during heart perfusion, of hydrogen donors for the peroxidase reaction used in the detection of H_2O_2.

The NO concentrations in the perfusion fluid that were able to inhibit O_2 uptake were 10–100 times higher than the ones that produce cellular effects through the guanylyl cyclase activation. Distribution of NO in the perfused myocardium seemed homogeneous and not limited by NO diffusion; the diffusion distance was calculated as 130 μm, considering a diffusion constant of $4 \times 10^{-6}\ cm^2 \cdot s$ (13, 27). The heart steady-state intracellular NO concentration reached during infusion will depend on NO concentration in the infusion fluid, on the perfusate flow, and on NO myocardial uptake, including NO binding to cellular and mitochondrial proteins like myoglobin and cytochrome oxidase. Particularly these two hemoproteins react with NO with great affinity, with diffusion-limited rate constants and, in the case of myoglobin, the binding is followed by immediate release of metmyoglobin (2). Consequently, myoglobin markedly shortens NO half-life and decreases NO concentration in the perfused heart (13). The tissue metmyoglobin content was found inversely related to NO concentrations, as shown by absorption spectrophotometry (Fig. 6) and recently by electron spin resonance (15).

According to Fick's law, the myocardial uptake of NO is expressed by the product of the difference between NO concentrations in the influent ($[NO]_{inf}$) and in the effluent ($[NO]_{eff}$) by the coronary perfusion flow

$$\text{NO myocardial uptake} = \text{coronary perfusion flow } ([NO])_{inf} - [NO]_{eff})$$

For example, at 2 μM NO in the perfusate solution

$$11.16\ nmol \cdot min^{-1} \cdot g^{-1} = 6\ ml \cdot min^{-1} \cdot g^{-1} \times (2.0\ nmol/ml - 0.14\ nmol/ml) \quad (1)$$

Considering the high O_2 concentrations in the experimental system (~800 μM), a minimal decay in NO availability by oxidation to NO_2/NO_2^- could be expected. Because the overall rate of reaction is given by the equation $d[NO]/dt = k_3[NO]^2[O_2]$ (where k_3 is a third-order rate constant), NO oxidation in the perfusate should not represent >10–30% of the respective infused 2–7 μM NO. However, these proportions could be lower than estimated because nitrite can be recon-

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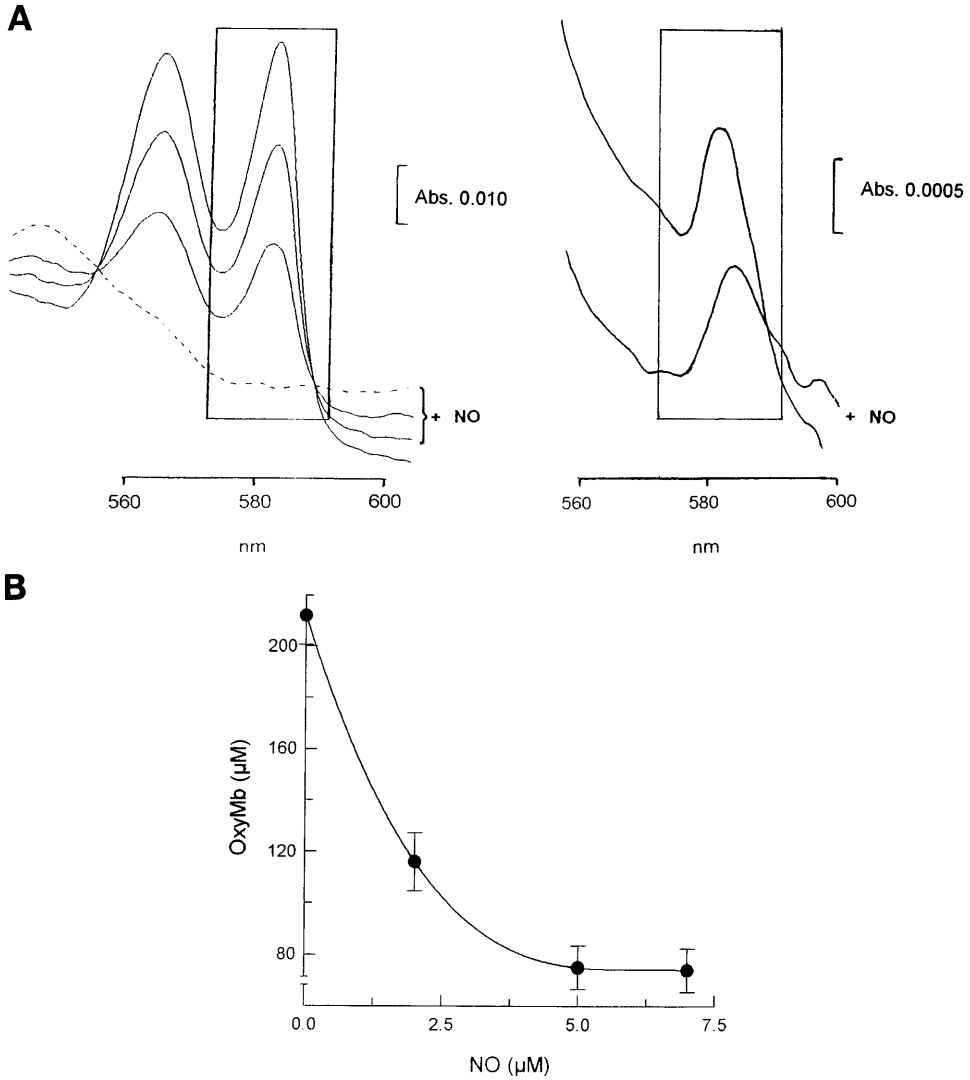

Fig. 6. Effect of infused NO on rat heart myoglobin. *A*: changes in oxymyoglobin (oxyMb) spectra in frozen myocardial slices after infusion of 7 μM NO (*right*) compared with similar changes achieved by the supplementation of an oxyMb solution with NO (*left*). *B*: decrease in perfused rat heart oxyMb concentrations produced by NO infusion and measured as in *A*. The length of the light path (the thickness of the tissue slice) was 6–7 μm. Data are mean values ± SE of 8 samples.

verted back to NO by the vascular structures. Hence, for a practical purpose, in *Eq. 1*, total NO uptake equals 112 nmol/g, which, considering the myoglobin content in the heart (~250 nmol/g; see Ref. 6), accounts for a 45% conversion of heart oxymyoglobin to metmyoglobin. This calculation is in complete agreement with the experimental 46% decrease in oxymyoglobin absorption in the heart slices (Fig. 6). Therefore, it ensues

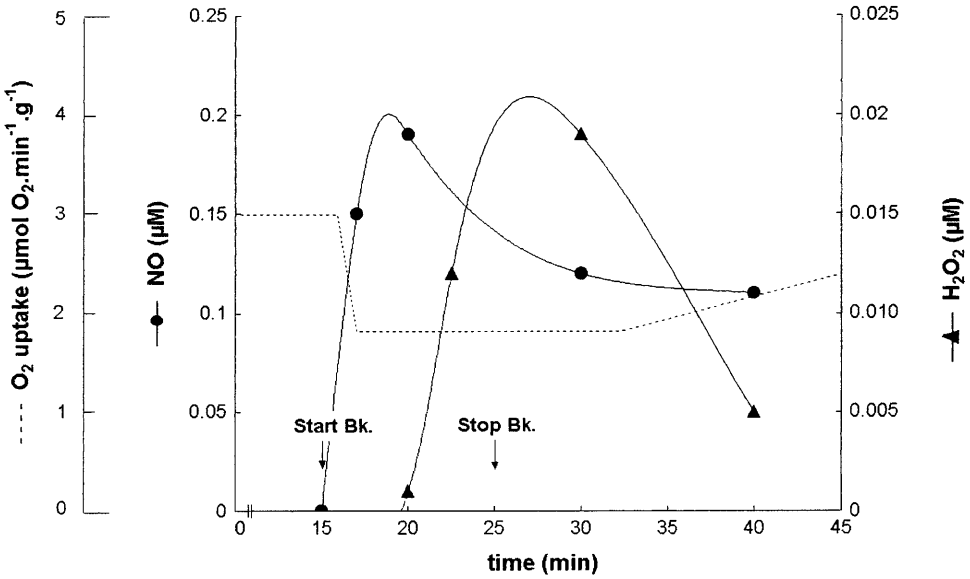

Fig. 7. Effects of 10 μM bradykinin (Bk) on NO and H_2O_2 release and O_2 uptake in the perfused rat heart.

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Table 1. *Effect of bradykinin on H_2O_2 and NO release and physiological functions in the perfused beating rat heart*

Condition	H_2O_2, μM	NO, μM	O_2 Uptake, %Change	Coronary Perfusion Pressure, %Change
Bradykinin (10 μM)	0.020 ± 0.002	0.15 ± 0.02	−38 ± 3	−27 ± 3
Bradykinin (10 μM) + L-NMMA	0.001 ± 0.000*	0.04 ± 0.02*	−17 ± 2*	−7 ± 2*
Bradykinin (1 μM)	0.016 ± 0.002	0.08 ± 0.03	−23 ± 3*	−21 ± 2
Bradykinin (1 μM) + enalapril	0.033 ± 0.004†	0.24 ± 0.00†	−51 ± 5†	−26 ± 3
Bradykinin (1 μM) + indomethacin	0.018 ± 0.004			−27 ± 1

Data are means ± SE of 3–5 experiments. NO, nitric oxide; L-NMMA, N^G-monomethyl-L-arginine. *$P < 0.05$ vs. 10 μM bradykinin and †$P < 0.05$ vs. 1 μM bradykinin.

that, under physiological conditions, the main part of endothelial NO is trapped by myoglobin, and the remnant free NO diffuses to mitochondria. The diffusion process is analogous to that of O_2, where O_2-myoglobin and O_2-cytochrome oxidase are the two steps of O_2 transport to mitochondria (14). Moreover, the buffering capacity of myoglobin probably maintains the mitochondrial concentration of NO in a relatively low and nontoxic range (Fig. 6). In this setting, the steady-state NO concentration in myocardial cells can be estimated as ~0.1–0.3 μM in a range between the NO concentration that effectively inhibits cytochrome oxidase by 50% (0.05–0.1 μM) and the NO concentrations measured in the effluent after bradykinin stimulation (0.3 μM NO). The biological effect of a steady-state concentration of 0.1–0.3 μM NO depends on the simultaneous O_2 tension; when O_2 tension is lowered, NO effects are increased and extended. Takehara et al. (24) reported competition between O_2 and NO for cytochrome oxidase. The inhibition of O_2 uptake in isolated rat liver and heart mitochondria by NO is consistent with a high affinity of NO for cytochrome oxidase, which is ~160 times higher than the corresponding O_2 affinity (8, 20, 24). This point is of physiological interest since O_2 concentrations in the myocardial cells have been reported as low as 3–8 μM O_2 (10), a condition in which an NO steady-state concentration of even 50 nM would effectively compete with O_2, producing a cytochrome oxidase inhibition of ~30%.

It is thought that a better O_2 myocardial distribution is developed when maximal mitochondrial state 3 O_2 uptake is lowered by endothelial NO release in response to hypoxia or ischemia (20). In this way, NO increases O_2 supply through coronary vasodilation and allows O_2 to diffuse further along its gradient, reaching more cells and mitochondria and lowering the steepness of the P_{O_2} gradient in the anoxic-normoxic transition (20). The same regulatory action has been proposed for skeletal muscle (22). These effects of NO could afford a generalized adaptive response; an increase in whole body O_2 uptake has been reported in the conscious dog after the administration of NO synthase inhibitors (23).

The role of NO in the regulation of O_2 uptake in the perfused heart is depicted in Fig. 8. The NO-induced production of H_2O_2 was not found to be deleterious for the heart performance as proposed by other studies (26). An increased NO concentration in the cell and mitochondria will sequentially inhibit cytochrome oxidase and electron transfer between cytochrome *b* and cytochrome *c;* the O_2^- production at the second site of inhibition sets a feedback mechanism in which an increased steady-state concentration of O_2^- will remove cytochrome oxidase inhibition by clearing NO to form $ONOO^-$ ($NO + O_2^- \rightarrow ONOO$; see Ref. 20). At an intracellular concentration of ~0.1 μM NO, O_2 could only insignificantly remove NO in mitochondria, by oxidation, in several hours (9). In contrast, according to Beckman (1), the very fast diffusion-controlled NO/O_2^- reaction assures a prompt regulatory NO clearance. It is apparent that NO and O_2^- are also able to interact in vivo; tolerance to NO donors, like nitrites, has been proposed to be based on an excessive O_2^- release by myocardium (18), and a protective myocardial action of

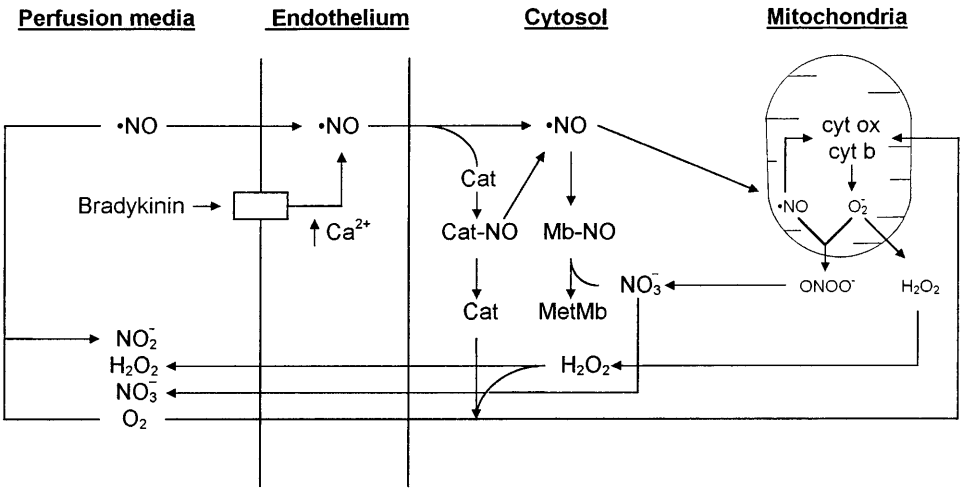

Fig. 8. Scheme of effects of NO on the O_2 of myocardial cells and mitochondria. Scheme integrates the release of endothelial NO and the regulation of NO steady-state concentration in the myocardial cells, the inhibition of mitochondrial O_2 uptake, and the production of O_2^- and H_2O_2 as a whole regulatory process in response to endothelial activation. Cat, catalase; Mb, myoglobin; MetMb, metmyoglobin; cyt ox, cytochrome oxidase; cyt b, cytochrome b.

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NO or of bradykinin has been suggested in ischemia-reperfusion (19, 29).

The interplay of NO and reactive O_2 species seems to constitute a suitable mechanism for physiological regulation of O_2 uptake in the heart and other tissues. The molecular details in terms of involved reactive species and the whole concept of oxidative stress have to be reexamined in terms of the interdependence of O_2^- and NO steady-state concentrations and of $ONOO^-$ formation.

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