Oxygen-Dependent Reversible Inhibition of Mitochondrial Respiration by Nitric Oxide

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ABSTRACT. Effects of nitric oxide (NO) and NO generating agents, on the electron transport system of mitochondria were examined in a study of the mechanism and physiological importance of NO in energy metabolism. In the presence of various substrates, uncoupled respiration was inhibited by NO in manner which was both dose- and oxygen tension-dependent. Simultaneously measuring changes in cytochrome absorption spectra and respiration showed that the site of action of NO is cytochrome oxidase. Similar inhibition was also brought about by 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC 18), an NO donor. Electron paramagnetic resonance (EPR) analysis revealed that inhibition of uncoupled respiration occurred only during the presence of NO in the reaction mixture. The inhibitory effect of NO was increased significantly by lowering the concentration of mitochondrial protein. No appreciable inhibition of respiration was observed in the presence of 3-morpholininosydnonimine (SIN-1), a peroxynitrite anion (ONOO⁻) generating reagent, but inhibition did occur in the presence of superoxide dismutase (SOD). These results indicate that NO reversibly interacts with mitochondria at complex IV thereby inhibiting respiration particularly under physiologically low oxygen tension and that *de novo* generated ONOO⁻ may have no significant effect under the present experimental conditions.

Nitric oxide (NO), generated from L-arginine, has been identified as an important regulatory molecule in various pathophysiological processes, involving, for example, neurotransmission, vasodilatation, inhibition of platelet aggregation and host defense (27, 31, 33). NO readily enters cells and affects functions of organelle, such as microsomes and mitochondria. In this context, endogenously generated NO has been shown to inhibit mitochondrial functions of various cells (12, 17, 28, 40, 44) through formation of nitrosyl ferrocyanide com-

plex (16, 24). NO deenergizes mitochondria, resulting in the disruption of cytosolic calcium homeostasis and thereby killing cultured hepatocytes (26, 38, 39). NO also inhibits protein kinase C and the calcium-dependent proteinase, calpain, reversibly (15, 32). NO, at sub-micromolar concentration, has been shown to reversibly inhibit the activity of isolated cytochrome (Cyt) oxidase as well as the enzyme present in mitochondria and brain synaptosomes (4, 5, 10, 45). This property of NO enables mitochondria to function as a sensor for detecting oxygen concentration over the physiological range (4).

Since the reactivity of NO with molecular oxygen is fairly high, the interaction of NO with the above mentioned enzymes might occur more efficiently at low rather than high oxygen tensions. Since oxygen concentrations in mitochondria (1–5 μ M) are significantly lower than those in the cytosol (10–60 μ M), mitochondrial metabolism might be affected by NO more significantly than that in the cytosol. We previously reported that a low concentration of NO reversibly inhibited mitochondrial respiration without affecting the activity of energy transducing enzymes and that the inhibition was more marked at physiologically low oxygen tensions than at

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Abbreviations used: DNP, dinitrophenol; EPR, electron paramagnetic resonance; Cyt. c, cytochrome c; GSH, reduced glutathione; GSNO, S-nitrosoglutathione; HbO₂, oxygenated hemoglobin; MGD, N-methyl-D-glucamine-dithiocarbamate; NO, nitric oxide; NO₂, nitrite; NO₃, nitrate; NOC 18, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene; O₂⁻, superoxide anion; $\cdot$ OH, hydroxyl radical; ONOO⁻, peroxynitrite anion; RCR, respiratory control ratio; SIN-1, 3-morpholininosydnonimine; SOD, superoxide dismutase; TMPD, tetramethyl-p-phenylenediamine.

high tensions (34, 43). We also reported that NO reversibly inhibited the respiration at the cellular level in a manner of oxygen tension-dependent (21). Our present work describes a site and mechanism for the inhibition of mitochondrial respiration by NO. Kinetic analysis confirmed that Cyt c oxidase is the primary site of NO interaction in mitochondrial respiration and that a fairly large dose of ONOO⁻ has negligible effects on mitochondrial functions. The physiological importance of changes in concentrations of NO and oxygen in the regulation of energy metabolism is discussed.

MATERIALS AND METHODS

Chemicals. Nitric oxide gas (98%) was obtained from Teisan Co. Ltd. (Tokyo). NOC 18, SIN-1 and N-methyl-D-glucamine-dithiocarbamate (MGD) were obtained from DOJINDO LABORATORIES (Kumamoto). All other chemicals were of analytical grade and obtained from Nacalai Tesque Co. (Kyoto).

Preparation of nitric oxide solution. Nitric oxide solution was prepared at room temperature by bubbling the medium used for analyzing mitochondrial energy transducing reactions with argon gas (10 min) and then with NO gas (30 min) which had been passed previously through a KOH column (2 × 2.5 cm) to remove nitrogen dioxide. Aliquots of the NO-containing medium (1.9 mM) were added to the mitochondrial suspensions using a gas-tight syringe (9).

Isolation of mitochondria. After fasting animals overnight, liver mitochondria were isolated from male Wistar rats weighing 200–250 g by the method of Hogeboom (19) using a medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA. EDTA was omitted in the final wash and the mitochondrial preparation was suspended in a concentration at 10–20 mg protein/ml in 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4, at 4°C. Mitochondrial protein was determined by Bradford's method using bovine serum albumin as a standard (3).

Measurement of oxygen uptake. Oxygen uptake was measured polarographically using a Clark type oxygen electrode fitted to a 2 ml water-jacketed closed chamber at 25°C (35). Isolated mitochondria (0.5–1 mg protein/ml) were suspended at 25°C in a medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM MgCl₂, 2 mM sodium phosphate and 10 mM Tris-HCl (pH 7.4). Uncoupled respiration was measured with either 5 mM glutamate, 5 mM succinate or 1 mM ascorbate-10 μM TMPD in the presence of either 1 mM malonate, 1 μM rotenone or 10 nM antimycin A, respectively. The respiratory control ratio (state 3/state 4 respiration, RCR) and the ADP/O ratio were determined as described by Estabrook (14).

Determination of the redox state of the mitochondrial electron transport chain. The oxidation-reduction state of cytochromes in mitochondria was determined by means of the difference spectrum (25) between NO-treated and untreated mitochondria using a dual beam spectrophotometer (Shima-

dzu UV 3000) in a thermostatically controlled cuvette equipped with an oxygen electrode and magnetic stirrer.

Electron paramagnetic resonance (EPR) analysis of MGD-Fe²⁺-NO complex. The concentration of NO in the reaction mixture was determined at room temperature by a spin trapping method (29). The reaction of NO with [MGD-Fe²⁺] was examined by using EPR spectroscopy (JES-FE-1X, JEOL, Tokyo, Japan). At various times before and after adding NO, the [MGD-Fe²⁺] complex in water was added to the reaction mixture containing mitochondria (final concentrations of MDG and FeSO₄ were 1 mM and 20 μM, respectively). Aliquots of the reaction mixture were transferred into a quartz flat cell (inner size, 60 × 10 × 0.3 mm) for EPR measurements. The instrument setting included 100 G field scan, 30 s scan time, 0.1 s time constant, 2.5 G modulation amplitude, 100 kHz modulating frequency and 25 mW microwave power.

RESULTS

Effect of NO on electron transport of mitochondria. We previously reported that phosphorylating and uncoupled respiration were reversibly inhibited by NO especially at low oxygen concentration; oxidative phosphorylation, the ADP/O ratio, and the respiratory control ratio returned fully to control levels after the disappearance of NO from the incubation medium. To investigate the mechanism of inhibition, the effect of NO on the mitochondrial electron transport system was examined. In the presence of glutamate and malonate, mitochondrial respiration was increased by adding dinitrophenol (DNP). The rate of uncoupled respiration was reversibly inhibited by 1 μM NO; the inhibitory effect was significantly stronger at low oxygen tension than at high tension (Fig. 1a). A similar inhibition was also observed with NO in the presence of either succinate and rotenone (Fig. 1b) or ascorbate, TMPD and antimycin A (Fig. 1c). In all cases, the inhibitory effect of NO was stronger at low oxygen tension than at high tension. A similar effect of NO was also observed with mitochondria which were uncoupled by repeated freezing and thawing (data not shown). In contrast, neither NO₂ nor NO₃ showed such an effect even at concentrations of 1 mM. These results indicated that the step of terminal electron transport in mitochondria, Cyt c oxidase, might be the primary site for the reversible inhibition of mitochondrial respiration by NO, which would confirm previous observations (34, 43).

NO-dependent reduction of mitochondrial cytochrome. To elucidate the mechanism of the reversible inhibition of respiration, the effect of NO on the absorption spectrum of cytochromes was examined. In the presence or absence of glutamate and DNP, oxygen uptake and the absorption spectrum were measured simultaneously. In the absence of NO, the mixture showed no absorption either in the presence or absence of the respira-

Fig. 1. Effect of NO on the uncoupled respiration of rat liver mitochondria. Oxygen consumption was measured polarographically at 25°C using a Clark type oxygen electrode fitted to a 2 ml water-jacketed closed chamber. Isolated mitochondria were suspended in a medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM MgCl₂, and 10 mM Tris-HCl (pH 7.4) at 25°C. a, b and c show the uncoupled respiration of mitochondria in the presence of 1 mM malonate and 5 mM glutamate, 1×10^{-7} M rotenone and 5 mM succinate, and 1×10^{-9} M antimycin A, 1 mM ascorbate and 1×10^{-5} M TMPD, respectively. Concentrations of mitochondria in a, b, and c were 1, 0.5 and 0.25 mg/ml, respectively. Concentrations of added NO ($\rightleftharpoons$) in a, b, and c were 1.2, 0.6 and 0.25 μ M, respectively. Concentration of DNP used in the experiments was 2×10^{-5} M. RLMt, rat liver mitochondria.

tory substrate (Fig. 2). However, various cytochromes were immediately reduced after adding NO. The spectra returned to the control state when respiration recovered. When the anaerobic state was obtained after consumption of all oxygen, all cytochromes were again reduced. The absorption spectra for the cytochromes were quite similar to that of the differential spectrum for the oxidized/reduced samples.

Effect of NO generating agent on mitochondrial respiration. To obtain an insight into the molecular mechanism for the reversible inhibition of mitochondrial respiration, the effect of NOC 18, an NO generating agent, on the uncoupled respiration was studied using succinate as substrate. Uncoupled respiration was inhibited by NOC 18 in a concentration-dependent manner. In the presence of 0.5 mg mitochondrial protein /ml, 2 mM NOC 18 completely inhibited the respiration

at an oxygen concentration of 6 μ M. The inhibited respiration recovered on adding oxygen to the medium. When the inhibitory effect of NO disappeared (20 min after introduction), the rate of oxygen uptake was slightly lower (by about 20%) than originally (Fig. 3). A similar decrease in the rate of oxygen uptake was also observed after incubation of untreated mitochondria for 20 min at 37°C, suggesting a nonspecific inactivation during the incubation.

Changes in NO level in mitochondrial suspension. To determine the change in NO concentration in the incubation, the EPR signal of the MDG-Fe²⁺-NO adduct was monitored during the experiments. The NO level in the reaction mixture rapidly decreased at high oxygen concentration but decreased slowly over a fairly long period at low oxygen concentrations (Fig. 4). The inhibitory effect of NO was not seen when the EPR signal dis-

Fig. 2. Simultaneous measurement of oxygen consumption and the oxidation-reduction state of mitochondrial cytochromes. Incubation conditions were as described in Fig. 1a except for the concentrations of added NO and glutamate which were $2.7 \mu\text{M}$ and 5 mM , respectively. Absorption spectra of mitochondrial cytochromes and oxygen uptake were measured using the dual beam spectrophotometer (Shimadzu UV 3000) equipped with an oxygen electrode in a thermostatically controlled cuvette holder with a magnetic stirrer. a, oxygen uptake in the presence or absence of added NO. Dotted lines show the control experiment without NO. Open arrow shows the time of NO addition. b, absorption spectrum at times 1, 2, 3, 4, and 5 in Fig. 2a; c, differential absorption spectra of mitochondria (1 mg/ml) between ferricyanide-oxidation and hydrosulfite-reduction; ABS, absorbance.

Fig. 3. Dose-dependent inhibition of mitochondrial respiration by NOC 18. Experimental conditions were as described in Fig. 1b. The concentrations of NOC 18 used was 0 (0), 1 (1), 2 (2), and 3 mM (3).

appeared.

Effect of mitochondrial concentration on the inhibitory action of NO. Interestingly, the extent of inhibition of uncoupled respiration by NO depended on the amount of mitochondrial protein in the assay mixture; the inhibition was more marked with low concentrations of mitochondria than with high concentrations (Fig. 5). Of course, we can see the inhibited respiration of mitochondria by higher concentration of NO, such as $2 \mu\text{M}$ NO, even when the mitochondrial concentration was 10 mg protein/ml . This observation suggested that NO might also interact with mitochondrial component(s) other than Cyt oxidase and undergo inactivation.

Effect of SIN-1 on mitochondrial respiration. It is well documented that NO rapidly reacts with superoxide ($\text{O}_2^{\cdot-}$) thereby generating stable ONOO^- (2). Mitochondria have been known to generate $\text{O}_2^{\cdot-}$ particular-

Fig. 4. Changes in NO level in reaction mixtures. Mitochondria (0.5 mg/ml) were suspended in 0.2 M sucrose, 10 mM KCl, 1 mM MgCl_2 , 2 mM sodium phosphate, and 10 mM Tris-HCl (pH 7.4) at 25°C. At the indicated times after adding 5 and 2 μM NO at high and low oxygen concentrations, respectively. NO concentration during the incubation was monitored by EPR method using 1 mM MDG and 20 μM FeSO_4 . Numbers (1–5) for EPR spectrum correspond to those in the oxygen consumption curves. a, effect of NO at high oxygen tension; b, the effect of NO at low oxygen tension.

ly when their electron transport chain is inhibited (8). To investigate the possible involvement of ONOO^- in the NO-dependent inhibition of mitochondrial respiration, the effect of SIN-1, an ONOO^- producing agent by releasing equimolar amounts of NO and $\text{O}_2^{\cdot-}$, on mitochondrial respiration was examined. The uncoupled respiration with succinate was not appreciably affected by fairly high concentrations of SIN-1 ($\sim 300 \mu\text{M}$). However, in the presence of exogenously added SOD (500 U/ml), the respiration was markedly inhibited by SIN-1 particularly at low oxygen concentrations; the inhibitory effect depended on SIN-1 concentration (Fig. 6). In the presence of SOD, generated $\text{O}_2^{\cdot-}$ from SIN-1 was dismutated to H_2O_2 and oxygen, and cytochrome oxidase was inhibited by generated NO. These results indicated that NO but not ONOO^- generated from SIN-1 might be responsible for the inhibition of mitochondrial respiration.

Fig. 5. Effect of mitochondrial protein concentration on the inhibitory effect of NO. Experimental conditions were as described in Fig. 2. The concentration of NO added was 0.95 μM . Concentrations of mitochondria were 0.1–1.0 mg protein/ml. Arrows show the time points of NO addition. Insert shows the dependency of the recovery time on mitochondrial protein concentration.

Fig. 6. Effect of SIN-1 on mitochondrial respiration. Experimental conditions were as described in Fig. 1b. Various concentrations of SIN-1 were added 5 min before incubation of mitochondria in the presence (2, 3, 4) or absence (1, 5) of 500 U/ml SOD. The concentrations of SIN-1 used were 0 (1), 0.1 (2), 0.2 (3) and 0.3 mM (4, 5). The dotted line (1) shows the control experiment without SIN-1.

DISCUSSION

The work presented in this paper demonstrated that NO reversibly inhibits energy transfer reactions of mitochondria in a dose- and oxygen concentration-dependent manner. The inhibition occurred more markedly and continued longer at physiologically low oxygen concentrations than at high oxygen concentration. Since both the ADP/O ratio and the respiratory control ratio (RCR) completely recovered to control levels at certain times after inhibition, the interaction of mitochondrial constituent(s) with NO and/or its metabolite(s) might have occurred reversibly. Since NO inhibited mitochondrial respiration either in the presence or absence of DNP, interaction with the electron transfer system might be responsible for the inhibition. Spectrophotometric analysis suggested that NO formed a dissociable complex(s) with heme-containing proteins in mitochondria. Consistent with this notion is the report that NO forms complexes with Cyt c oxidase and complexes I and II (11, 13, 18, 40, 47). Since NO equally inhibited respiration in the presence of either rotenone or antimycin A, Cyt c oxidase might be the primary site for inhibition. It should be noted that the inhibitory effect of NO increased with concomitant decreases in protein concentrations; the inhibition by 1 nmol NO was negligible at 1 mg of mitochondrial protein but became apparent at concentrations lower than 0.5 mg. Mitochondria contain about 0.8 nmol of heme-proteins/mg proteins (7). Thus, mitochondrial component(s) other than heme-proteins might also interact with NO. In this context, NO has been shown to form nitrosothiols (42). Since GSH is the major thiol in mitochondria and has high affinity for NO, it might form S-nitrosoglutathione (GSNO). Assuming values of 5 mM and 1 μ l/mg protein for mitochondrial GSH and matrix volume, respectively (22, 30), about 5 nmol of GSH would exist in 1 mg of mitochondrial protein. Hence, significant amounts of NO used in the experiments in Fig. 5 (1 μ M) may have been trapped by mitochondrial GSH, particularly at protein concentrations higher than 0.2 mg/ml. This might explain why the inhibitory effect of NO increased with a concomitant decrease in mitochondrial concentration. It should be noted that GSNO is also an NO donor which slowly releases NO (36). Thus, *de novo* generated GSNO might slowly release NO thereby inhibiting Cyt c oxidase (10) for a prolonged period of time. However, preliminary experiments revealed that 1 μ M GSNO had no appreciable effect on respiration. Although active transport of GSH into the mitochondrial matrix has been well documented, transport of GS-conjugates, including GSNO, across the inner-mitochondrial membrane has not been demonstrated. Decomposition of GSNO to NO and GSSG requires strong reducing agents, such as thiolate anions (36). Thus, the possi-

ble occurrence of GSNO and its homolytic cleavage within the mitochondrial matrix needs to be studied further.

It should be noted that mitochondria generate $O_2^{\cdot-}$, particularly when the electron transfer system is inhibited (46). Preliminary experiments revealed that NO-treated mitochondria also generated $O_2^{\cdot-}$ (2.2 pmol/mg protein/min). Since NO rapidly reacts with $O_2^{\cdot-}$ to forms toxic ONOO $^-$ (1, 20, 23), the inhibition of mitochondrial respiration may be caused by ONOO $^-$. In fact, ONOO $^-$ has been shown to inhibit functions of mitochondrial components, such as succinate dehydrogenase, ATPase and aconitase (6, 37). However, inhibition of these proteins by ONOO $^-$ occurred irreversibly (11, 32). Furthermore, cytochrome c oxidase is highly resistant to ONOO $^-$ (37). Since the inhibition by NO was completely reversible under the present experimental conditions, NO and/or its metabolite(s), other than ONOO $^-$, might be principally responsible for the inhibition of mitochondrial respiration.

It should be noted that the lifetime of NO is significantly longer under physiologically low intracellular oxygen tensions (10–60 μ M) (13) than at its high concentrations. However, most experiments *in vitro* describing the inhibitory effects of NO were carried out under air atmospheric conditions where the oxygen concentration (235 μ M) is very much higher than intracellular levels. Furthermore, under aerobic conditions, NO rapidly disappears and forms various reactive species, such as NO $_2$, ONOO $^-$, and $\cdot$ OH. These hazardous metabolites of NO but not NO itself might result in the irreversible inactivation of mitochondrial constituents under highly aerobic conditions. However, the effect of NO might be reversible under physiological conditions and more potent, longer lasting and specific *in vivo* than previously observed with *in vitro* experiments performed under air atmospheric conditions. Since oxygen concentrations in ischemic tissues are extremely low, NO might inhibit mitochondrial energy metabolism more potently in and around ischemic areas than in non-ischemic areas. Critical roles of NO in energy metabolism in and around normal and ischemic tissues should be studied further.

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