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Oxygen-Dependent-Regulation of Ehrlich Ascites Tumor Cell Respiration by Nitric Oxide

Yoko Inai[1,4], Yoshiki Takehara[2], Munehisa Yabuki[1,2], Eisuke F. Sato[3], Jitsuo Akiyama[4], Tatsuji Yasuda[1], Masayasu Inoue[3], Alan A. Horton[5], and Kozo Utsumi[2],*

[1]Department of Cell Chemistry, Institute of Molecular and Cell Biology, Okayama University Medical School, Okayama 700, [2]Institute of Medical Science, Center for Adult Diseases, Kurashiki, Kurashiki 710, [3]Department of Biochemistry, School of Medicine, Osaka City University Medical School, Osaka 545, and [4]Donan Institute of Medical Science, Hakodate 041, Japan, and [5]School of Biochemistry, The University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom.

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ABSTRACT. **Effects of nitric oxide (NO) on oxygen uptake of Ehrlich ascites tumor cells (EATC) were examined in a study of the biological actions of NO on respiration and energy metabolism at the cellular level. Endogenous respiration of EATC was inhibited reversibly by NO in a dose dependent manner. Oxyhemoglobin, an NO trapping agent, restored the respiration promptly. The inhibitory action of NO also depended on oxygen-concentration, and the duration of suppression was prolonged remarkably at low oxygen tension. Similar inhibition was also observed in the presence of glucose. In this case, both lactate production and glucose consumption were promoted by NOC 18, an NO generating agent, and the activation was enhanced by lowering the oxygen-concentration. Furthermore, the membrane potential of EATC was depolarized transiently by adding NO, and the degree of depolarization was decreased in the presence of glucose. These results suggest that at physiologically low oxygen tension in ascites fluid, NO acts not only as a cytotoxic respiratory inhibitor but also as a regulatory factor in the energy metabolism of EATC.**

Nitric oxide (NO) has become a subject of considerable interest recently because of its multiple biological functions, including vasodilatation (8, 9, 21), inhibition of platelet aggregation (10), and neurotransmission (11, 22). Alternatively NO may also mediate antitumor effects of activated macrophages. At least part of the antitumor effects of NO is believed to be mediated by inhibition of mitochondrial respiration (12, 13, 27, 30). In addition, mitochondrial respiration in hepatocytes (15, 25) and astrocytes (3) is also suppressed by NO.

Although the disappearance of NO has been reported to be a second order reaction in the presence of biological materials (28), Takehara *et al.* showed that NO had inhibitory effects on isolated mitochondrial respiration for several minutes at low oxygen tension (29), and that its inhibition was completely reversible. This suggested that the effects of NO on mitochondrial respiration *in vivo* are greater than *in vitro* (in the air).

The present study shows the effects of NO on the respiration of Ehrlich ascites tumor cells (EATC) at varying oxygen tensions using NO solution and NOC 18, an NO generating agent. The authors also report on the effects of NO on glycolysis in Ehrlich ascites tumor cells to gain an understanding of the regulatory functions of NO *in vivo* on the energy metabolism of tumor cells.

* To whom correspondence should be addressed.
TEL: 086–422–2111, FAX: 086–426–8616

Abbreviations: NO, nitric oxide; EATC, Ehrlich ascites tumor cells; HbO_2, oxyhemoglobin; KRP, Krebs-Ringer phosphate buffer; DNP, 2,4-dinitrophenol; TMPD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; Glc, glucose

MATERIALS AND METHODS

Chemicals. Nitric oxide gas (98%) was obtained from Teisan Co. Ltd. (Tokyo). Cyanine dye, NK-1507, was donated by The Japan Research Institute for Photosensitizing Dyes Co. Ltd. (Okayama). NOC 18 was obtained from Dojindo Laboratory (Kumamoto) and dissolved in NaOH solution as a stock solution just prior to use. All other chemicals were of analytical grade and purchased from Nacalai Tesque Co. (Kyoto).

Preparation of tumor cells. Ehrlich ascites tumor cells (EATC) were supplied by the Japanese Cancer Research Resources Bank (Tokyo). The tumor cells, as ascites fluid from donor tumor-bearing animals, were inoculated into the murine peritoneal cavity (0.2 ml/mouse). Young adult ICR mice were used as tumor-bearing animals. EATC were harvested 7–12 days after inoculation and washed 3–5 times by centrifugation in calcium-free Krebs-Ringer phosphate buffer (KRP),

pH 7.4, containing 10 U/ml heparin. They were finally washed twice in heparin- and calcium-free KRP, and resuspended at $0.8–1.2 \times 10^8$/ml.

Preparation of nitric oxide solution. Calcium-free KRP (Pi=20 mM) was deoxygenated by bubbling with argon gas for 10 minutes, and then saturated by bubbling with NO gas for at least 30 minutes at 25°C. NO gas was first passed over KOH pellets and through a filter to remove nitrogen dioxide and impurities. The concentration of NO was measured using the oxyhemoglobin method (20).

Measurement of respiration. Oxygen consumption was measured polarographically using a Clark type oxygen electrode fitted to a 2 ml water-jacketed closed chamber. This system was kept at 37°C and equipped with a magnetic stirrer (29). The tumor cells were used at $0.75–1.5 \times 10^7$/ml in calcium-free KRP.

Preparation of permeabilized EATC with digitonin. EATC were collected, washed, and resuspended in calcium-free KRP as described above. Small aliquots (15 μl) of 10% (w/v) digitonin dissolved in dimethyl sulfoxide were added to the cell suspension and stirred slowly with a magnetic stirrer. After addition of each aliquot of digitonin, a small sample of cells was taken and assessed for their permeability by their ability to exclude trypan blue dye. Treatment with digitonin was terminated when more than 90% of the cells became permeabilized. The final concentration of added digitonin was 0.10–0.15% (19, 25). After the treatment, calcium-free KRP was added immediately to the cell suspension to dilute the added digitonin. The cell suspension was washed three times and resuspended at about 1×10^8 cells/ml calcium-free KRP.

Measurement of the membrane potential of EATC. The membrane potential of EATC was monitored by a cyanine dye method in KRP containing 1 mM calcium chloride, as described elsewhere (14). Changes in fluorescence intensity were measured with a fluorospectrophotometer (Hitachi 650-10LC) equipped with a thermostatically controlled cuvette holder and magnetic stirrer. The wavelengths for excitation and emission were 530 and 570 nm, respectively.

Determinations of glucose and lactate. The tumor cells were suspended in calcium-free KRP containing 200 U/ml of penicillin and 200 μg/ml of streptomycin and 1 mM of glucose. NOC 18 or its solvent was added to cell suspensions and the cells maintained for 6 hours at 37°C in a 5% CO_2/20% O_2 incubator or a 5% CO_2/5% O_2 incubator.

L-Lactate was determined using the L-Lactic Acid Food Analysis Kit of Boehringer Mannheim (Mannheim). Glucose was quantified using the Iatro-Chrome-GLU-LQ Kit of the IATRON Co. (Tokyo).

RESULTS

Effects of NO on the endogenous respiration of EATC. EATC consumed oxygen without adding respiratory substrate to the KRP medium at 37°C. Endogenous respiration by oxidative phosphorylation forms ATP in the cells. The endogenous respiration was inhibited transiently by NO in a dose-dependent manner. At a certain time after adding NO, the inhibited respiration recovered completely (Fig. 1). The respiration inhibited by NO was inversely related to the number of EATC incubated. The endogenous respiration was increased by adding 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation. A similar inhibition of uncoupled EATC respiration was observed by adding NO (data not shown).

Effect of oxygen concentration on the NO-induced inhibition of EATC respiration. In a previous paper (29), we stated that the mitochondrial respiration inhibited by NO depends on the oxygen concentration of the incubation medium. In order to assess the effect of oxygen tension on the inhibitory activity of NO, a fixed concentration of NO was examined at different oxygen tensions. The inhibitory activity of NO was pronounced at oxygen tension lower than air saturated medium, and was oxygen tension-dependent (Fig. 2a). Although the recovery of the inhibited respiration was not complete at low oxygen tension, the inhibited respiration recovered completely after adding oxygen to the medium (Fig. 2b).

Suppression of NO-inhibited EATC respiration by oxyhemoglobin (HbO_2). To confirm that the added NO inhibits respiration of EATC by direct action, the effects of HbO_2 on the inhibited respiration were examined. The inhibited respiration recovered immediately

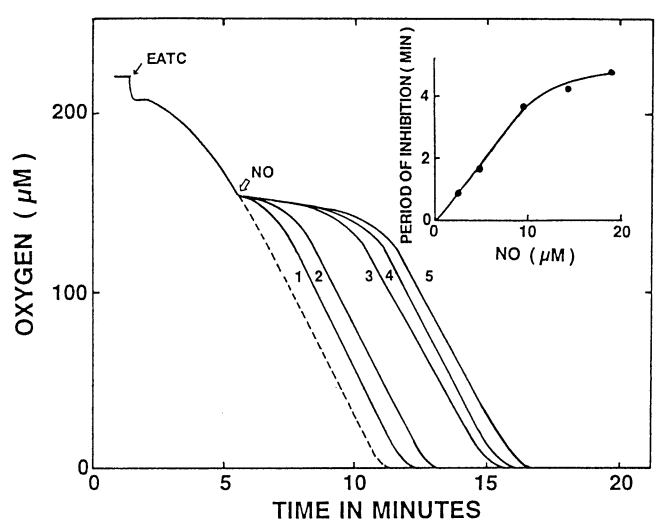

Fig. 1. Effect of NO on the endogenous respiration of EATC. Oxygen consumption was measured polarographically using a Clark type oxygen electrode equipped with a thermostatically controlled water-jacketed chamber at 37°C. EATC (8×10^6 cells/ml) were suspended in calcium-free KRP (pH 7.4). NO was added to the incubation medium as NO saturated medium at the indicated oxygen tension. Insert shows the dose dependent curve for the period of inhibition. 1, 2, 3, 4 and 5 correspond to the 2.4, 4.8, 9.5, 14.3 and 19.0 μM of NO.

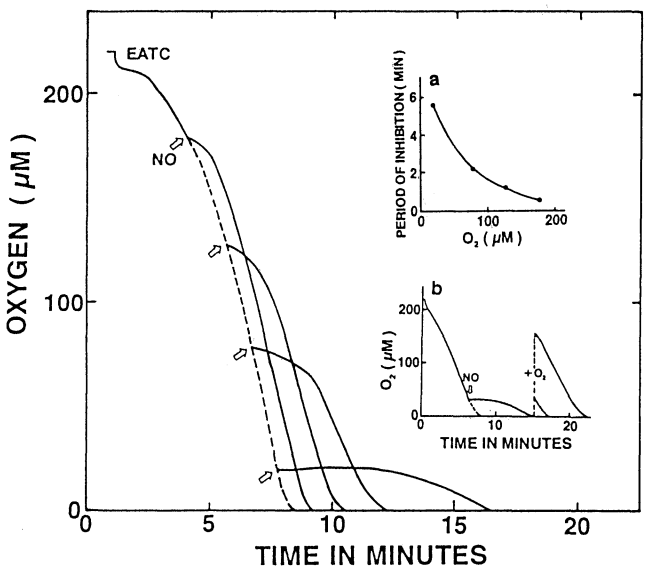

Fig. 2. Oxygen-dependent inhibition of EATC respiration by NO. Experimental conditions were as described in Fig. 1 except for the number of EATC (1×10^7 cells/ml). NO was added at different oxygen concentrations to the incubation medium at final concentration of 3.7 μM. Open arrows show the points of NO addition. Insert a shows the relationship between oxygen concentration and the duration of inhibition. Insert b shows the reversibility of NO-inhibition.

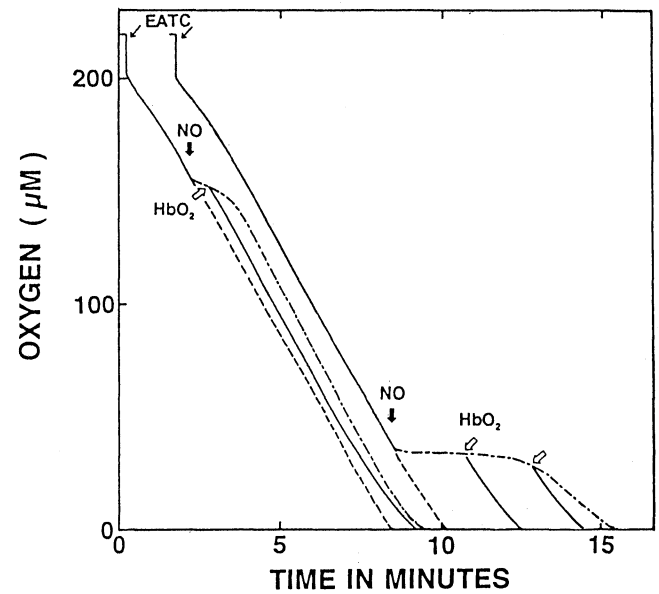

Fig. 3. Inhibition of EATC respiration by NO and its recovery by treatment with oxyhemoglobin. Experimental conditions were as described in Fig. 2. Closed arrows show the points of NO addition, (4.8 μM) and open arrows indicate the points of oxyhemoglobin addition (14.4 μM). HbO_2, oxyhemoglobin.

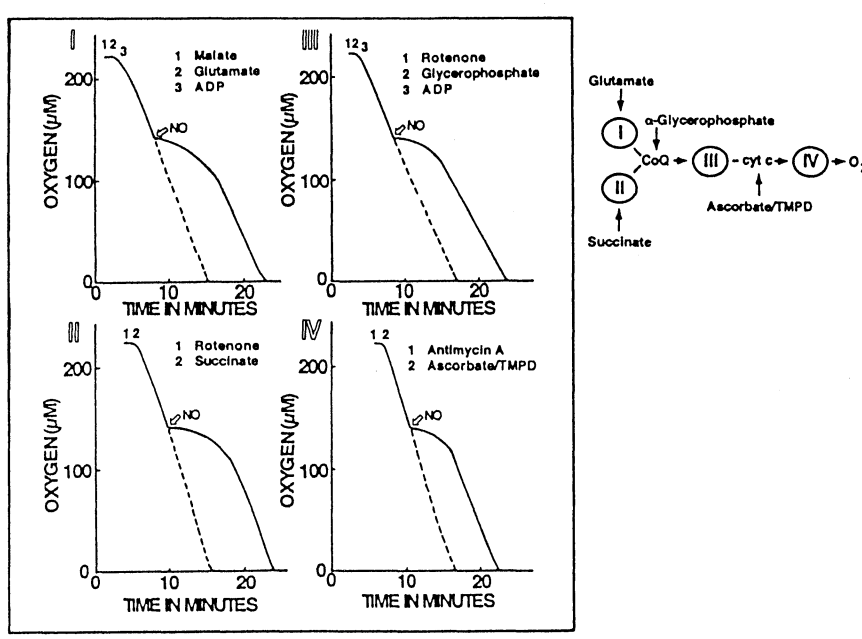

Fig. 4. Effect of NO on the oxygen consumption of digitonin treated EATC. EATC were permeabilized by exposure to 0.10–0.15% digitonin for a few minutes and were then washed 3 times with calcium-free KRP (pH 7.4). Permeabilized EATC (7.5×10^6 cells/ml) were suspended in calcium-free KRP and respiratory inhibitors of each complex were added. Oxygen consumption was started by addition of respiratory substrate and then NO (4.8 μM) was added at the points indicated by open arrows. Concentrations of various respiratory substrates and inhibitors were: complex I, 25 mM glutamate and 0.2 mM ADP and 10 mM malate; complex II, 2.5 mM succinate and 1 μM rotenone; complex III, 35 mM alpha-glycerophosphate, 0.2 mM ADP and 1 μM rotenone; complex IV, 0.09 mM TMPD, 0.45 mM ascorbate and 60 μg/ml antimycin A. Insert shows the mitochondrial electron transport chain. TMPD, tetrametyl-*p*-phenylenediamine.

on addition of HbO_2 at either air saturated or low oxygen tensions (Fig. 3).

Effect of NO on the respiration of digitonin treated EATC in the presence of various substrates. The endogenous respiration of EATC was inhibited on the treatment with low concentrations of digitonin which selectively permeabilizes the cell membrane and releases low molecular weight components of the cytoplasm. The respiratory activity of digitonin-treated EATC was restored by adding respiratory substrates such as glutamate, succinate, alpha-glycerophosphate and ascorbate-2,3,5,6-tetramethyl-*p*-phenylenediamine (TMPD) in the presence of various electron transport inhibitors (Fig. 4). The respiration is coupled and has the capacity to form ATP. This respiration was also inhibited reversibly by exogenous added NO (Fig. 4). Furthermore, the inhibitory effect of NO was pronounced at low oxygen concentrations (data not shown).

Inhibition of EATC-respiration by NO in the presence of glucose. It was generally accepted that the endogenous respiration of certain tumor cells was inhibited by addition of glucose. This phenomenon is called a Crabitree effect. In the case of EATC, the endogenous respiration was lowered by adding glucose coupled with increased glycolysis (Fig. 5). This suppressed oxygen

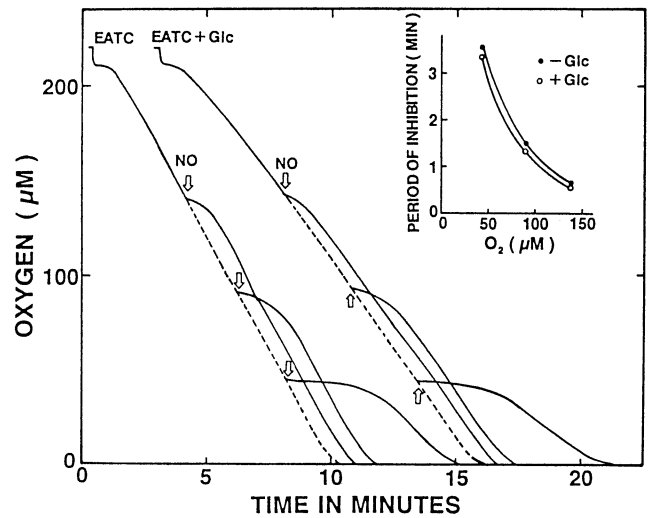

Fig. 5. Effect of NO on the oxygen consumption of EATC in the presence or absence of glucose. Experimental conditions were as described in Fig. 2. Open arrows show the points of NO addition (1.4 μM). Insert indicates the relationship of between the periods of inhibition and oxygen concentration. Glc, 10 mM glucose.

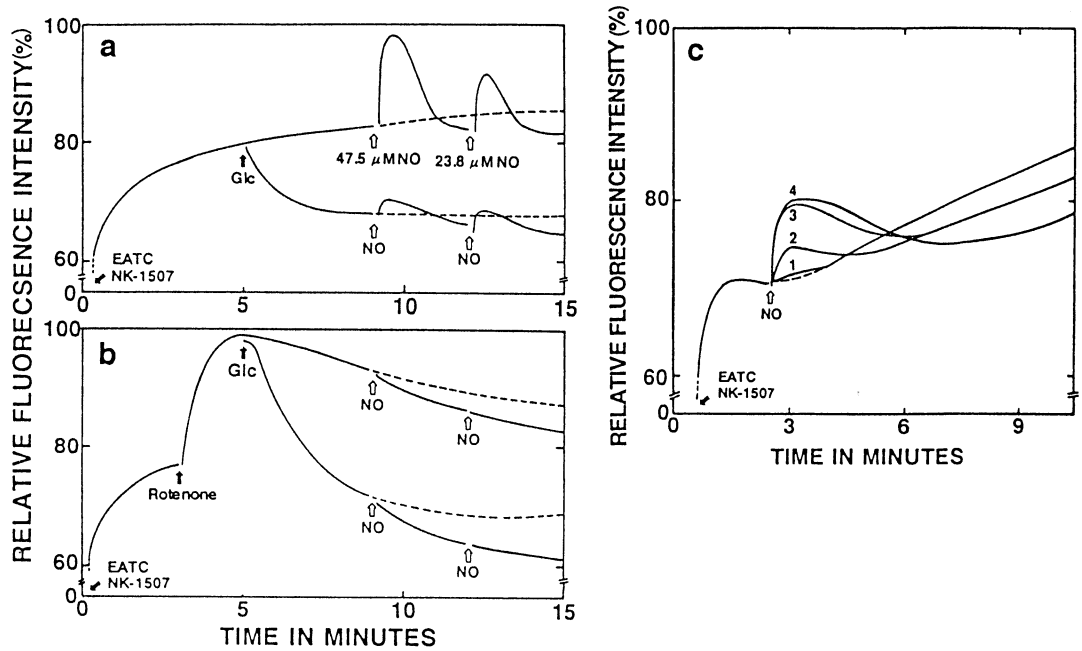

Fig. 6. Transient depolarization by NO of the EATC membrane potential. The membrane potential of EATC in KRP containing 1 mM of $CaCl_2$ was monitored by changes in the fluorescence intensity of cyanine dye using a fluorospectrophotometer (Hitachi 650-10LC). a, EATC (5×10^5 cells/ml) were incubated in KRP containing cyanine dye NK-1507 (50 ng/ml) in the presence or absence of glucose. The wavelength for excitation and emission was set at 530 and 570 nm respectively. Glc, 10 mM glucose; NO, 47.5 and 23.8 μM NO. b, Effect of NO on the membrane potential of EATC in the presence of rotenone and glucose. Experimental conditions were as described in a except for the addition of rotenone. Rotenone, 1 μM rotenone. c, Effect of oxygen concentration on the membrane potential changes induced by NO. 1, 2, 3, and 4 correspond to 220, 130, 86 and 57.9 μM oxygen respectively. NO, 1.4 μM NO; EATC 5×10^5 cells/ml EATC; NK-1507, a cyanin E dye NK-1507, 0.1 μg/ml.

consumption by glucose was also inhibited by NO as observed in the absence of glucose. The inhibition was maximal at low oxygen tension and the inhibited respiration was reversed completely at a certain time after adding NO (Fig. 5).

Effect of NO on the membrane potential of EATC. It has been found that changes in the transmembrane potential are coupled to the energy metabolism of cells. In the absence of glucose, EATC maintained a steady state potential. EATC were depolarized transiently in a dose dependent manner by adding NO (Fig. 6a). Moreover, the degree of depolarization brought by NO depended on the oxygen concentration of the incubation medium. The depolarization was more marked at low oxygen tension than at high tension (Fig. 6c).

The steady state membrane potential of EATC was further polarized by adding glucose, and the degree of depolarization by NO decreased. The steady state membrane potential of EATC was depolarized by adding rotenone, a respiratory inhibitor, and a remarkable polarization was induced by adding glucose. When the respiration of EATC was inhibited by rotenone, depolarization by NO was not induced in the presence or absence of glucose (Fig. 6b).

Effect of NO on glycolysis of EATC. Glycolytic activity of EATC monitored by glucose consumption and lactate formation was not affected by oxygen concentrations (20% or 5% oxygen in the atmosphere). However, the glycolytic activity was increased by adding NOC 18, an NO generating agent, and the increased glycolysis was further enhanced in a low oxygen atmosphere (5% oxygen) (Fig. 7).

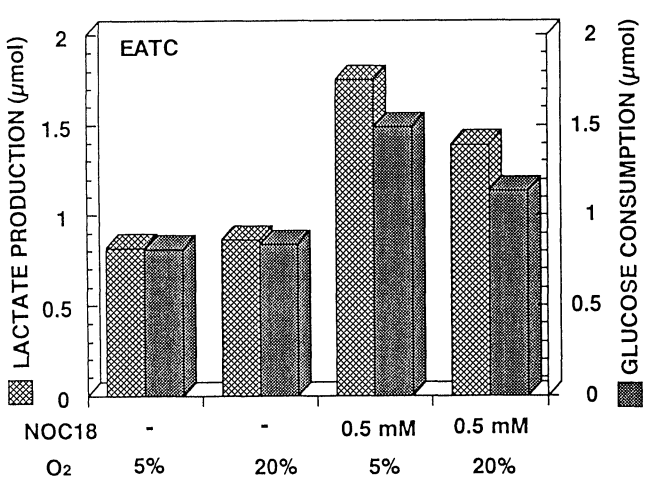

Fig. 7. Changes in lactate production and glucose consumption of EATC by treatment with NOC 18. EATC (5×10^5 cells/ml) were incubated in KRP containing 1 mM of glucose in the presence or absence of NOC 18 (0.5 mM) in 5% or 20% O_2/5% CO_2. After 6 hours glucose and lactate in the medium were measured. Lactate production and glucose consumption were expressed as the values per 1×10^6 cells.

DISCUSSION

It has been reported that NO not only has physiologically important roles, such as vasodilatation (8, 9, 21) and neurotransmission (11, 22), but also cytotoxic effects on various cells, such as the inhibition of mitochondrial respiration (12, 13, 27, 30) and DNA replication (13, 16, 18, 23). Its inhibitory effect on mitochondrial function, however, is reversible and has no effect on oxidative phosphorylation after recovery from inhibition. Moreover, it was found that the inhibitory activity of NO against mitochondrial functions depended not only on the NO dose but also on the oxygen concentration of the incubation medium (29).

From this evidence, the effects of NO on respiration and energy metabolism of EATC were examined at the cellular level. The present experiments demonstrated that the endogenous respiration of EATC was inhibited reversibly by NO in the presence or absence of DNP and that the inhibitory activity of NO was markedly suppressed by HbO_2, an NO trapping agent (5, 7). In addition, it was found that the respiration of digitonin-permeabilized EATC was reversibly inhibited by NO in the presence of various exogenous substrates, such as glutamate, succinate, alpha-glycerophosphate, and ascorbate-TMPD.

It has been suggested that the site of inhibition of mitochondrial electron transport by NO is complex IV (29). The inhibition of oxygen consumption by NO was observed in digitonin-treated EATC in the presence of ascorbate-TMPD. Although the precise mechanism of the inhibition by NO remains unknown, it was shown that at least one of the sites of inhibition of NO is complex IV in the mitochondrial electron transport system. However, many inconsistent results have been reported on the sites of NO inhibition, such as complexes I and II (3, 25, 26). Further experiments are required to determine the site of inhibition of NO in EATC.

We found that the duration of inhibition of endogenous respiration in EATC by NO was prolonged at physiologically low oxygen concentrations since the lifetime of NO is significantly longer at physiologically low oxygen tension (20–50 μM) than air saturated solution (220 μM). The oxygen tension is fairly low (less than 10 μM when measured with the oxygen electrode) in ascites fluid of EATC bearing mice, and many macrophages are also localized in the fluid. Thus NO generated from macrophages might inhibit the endogenous respiration of EATC in ascites fluid, and decrease ATP formation by mitochondrial respiration.

Many investigators have described the inhibition effect of NO on respiration as a cytotoxic action (2, 4, 6, 13, 17, 31). Actually we have shown in our laboratory

that NOC 18, an NO-generating drug can induce apoptosis of another cell line (HL60), and another laboratory also reported NOC-induced apoptosis of macrophages (24). However, apoptosis of EATC was not induced by NO under similar experimental conditions. Thus macrophage-generated NO might not cause apoptosis of EATC in ascites fluid. As described above, we have shown that the inhibitory effect of NO on EATC respiration is reversible, and that NO can promote glycolysis of EATC which is inversely related to the oxygen concentration. Similar results have been reported in another cell line in which NO stimulates glycolysis (1, 3). From the changes in membrane potentials, it is suggested that EATC would compensate for the respiratory inhibition by NO by activation of glycolysis. Therefore it is hypothesized that in certain kinds of cells NO has an important role as a metabolic regulatory factor rather than a cytotoxic factor. Thus, NO generated by peritoneal macrophages might play a critical role on the energy metabolism of ascites tumor cells.

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