Oxygen-dependent regulation of the respiration and growth of *Escherichia coli* by nitric oxide

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Abstract To elucidate the role of nitric oxide (NO) in the metabolisms of enteric bacteria, its effect on the respiration and growth of *Escherichia coli* was examined. Respiration of *E. coli* was reversibly inhibited by NO particularly under low oxygen tensions. Growth of *E. coli* was also inhibited by NO more strongly under low oxygen tension than at its high concentration. Because the intestinal lumen is anaerobic, even a small amount of NO might strongly inhibit the energy metabolism and growth of *E. coli* and other enteric bacteria in vivo than in air atmospheric conditions in which oxygen tension is unphysiologically high.

Key words: Nitric oxide; Oxygen; Respiration; Growth; *Escherichia coli*

1. Introduction

Nitric oxide (NO) is a short-lived gaseous radical synthesized by NO synthase (NOS) and has a wide variety of functions, such as vasodilatation, neuronal transmission, and inhibition of leukocyte adhesion and platelet aggregation [1]. Recent studies revealed the presence of several NOS isozymes, such as the constitutive type enzyme (cNOS) in vascular endothelial cells [2] and NO neurons [3] and the inducible type enzyme (iNOS) in macrophages [4] and neutrophils [5]. It has been reported that NO derived from activated macrophages and neutrophils exhibits bactericidal and tumoricidal actions [6–8]. The effects of NO are mediated, at least in part, through interaction with iron–sulfur clusters and heme-containing enzymes [9]. NO also inhibits ribonucleotide reductase thereby suppressing DNA replication [10].

Because NO also reacts rapidly with other compounds, such as O_2, superoxide radicals and thiols, its fate and functions might be affected by these reactants. In fact, the lifetime of NO is fairly short under air atmospheric conditions (ca. several seconds) predominantly due to rapid reaction with molecular oxygen ($k = 6 \times 10^6$ M^{-2} s^{-1}) [11]. Thus, the lifetime of NO and its functions might differ significantly depending on the oxygen tensions in and around cells and tissues. Although effects of NO on various functions of enteric bacteria have been studied extensively [12–14], most of in vitro experiments were carried out under air atmospheric conditions in which oxygen concentration is significantly higher than those in vivo. Because the intestinal lumen is anaerobic, in vivo effects of NO on the metabolism of enteric bacteria might be stronger than those expected from in vitro experiments performed under non-physiologically high oxygen tensions. The present work describes the effect of NO on the respiration and growth of *E. coli* under different oxygen tensions.

2. Materials and methods

2.1. Chemicals

NO and argon gases were obtained from Kinkisanki Co. (Osaka). *N*-Ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)-ethanamine (NOC12) was purchased from Dojindo Co. (Kumamoto, Japan). All reagents used were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of NO solution

NO solution was prepared after bubbling NO gas through 50 mM HEPES–NaOH buffer, pH 7.4, as described previously [15]. Two small tubes were fitted with an air-tight septum with glass tubes inserted for delivery and escape of gases with a first tube containing 5 M KOH and a second tube containing the HEPES–NaOH buffer. Argon was delivered into two tubes at a flow rate of 100 ml/min. After 15 min, argon was replaced with NO at a flow rate of 100 ml/min. After 15 min, the saturated NO solution (1.9 mM) was kept on ice and used for experiments within 3 h; the concentration of NO in the stock solution remained unchanged during the experiments.

2.3. Bacteria

E. coli K12 strain JM 109 was used throughout the experiments. *E. coli* was cultured at 37°C in LB broth. All solutions were sterilized by autoclaving. One-hundred microlitres of an overnight broth culture was inoculated into a 50 ml flask containing 10 ml of nutrient broth and incubated at 37°C with shaking for 5–6 h. Early-log-phase cultures were obtained as described previously [16]. All experiments were performed using *E. coli* at this phase. Cultured cells were centrifuged at $3000 \times g$ and 4°C for 10 min, washed once with 10 ml of saline, and resuspended in LB broth at a titer of 1×10^5 colony-forming units (CFU)/ml. An optical density (OD) of 0.1 at 550 nm corresponds to approximately 1×10^8 CFU/ml. Growth curves were determined by subculturing LB broth both in air atmospheric conditions and at low oxygen tension (25 μM).

2.4. Analysis of oxygen consumption

Oxygen consumption by *E. coli* (1×10^8 cells/ml) was determined polarographically using a Clark type oxygen electrode fitted to a 2 ml water-jacketed closed chamber [17]. Cellular respiration was measured at 37°C in HEPES–KRP (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM each of $MgCl_2$, NaH_2PO_4, D-glucose and $CaCl_2$). Aliquots of an NO-saturated solution were added to the reaction mixture at varying oxygen tensions.

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Abbreviations: NO, nitric oxide; NOS, NO synthase; NOC12, *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)-ethanamine; CFU, colony-forming units; OD, optical density

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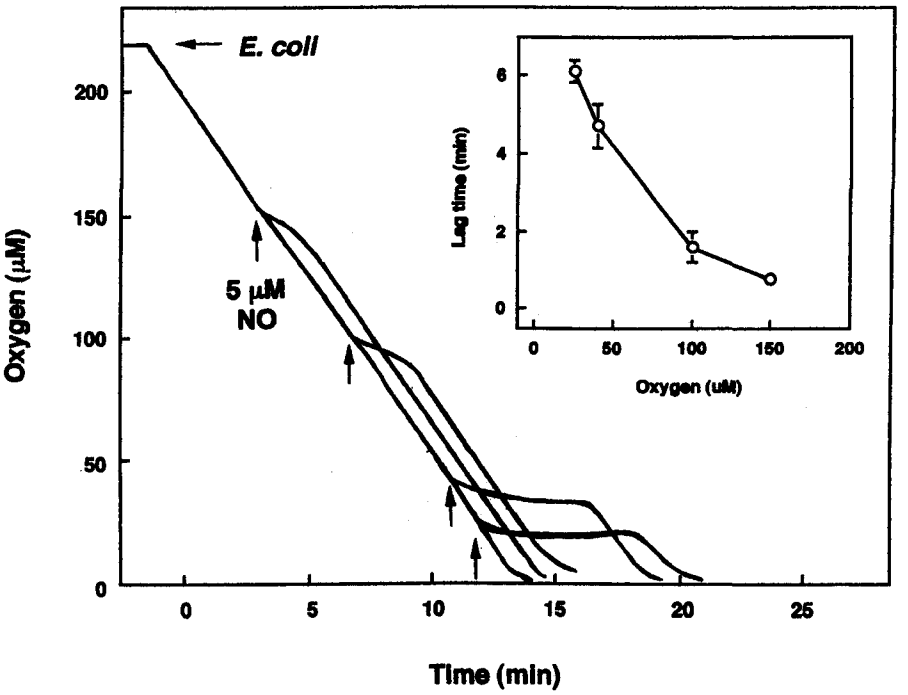


Fig. 1. Effect of NO and oxygen on the respiration of *E. coli*. *E. coli* (1×10^8 cells/ml) were incubated in a closed chamber containing 2 ml of HEPES–KRP (pH 7.4) at 37°C. At the indicated times (arrows), NO was added to the reaction mixture to give a final concentration of 5 μM. During the incubation, the oxygen consumption in the medium was monitored as described in the text. Inset shows the effect of oxygen tension on the time required for the disappearance of the inhibitory effect of NO (mean ± SE). The experiments were carried out at least 5 times with similar results.

3. Result

3.1. *Effect of NO and oxygen tension on the respiration of E. coli*

E. coli revealed a marked respiration in the culture medium as measured by an oxymeter. Cellular respiration was transiently inhibited by a low concentration of NO and recovered completely after certain periods of incubation (Fig. 1). The inhibitory effect of NO significantly increased with concomitant decrease in oxygen tension. The inhibitory effect of NO depended on its concentration both at high and low oxygen tensions (Fig. 2). The time required for the recovery of the respiration depended on the concentrations of both NO and oxygen (Fig. 3).

3.2. *Effects of NO metabolites and hemoglobin on the respiration of E. coli*

To elucidate the possible involvement of NO metabolites in the inhibition of *E. coli* respiration, effect of nitrite and nitrate on cellular respiration was examined (Fig. 4). A fairly high concentration (100 μM) of both nitrite and nitrate had no appreciable effect on the respiration of *E. coli* at any oxygen concentrations tested.

Because NO has an extremely high affinity for oxyhemoglobin (HbO_2), this protein has been used for testing the specificity of NO action. To elucidate the inhibitory mechanism of NO, its effect was also studied in the presence of either erythrocytes or HbO_2. A fairly small amount of erythrocytes (1.0% hematocrit) completely reversed the respiration inhibited by NO. The inhibitory effect of NO was also suppressed by HbO_2 in a dose-dependent manner. An equimolar concentration of HbO_2 completely blocked the inhibitory effect of NO.

3.3. *Effect of NO and oxygen tension on the growth of E. coli*

NOC12 spontaneously and stoichiometrically releases NO (1.5 mol NO from 1 mol of the agent) and, hence, this compound has been used as an useful NO donor. The growth of *E. coli* was inhibited by the presence of NOC12 (Fig. 5). The inhibitory effect depended on the concentration of NOC12. Again, the inhibitory effect of NOC12 was significantly stronger under low oxygen tension than under air atmospheric conditions.

4. Discussion

The present work demonstrates that NO but not nitrite and nitrate reversibly inhibited the respiration of *E. coli* in a concentration-dependent manner by some mechanism which was suppressed by either erythrocytes or HbO_2. The inhibitory effect of NO lasted significantly longer under low oxygen tensions than at its high concentrations. Preliminary experiments revealed that similar doses (~10 μM) of peroxynitrite had no appreciable effect on the respiration of *E. coli*. These results indicate that NO rather than its reactive metabolites is responsible for the reversible inhibition of cellular respiration. Consistent with this notion is the reports that NO reversibly interacts with cytochrome *c* oxidase and other proteins in the electron transport systems thereby inhibiting the respiration and energy transduction of mitochondria [18,19] and ascites tumor cells [20,21]. The effects of NO on other functions of cells and tissues also increased with concomitant decrease in oxygen tensions [19–21]. Thus, NO effects are principally stronger under physiologically low oxygen tensions than previously expected from in vitro experiments performed under air atmospheric conditions. Because the origin of mitochon-

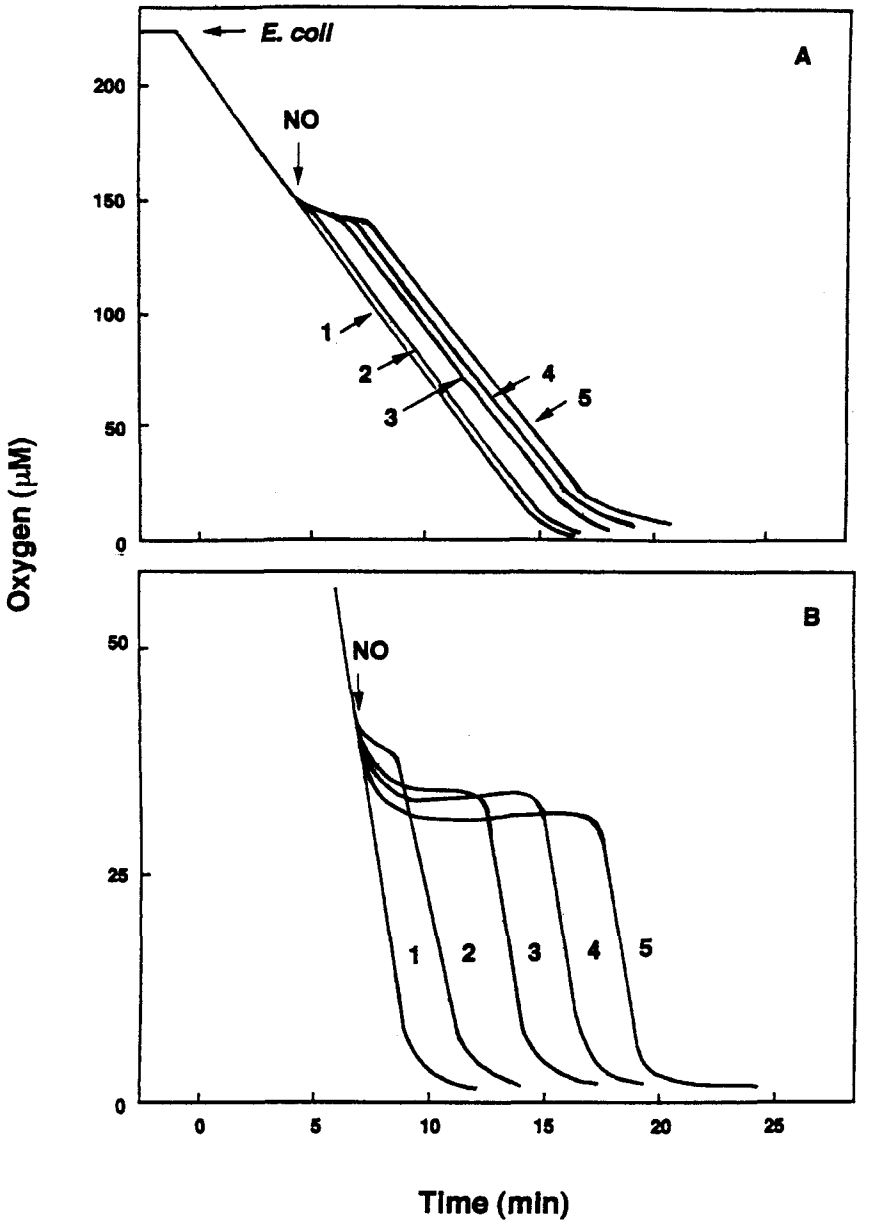
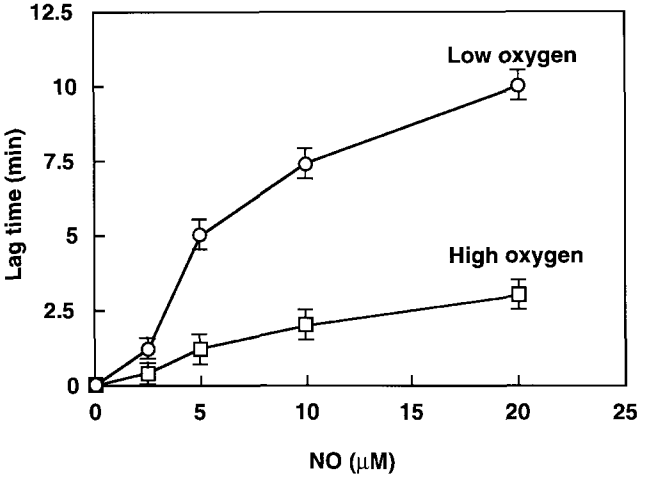


Fig. 2. Dose-dependent inhibition of *E. coli* respiration at different oxygen tensions. At oxygen tensions of 150 (A) and 40 μM (B), NO was added to the reaction mixture to give final concentrations of 0 (1), 2.5 (2), 5 (3), 10 (4) and 20 μM (5). Other conditions were as descried in Fig. 1. Each experiment was carried out at least 5 times with similar results.



dria has been postulated to be some bacteria with aerobic nature [22], it is not surprising that the respiration of both mitochondria and *E. coli* was inhibited by NO in a similar fashion.

The present work also demonstrates that the growth of *E. coli* was inhibited by NOC12 more strongly under low oxygen tensions than under air atmospheric conditions. Although, the lifetime of NO has been postulated to be extremely short (several seconds) particularly under air atmospheric conditions [11], NO is fairly stable under low oxygen tensions

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Fig. 3. Effect of oxygen tension on the inhibitory effect of NO. NO was added to the incubation mixtures at oxygen tension of 40 (○) or 150 μM (□). Effect of oxygen tension on the time required for the disappearance of the inhibitory effect of NO (mean ± SE) was shown. Other conditions were the same as in Fig. 2.

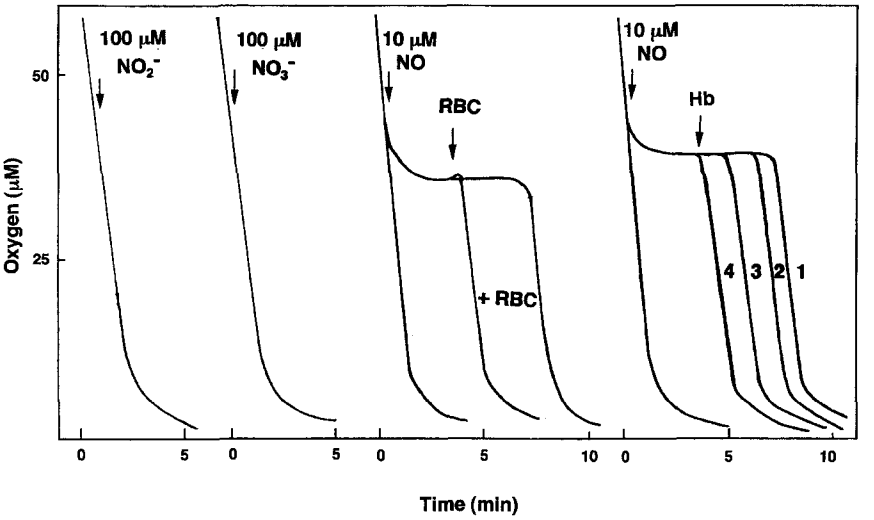

Fig. 4. Effect of NO metabolites and hemoglobin on the respiration. At the indicated times, either 100 μM of nitrite, nitrate or 10 μM of NO was added to the reaction mixture. Then, either erythrocytes (RBC, 1.0% hematocrit) or oxyhemoglobin (Hb) was added to the reaction mixture. The final concentrations of Hb were 0 (1), 2.5 (2), 5 (3), and 10 μM (4). The experiments were carried out at least 3 times with similar results.

[21]. This might be one of the reason why NO strongly inhibited the respiration and growth of *E. coli* under low oxygen tensions.

It should be noted that oxygen is the substrate for NOS and, hence, the rate of NO synthesis may decrease under low oxygen tensions. Thus, the enhancement of NO action by low oxygen tensions might not occur in completely anaerobic compartments, such as in the large intestinal lumen, where substrate oxygen for NOS is lacking. In this context, the K_m value of iNOS for molecular oxygen has been reported to be about 6.3 ± 0.9 μM [23]. Therefore, macrophages and neutrophils not only in the circulation but also in the interstitial space of the intestine might generate reactive oxygen species including NO [24]. Furthermore, because of gaseous nature of NO, it might easily diffuse from the site of generation in intestinal walls into their lumenal compartments. Thus, NO generated by the activated leukocytes in an inflammatory lesion of the intestine might reach to its lumenal space where a large number of enteric bacteria are living.

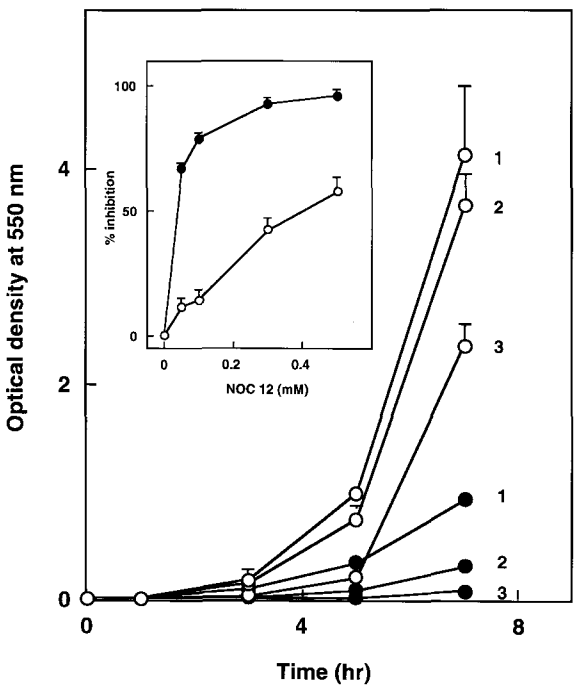

Fig. 5. Effect of NO and oxygen concentration on cell growth. *E. coli* were cultured at 37°C in 10 ml of LB broth either under air atmospheric conditions (○) or under 25 μM oxygen (●). The growth of *E. coli* was determined by measuring optical density at 550 nm. An OD value of 0.1 corresponded to approximately 1×10^8 CFU/ml. The concentrations of NOC12 used were 0 (0), 0.05 (1) and 0.3 mM (2). The experiments were carried out at least 3 times with similar results. The insert shows % inhibition of the growth by NOC12 at 7 h (mean+SE).

Although cNOS has been reported to localize in gastrointestinal tracts, such as in myenteric plexus [25] and epithelial cells [26], enteric activity of iNOS in normal subjects is low. However, iNOS is induced in macrophages and neutrophils which are stimulated by various ligands including bacterial lipopolysaccharide (LPS) and inflammatory cytokines. In fact, patients with inflammatory bowel disease, such as ulcerative colitis and Crohn's disease, have high activity of iNOS in the large intestine [27,28]. Furthermore, plasma levels of nitrite and nitrate in these patients are significantly higher than those in normal subjects. Although NO derived from inflammatory cells often increases vascular permeability and tissue injury, it principally plays protective roles in host defense mechanism against bacterial translocation [29–32]. Preliminary experiments revealed that the sensitivity of bacteria to NO might seem to differ from one species to another. Thus, NO might play important roles in the regulation of metabolisms and growth of NO-sensitive bacteria in the anaerobic lumen of gastrointestinal tracts. The mechanism by which NO inhibits respiration and cell growth of *E. coli* should be studied further.

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