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Cross-Talk of NO, Superoxide and Molecular Oxygen, A Majesty of Aerobic Life

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Because nitric oxide (NO) reacts with various molecules, such as hemeproteins, superoxide and thiols including glutathione (GSH) and cysteine residues in proteins, biological effects and metabolic fate of this gaseous radical are affected by these reactants. Although the lifetime of NO is short particularly under air atmospheric conditions (where the oxygen tension is unphysiologically high), it increases significantly under physiologically low oxygen concentrations. Because oxygen tensions in human body differ from one tissue to another and change depending on their metabolism, bioloical activity of NO in various tissues might be affected by local oxygen tensions. To elucidate the role of NO and related radicals in the regulation of circulation and energy metabolism, their effects on arterial resistance and energy metabolism in mitochondria, mammalian cells and enteric bacteria were studied under different oxygen tensions. Kinetic analysis revealed that NO-dependent generation of cGMP in resistance arteries and their relaxation were strongly enhanced by lowering oxygen tensions in the medium. NO reversibly suppressed the respiration and ATP synthesis of isolated mitochondria and intact cells particularly under low oxygen tensions. Kinetic analysis revealed that cross-talk between NO and superoxide generated in and around endothelial cells regulates arterial resistance particularly under physiologically low oxygen tensions. NO also inhibited the respiration and ATP synthesis of *E. coli* particularly under low oxygen tensions. Because concentrations of NO and H^+ in gastric juice are high, most ingested bacteria are effectively killed in the stomach. However, the inhibitory effects of NO on the respiration and ATP synthesis of *H. pylori* are extremely small. Kinetic analysis revealed that *H. pylori* generates the superoxide radical thereby inhibiting the bactericidal action of NO in gastric juice. Based on such observations, critical roles of the cross-talk of NO, superoxide and molecular oxygen in the regulation of energy metabolism and survival of aerobic and microaerophilic organisms are discussed.

Keywords: Superoxide, circulation, nitric oxide, energy metabolism, oxygen toxicity, infection

INTRODUCTION

NO (nitric oxide) is a multifunctional gaseous radical that plays critical roles in the regulation of

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circulation, neuronal transmission, and defense mechanisms.[1–3] Because of the gaseous nature of NO and its high affinity for various molecules, such as hemeproteins, thiols, and related radicals, it easily penetrates through cell membrane/lipid bilayers and modulates cellular metabolism and functions through the formation of dissociable complexes with these molecules. NO synthesized in vascular endothelial cells binds to guanylate cyclase in smooth muscle cells thereby decreasing vascular resistance and enhancing oxygen delivery to peripheral tissues. NO also binds to other hemeproteins, such as electron transfer complexes in mitochondria[4–6] and cytochrome P450,[7,8] thereby inhibiting respiration, ATP synthesis and detoxification of xenobiotics.

Based on experiments *in vitro*, the lifetime of NO has been postulated to be extremely short (~8 s). Because NO also reacts with molecular oxygen, it rapidly loses biological activity particularly under air, a condition where oxygen tension is unphysiologically high (220–250 μM).[9] NO is fairly stable under physiologically low intracellular oxygen tensions.[10] Although effects of NO on the functions of proteins, organelles and cells have been studied extensively *in vitro*, most experiments were carried out under air, where the lifetime of NO is minimized.

Because oxygen tension in tissues changes depending on their metabolism and circulatory status, the lifetime and biological effects of NO in a tissue might be affected significantly by these factors. For example, the energy requirement in the muscle dramatically increases during exercise and, hence, their blood flow and ATP generation are strongly enhanced. Oxygen tensions in gastric juice also change depending on the ingested solutions, such as cold water, hot tea and beer. We describe here the physiological importance of local concentrations of molecular oxygen and superoxide radical for NO-dependent regulation of circulatory status, energy metabolism and survival of aerobic and microaerophilic organisms.

Effect of Oxygen Tension on NO-dependent Regulation of Arterial Resistance and Guanylate Cyclase Activity

Infusion of high concentrations of oxygen (~100%) into a medium has been used frequently to maintain ATP levels in excised tissues. Therefore, oxygen tensions in a medium used for *in vitro* and *exo vivo* experiments are generally maintained under hyperoxic conditions. In order to elucidate the effect of oxygen tensions on NO-dependent relaxation of arteries, aortic specimens were exposed to NO under varying oxygen tensions. When exposed to 100% oxygen at 37°C, the steady-state concentration of oxygen in a medium reaches as high as 690 μM. Under such unphysiologically hyperoxic conditions, low concentrations of NO induced arterial relaxation only slightly (Figure 1). When the medium was saturated with air, its oxygen concentration

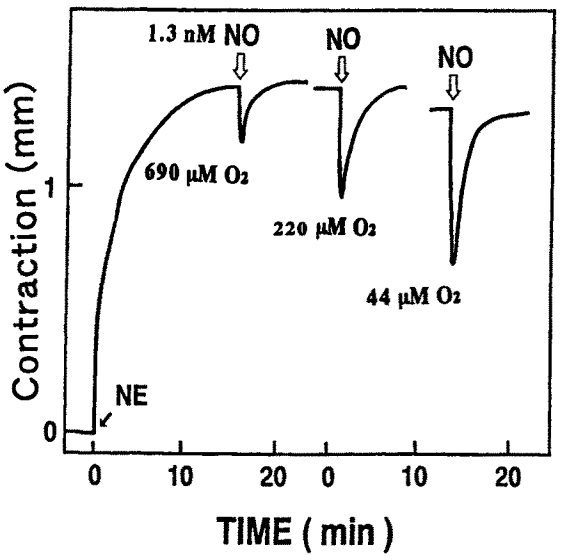

FIGURE 1 Effect of oxygen on NO-dependent relaxation of arteries. Endothelium-denuded aortic specimens were incubated in Krebs–Henseleit solution (KRP) at 37°C for 5 mm under varying concentrations of oxygen. After contraction with norepinephrine, the specimens were treated with 1.3 nM NO. Oxygen concentrations in the medium infused with 100% oxyen and air were 690 and 220 μM, respectively. NO was also added at an oxygen concentration of 44 μM. The ability of NO to induce relaxation of arteries strongly depends on oxygen concentration in the medium and increases under physiologically low oxygen tensions.

decreased to 220 μM. Under such conditions, the same dose of NO induced arterial relaxation more strongly than when saturated with pure oxygen. The extent of relaxation further increased by decreasing oxygen concentrations to physiologically low levels (~40 μM). Thus, the ability of NO to induce relaxation of resistance arteries strictly depends on the oxygen tension in and around vascular walls. Kinetic analysis revealed that the enhanced action of NO was predominantly due to increase in its lifetime under low oxygen tensions.[9,10]

Because the ability of NO to induce vascular relaxation is enhanced by low oxygen tension, NO-dependent metabolism in arterial walls might also be affected by the change in oxygen tensions. Thus, we studied the effect of oxygen tension on NO-dependent activation of guanylate cyclase. The amount of cGMP formed in arterial specimens was increased by NOC 18, a NO donor, in a concentration-dependent manner (Figure 2). Lowering oxygen tension in the medium also increased arterial levels of cGMP. Kinetic analysis revealed that the enhancement of NO-dependent relaxation of arterial specimens by lowering oxygen tension was due to increase in the generation of cGMP. Thus, arterial resistance might principally be determined by cross-talk of NO, superoxide and molecular oxygen in and around vascular walls.

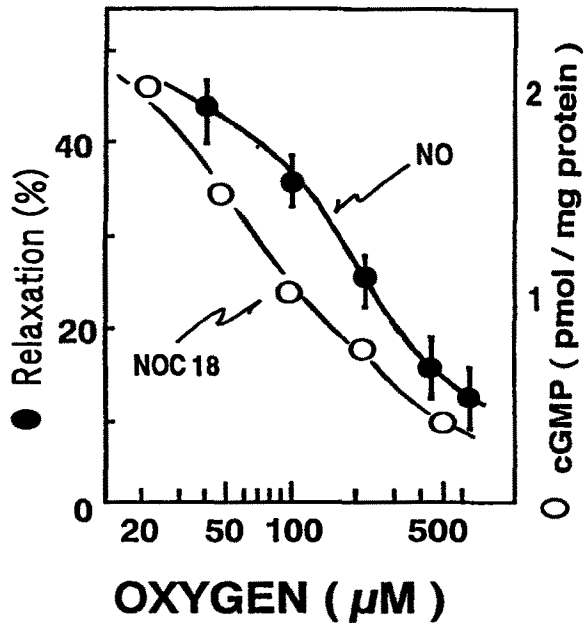

FIGURE 2 Effect of oxygen on NO-dependent generation of cGMP. Endothelium-denuded aortic specimens were incubated at 37°C for 5 min under varying concentrations of oxygen. After incubation for 1 min with 10 μM NOC 18, arterial levels of cGMP were determined. The effect of NO to activate guanylate cyclase strongly depends on oxygen concentration in the medium and increases under physiologically low oxygen tensions.

Effect of NO on the Energy Metabolism of Mitochondria and Cells

Because NO has high affinity for hemeproteins, it reacts with not only guanylate cyclase but also with other proteins, such as hemoglobin,[11] mitochondrial electron transfer complexes,[4,6] and cytochrome P450.[7,8] Thus, we studied the effect of NO on the respiration of mitochondria. In the presence of a respiratory substrate and inorganic phosphate, ADP initiated the state-3 respiration of mitochondria. The state-3 respiration was reversibly inhibited by NO (Figure 3). The inhibitory effect of NO increased with concomitant decrease in oxygen tension. NO also inhibited the dinitrophenol-uncoupled respiration of mitochondria in a concentration-dependent manner. Similar inhibition was also observed with other substrates, such as α-ketoglutarate and ascorbate. Thus, reversible inhibition of ATP synthesis might principally reflect the inhibition of cytochrome *c* oxidase by NO.

Some tumor cells reveal a marked respiration without adding respiratory substrates. Although a physiologically low level of NO had minimum effect on the respiration of tumor cells under hyperoxic conditions, it strongly inhibited the respiration when oxygen tension in the medium decreased (Figure 4).[12,13] Similar effects of NO were also observed with other types of cells, such as gastrointestinal epithelial cells and *E. coli*.[14,15] Thus, cellular production of ATP might be regulated pivotally by the coordination of vascular

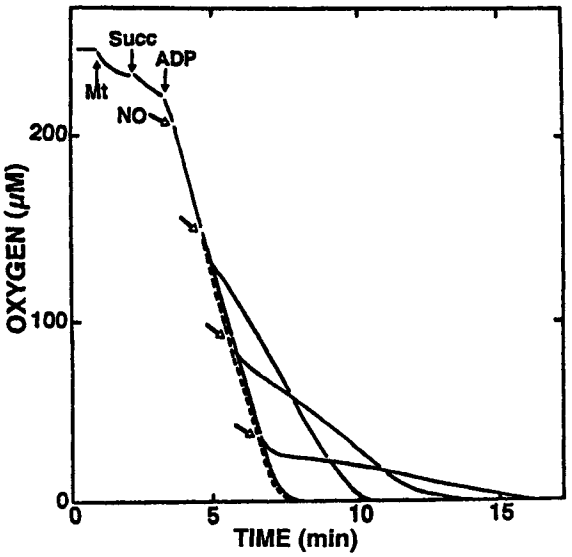

FIGURE 3 Effect of NO on mitochondrial respiration is enhanced by low oxygen tension. Isolated mitochondria were suspended in a medium (0.5 mg protein/ml) consisting of 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 2 mM sodium phosphate, 10 mM Tris-HCl (pH 7.4), and 5 mM succinate 25°C. Oxidative phosphorylation was measured polarographically using a Clark-type oxygen electrode fitted to a 2 ml water-jacketed closed chamber at 25°C. State-3 respiration was initiated by adding 600 μM ADP. NO was added at different oxygen tensions (arrow) at a final concentration of 0.8 μM. The inhibitory effect of NO is enhanced by decreasing oxygen tension.

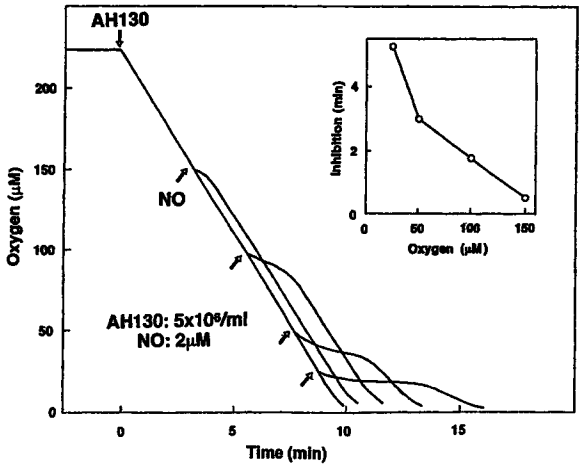

FIGURE 4 Effect of NO on the respiration and ATP levels of AH130 cells. AH130 cells (5×10^6/ml) were incubated in 2 ml of KRP at 37°C. At the indicated times (arrow), NO was added to give a final concentration of 2 μM. During the incubation, oxygen concentration in the medium was monitored polarographically. Inset shows the effect of oxygen tension on the time required for the disappearance of the inhibitory effect of NO. Nitric oxide functions as a regulator for energy production by mitochondria particularly under physiologically low oxygen tensions. During the time when cellular respiration was inhibited by NO, ATP level decreased in a reversible manner.

and mitochondrial actions of NO both of which depend on local oxygen tensions (Figure 5).

Effect of NO and Oxygen Tension on Membrane Potential and Apoptosis

Intact mitochondria generate membrane potential either by substrate oxidation or by ATP hydrolysis.[16] We recently reported that the membrane potential of mitochondria was depolarized by NO in a reversible manner (Figure 6). The extent of depolarization depends on the concentration of NO and is larger at low oxygen concentrations than at high tensions.[12] When cells are depolarized by NO, fairly large amounts of Ca^{2+} come out from mitochondria into the cytosolic compartment. Thus, Ca^{2+}-dependent cellular processes would be enhanced by NO particularly under low oxygen tensions. Such properties of NO are important not only for the regulation of energy metabolism in normal cells but also for the determination of the viability of cancer cells. In fact, when mitochondrial energy transduction in cancer cells is inhibited by NO under physiologically low oxygen tensions for a long time, cytochrome is released from depolarized mitochondria and cells undergo apoptosis (Figure 7). Thus, NO-induced apoptosis might underlie the mechanism by which the growth of cancer cells is inhibited by infiltrated macrophages in and around tumors.

Because NO has high affinity for hemoglobin, this protein has been used for testing the specificity of NO-dependent processes. In fact, a fairly small amount of either erythrocytes (0.1%) or equimolar hemoglobin completely abolished the NO-inhibited respiration of cells and mitochondria (Figure 8). These properties of NO and

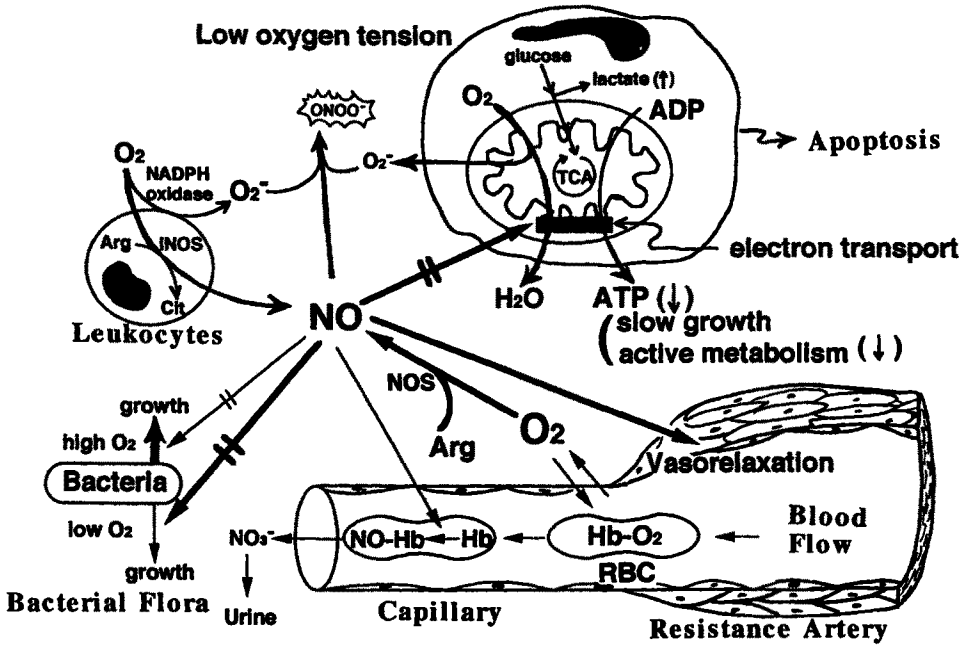

FIGURE 5 Supersystem for the regulation of energy production by cross-talk of NO, superoxide and molecular oxygen. Nitric oxide induces vasorelaxation at resistance arteries and enhances oxygen delivery to peripheral tissues and their energy production while it suppresses ATP synthesis by mitochondria. Both effects of NO are inhibited by superoxide radical and enhanced by lowering oxygen tension. Such pivotal action of NO might play important role in the regulation of energy metabolism in tissues in which oxygen tension changes strongly, such as in skeletal muscles.

hemoglobin might explain why the growth of cancer cells is fairly slow in avascular tumors while it abruptly increases after neovascularization and/or bleeding in and around tumors.

Effect of NO and Low Oxygen Tensions on the Metabolism of Bacteria

Although effects of NO on various bacteria have been studied extensively,[15,17–19] most *in vitro* experiments were carried out under air atmospheric conditions in which oxygen concentration is fairly high. 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 air atmospheric conditions. Thus, we studied the effects of NO and oxygen tension on the electron transfer reaction and growth of *E. coli*. The respiration of *E. coli* was transiently inhibited by NO and recovered completely (Figure 9). The inhibitory effect of NO also increased with concomitant decrease in oxygen tension. Both nitrite and nitrate have no appreciable effect on the respiration of *E. coli*. A fairly small amount of erythrocytes (0.1%) completely abolished the inhibitory effect of NO. The inhibitory effect of NO was also suppressed by an equimolar amount of hemoglobin.

The growth of *E. coli* was also inhibited by NOC 12, a NO donor, in a concentration-dependent manner. The inhibitory effect of NOC 12 increases with concomitant decrease in oxygen. Because the intestinal lumen is anaerobic, this property of NO might play critical roles in the regulation of the number and species of bacterial flora in normal and pathologic subjects. Antibiotics have been used for patients with intestinal bleeding to prevent abnormal growth of bacteria. The reason why antibiotics are effective in treating patients with intestinal bleeding could be explained on this basis.

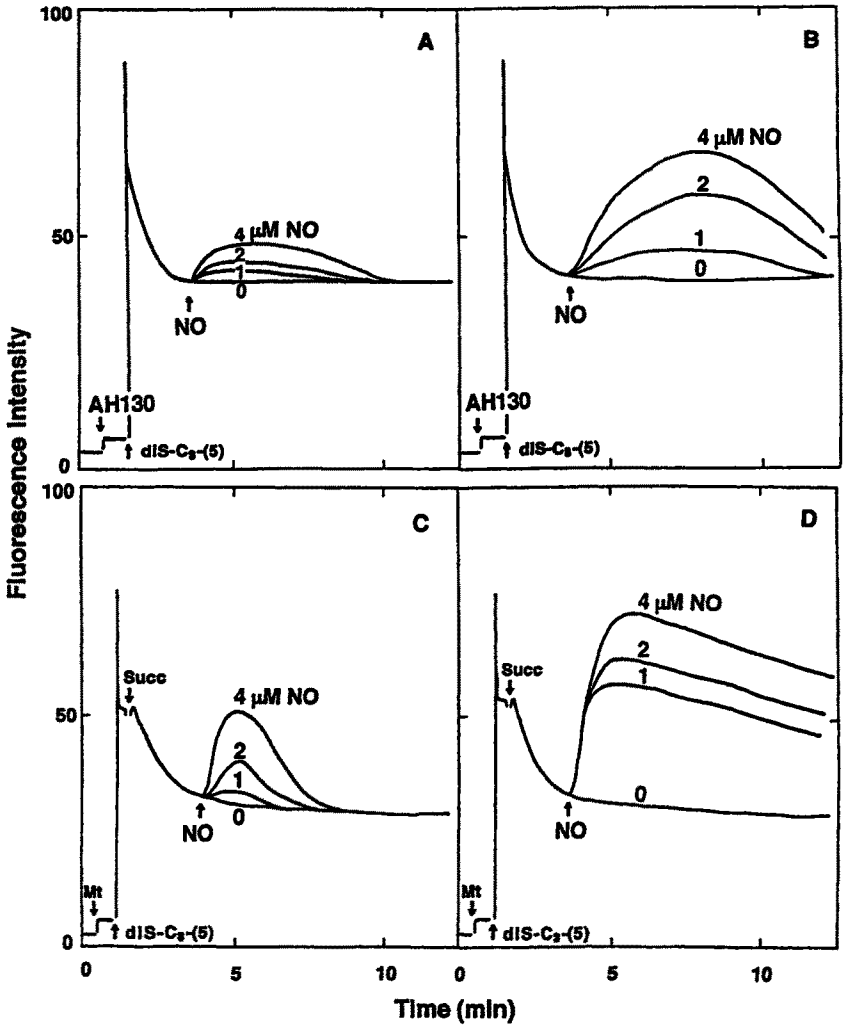

FIGURE 6 Effect of NO and oxygen tension on the membrane potential of cells and mitochondria. At oxygen concentrations of 150 (A, C) and 25 μM (B, D), 1, 2 or 4 μM of NO was added to AH130 cells and mitochondria. Experiments with mitochondria were carried out in the presence of 5 mM succinate (Succ). A and B, AH130 cells; C and D, mitochondria (Mt); diS-C3-,[5] cyanine dye (1 μM). $[Ca^{2+}]_i$ is increased by NO particularly under low oxygen tensions.

NO is generated in the oral cavity and gastric juice from nitrite and nitrate contained in saliva and foods (Figure 10). Thus, in addition to strong acidity of gastric juice, bactericidal actions of NO might also play important roles in the defense mechanism against bacterial infection. However, some bacteria escape from the bactericidal action of gastric NO. *H. pylori*, a Gram-negative microaerophilic bacterium that resides in the mucus layer of the stomach, is one such example. Partly because of high activity of urease on the membranes of *H. pylori*, they can survive even in an acidic environment in the stomach. However, the effect of NO on *H. pylori* metabolism is not known. Thus, we also studied the effect of NO on the respiration of *H. pylori*. Although NO inhibited the respiration of *H. pylori*, its effect was substantially smaller than that observed with *E. coli* (Figure 11). The inhibitory effect of NO is irreversible and insensitive to erythrocytes, oxyhemoglobin, and the NO-trapping agent carboxy-PTIO. Thus, NO seems to interact rapidly with

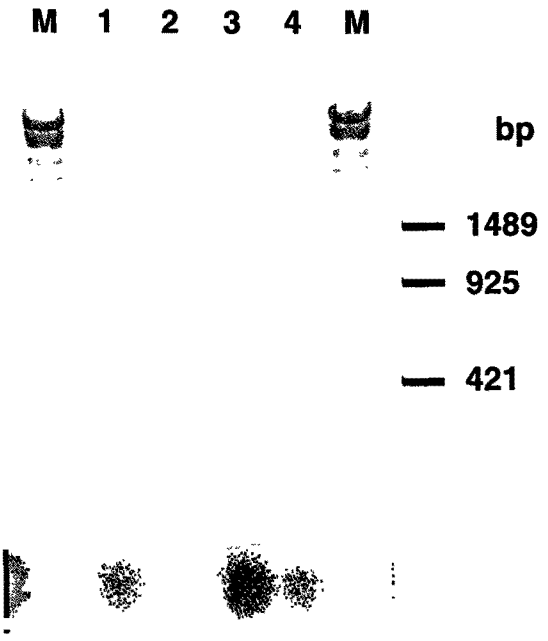

FIGURE 7 NO elicits apoptosis of cancer cells under low oxygen tension. On day 4 (lane 1), 7 (lane 2), 11 (lane 3) and 14 (lane 4) of intraperitoneal inoculation of AH130 cells, they were collected and washed in PBS. DNA samples were obtained and subjected to agarose gel electrophoresis.

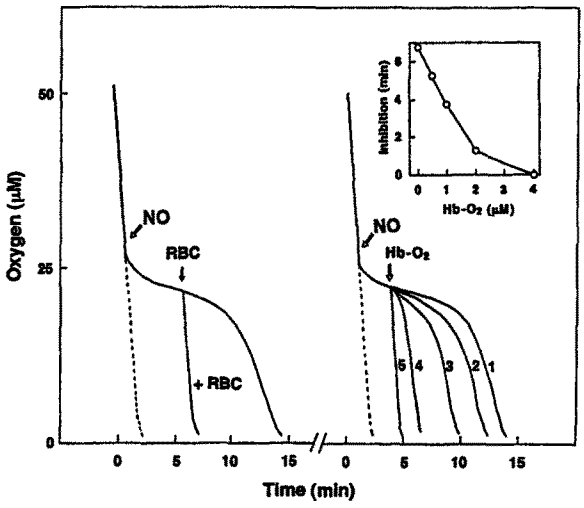

FIGURE 8 Effect of RBC and HbO_2 on the inhibitory action of NO. Respiration of cells was inhibited by 4 μM NO. At the indicated times, RBC (0.1% Ht) were added to the reaction mixture. HbO_2 was also added to give final concentrations of 0 (1), 0.5 (2), 1 (3), 2 (4) or 4 μM (5). The presence of either RBC or HbO_2 instantaneously inhibits the biological activity of NO.

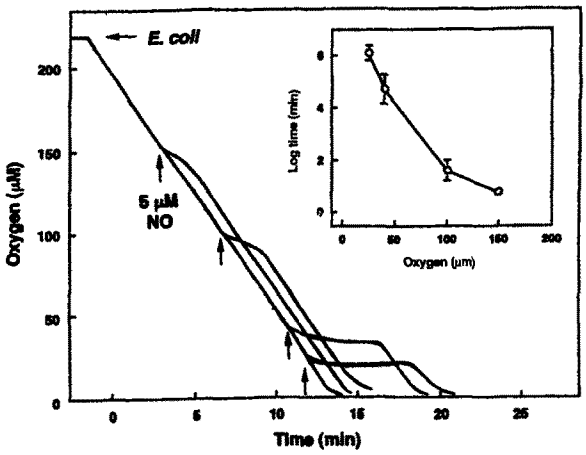

FIGURE 9 Effect of NO on the respiration of *E. coli*. *E. coli* (1×10^8 cells/ml) were incubated in a closed chamber containing 2 ml of Hepes–KRP (pH 7.4) at 37°C. At the indicated times (arrows), NO was added to the reaction mixture to give a final concentration of 5 μM. During the incubation, the 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. The experiments were carried out at least 5 times with similar results.

some cellular component(s) and weakly but irreversibly inhibits the respiration of *H. pylori*.[20]

NO rapidly reacts with the superoxide radical thereby forming peroxynitrite, a metabolite that may be converted to hazardous hydroxy radical. Thus, we tested the effect of peroxynitrite on the respiration of both *E. coli* and *H. pylori*. The respiration of both *E. coli* and *H. pylori* was inhibited irreversibly by peroxynitrite. This observation drove us to speculate that peroxynitrite might be responsible for the irreversible inhibition by NO of *H. pylori* respiration. In fact, *H. pylori* but not *E. coli* generates substantial amounts of superoxide radicals as determined by luminol chemiluminescence (Figure 12). The *H. pylori*-dependent chemiluminescence was completely inhibited by adding SOD in the medium. Thus, exogenously added NO rapidly reacts with the superoxide radical generated by *H. pylori* and the resulting peroxynitrite irreversibly inhibits their respiration.[20]

H. pylori shows unusually large genomic diversity.[21] Although the genomic diversity of *H. pylori* has been postulated to contribute to the

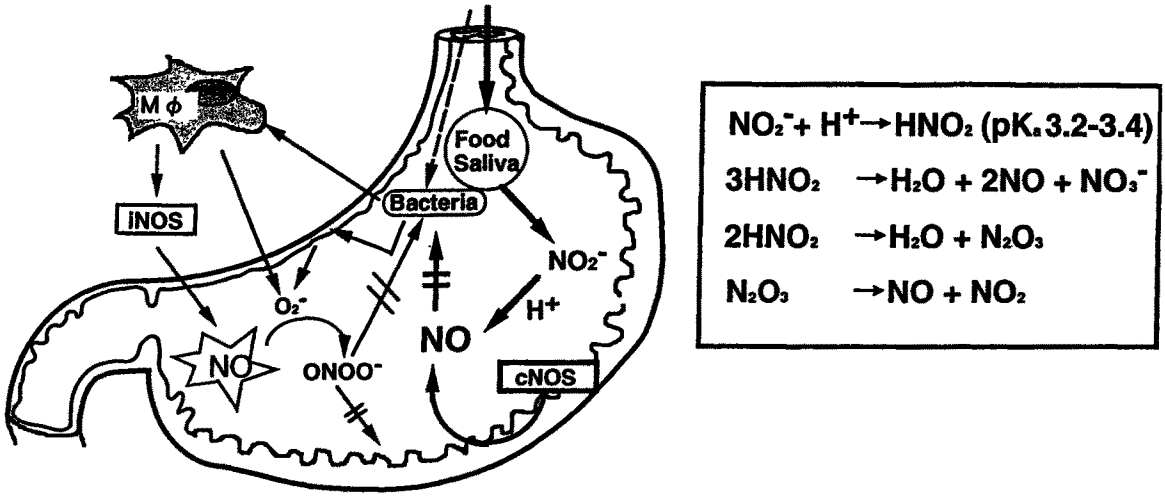

FIGURE 10 Gastric occurrence of NO and its bactericidal action.

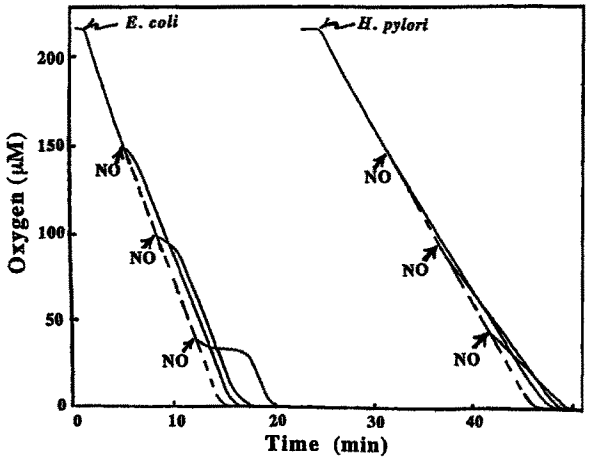

FIGURE 11 Effect of NO on the respiration of *E. coli* and *H. pylori*. Respiration of *E. coil* and *H. pylori* (1×10^8 cells/ml) was monitored polarographically with a Clark-type oxygen electrode at 37°C in 10 mM Hepes-NaOH (pH 7.0) containing 0.9% NaCl and 5 mM succinate. At the indicated times (arrow), NO was added to give a final concentration of 5 μM. Dotted lines, control experiments without NO.

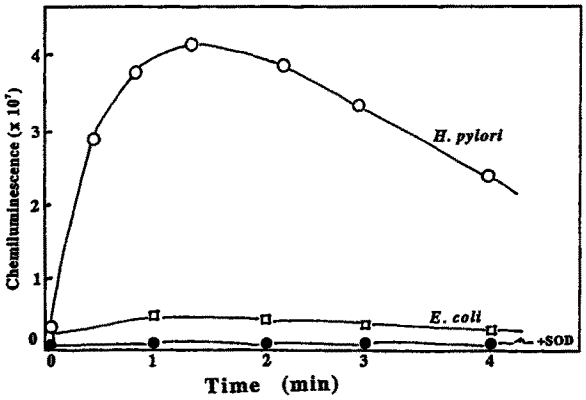

FIGURE 12 Superoxide generation by *H. pylori*. Incubation mixtures (1 ml) contained 1 μM MCLA and 10^8 cells/ml of either *H. pylori* (circles) or *E. coli* (squares). Chemiluminescence intensity was monitored continuously in the absence (open symbols) or presence (closed circles) of Mn-SOD (100 U/ml). Other conditions were as in Figure 9.

success of this species as a ubiquitous pathogen, the molecular mechanism for its occurrence is not known. Because peroxynitrite interacts with transition metals and forms hazardous products, it might irreversibly modify membranes, proteins and DNA of *H. pylori*. Thus, the mutagenic properties of peroxynitrite and related metabolite(s) may underlie the mechanism for increasing the genomic diversity of *H. pylori* and etiology of gastric inflammation, ulceration and carcinogenesis (Figure 13). The supersystem driven by the cross-talk of NO, superoxide, molecular oxygen and related enzymes might underlie the mechanism for the regulation of energy metabolism

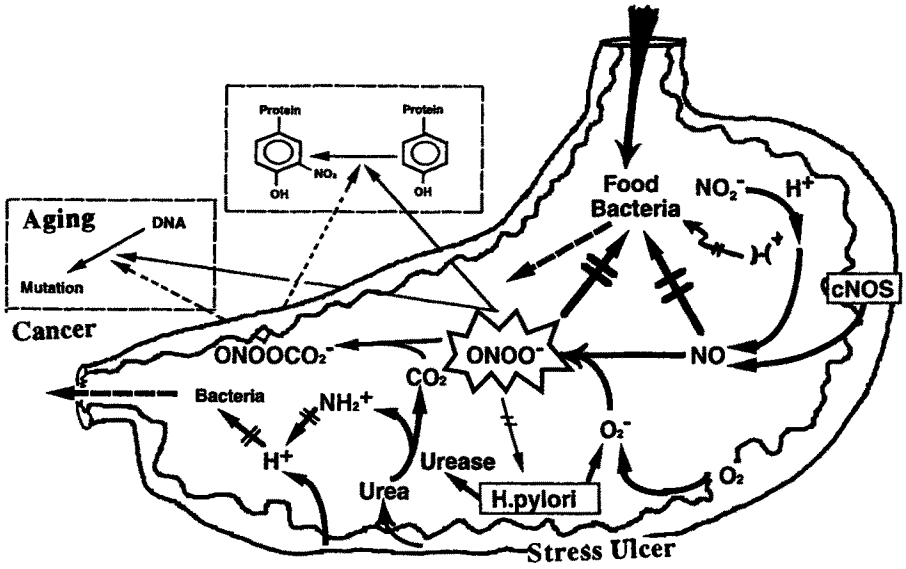

FIGURE 13 Bactericidal action and metabolism of nitric oxide in the stomach.

and determine the survival of not only aerobic organisms but also for microaerophilic prokaryotes.

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