Free Rad. Res., Vol. 33, pp. 757-770
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Cross-Talk Between NO and Oxyradicals, a Supersystem that Regulates Energy Metabolism and Survival of Animals

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Accepted for publication by Prof. N. Taniguchi

(Received 20 June 2000)

Mammalian tissues have large amounts of available ATP which are generated by oxidative phosphorylation in mitochondria. For the maintenance of the human body, a large amount of oxygen is required to regenerate these ATP molecules. A small fraction of the inspired oxygen is converted to superoxide radical and related metabolites even under physiological conditions. Most reactive oxygen species react rapidly with a variety of molecules thereby interfering with cellular functions and induce various diseases.

Nitric oxide (NO) is an unstable gaseous radical with high affinity for various molecules, such as hemeproteins, thiols, and related radicals. NO easily penetrates through cell membrane/lipid bilayers, forms dissociable complexes with these molecules and modulates cellular metabolism and functions. Because NO has an extremely high affinity for the superoxide radical, the occurrence of the latter might decrease the biological function of NO. Thus, superoxide radicals in and around vascular endothelial cells play critical roles in the pathogenesis of hypertension and vasogenic tissue injury. Because NO also reacts with molecular oxygen, it rapidly loses its biological activity, particularly under ambient atmospheric conditions where the oxygen tension is unphysiologically high. Thus, biological functions of NO are determined by the local concentrations of molecular oxygen and superoxide radicals.

NO also inhibits electron transfer reaction and ATP synthesis in mitochondria and aerobic bacteria, such as *E. coli*; the inhibitory effects are also enhanced by hypoxia. Thus, the cross-talk between NO, molecular oxygen and oxyradicals play critical roles in the regulation of energy metabolism, fates and the survival of aerobic organisms. The present work describes the pathophysiological significance of the supersystem driven by the cross-talk between NO and oxyradicals.

Because of their ability to carry out aerobic metabolism, mammalian tissues have large amounts of available ATP which are generated by oxidative phosphorylation in mitochondria. Assuming that the amount of energy daily consumed by healthy adult humans is about 2100 Cal and the over-all efficiency of ATP synthesis is 50%, about 150 mol of ATP (75 kg) would be utilized every day. Since about 100 g of ATP is present in man, each ATP molecule would need to be regenerated ~800 times per day predominantly by oxidative phosphorylation. For the maintenance of the human body, a large amount

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of oxygen (about 500 liters of O_2/day) is required to regenerate these ATP molecules. Under physiological conditions, a small fraction of the inspired oxygen is converted to superoxide radical (O_2^-) which is further converted to hydrogen peroxide and related metabolites. Under physiological conditions, the steady-state levels of superoxide and hydrogen peroxide are postulated to be 10^{-12}~10^{-11} and 10^{-9}~10^{-7}M, respectively [1]. Most, but not all, reactive oxygen species react rapidly with a variety of molecules thereby interfering with cellular functions [2]. Based on experiments using agents that scavenge reactive oxygen species and related free radicals, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and endogenous antioxidants, such as vitamin E, reduced glutathione (GSH), and ascorbic acid, these species have been postulated to underlie the pathogenesis of various diseases [3–5]. Superoxide radical is also generated by electron transfer systems in mitochondria and microsomes. The extent of superoxide formation is increased when the respiratory chain is under fully reduced conditions. This is one of the reasons for why tissue injury occurs more severely during reperfusion rather than during ischemia [6–8].

Nitric oxide (NO) is an unstable radical with a half-life of 5 to 8 seconds in air atmospheric conditions [9]. Because of its gaseous nature and high affinity for various molecules, such as hemeproteins, thiols, and related radicals, NO easily penetrates through cell membrane/lipid bilayers, forms dissociable complexes with these molecules and modulates cellular metabolism and functions [10–13]. The NO radical is a multifunctional intermediate, in that it regulates vascular resistance [14–17], inhibits platelet aggregation [18], and functions as an neurotransmitter [19,20]. NO synthesized in vascular endothelial cells binds to guanylate cyclase in smooth muscle cells, decreases vascular resistance (known as EDRF) and enhances oxygen delivery to peripheral tissues. NO also binds to other hemeproteins, such as electron transfer complexes in mitochondria [21–23] and cytochrome P450 [24,25], thereby inhibiting respiration, ATP synthesis and detoxification of xenobiotics.

It should be noted that NO has an extremely high affinity for the superoxide radical ($k = 6.7 \times 10^9 M^{-1}s^{-1}$). Thus, the occurrence of the superoxide at or near the site of NO generation significantly decreased the biological function of the latter. Furthermore, when reacted with superoxide radicals, NO is converted to a toxic peroxynitrite $ONOO^-$[26]. In this context, Inoue et al. [27–29] developed a fusion gene encoding human Cu/Zn-SOD and a C-terminal heparin binding domain which has a high affinity for heparan sulfates. When injected intravenously, the fusion SOD binds to the heparan sulfate molecules on the vascular endothelial cells of arteries [27–30]. The heparin binding fusion SOD markedly inhibits vasogenic tissue injury including postischemic reperfusion injury of the liver [27,28]. Kinetic analyses have revealed that the protective action was predominantly due to scavenging of the superoxide radical in and around endothelial cells, thereby enhancing the vascular function of NO. The blood pressure of spontaneously hypertensive rats (SHR) was also normalized by endothelially targeted SOD predominantly by inhibiting the disappearance of NO in vascular walls [30,31]. These findings suggest that superoxide radicals in and around vascular endothelial cells play critical roles in the pathogenesis of hypertension and vasogenic tissue injury.

Because NO also reacts with molecular oxygen, it rapidly loses its biological activity, particularly under ambient atmospheric conditions where the oxygen tension is unphysiologically high (220~250 μM)[32]. In contrast, NO is fairly stable under physiologically low intracellular oxygen tensions [33]. Thus, biological functions of NO are determined by the local concentrations of molecular oxygen and superoxide radicals.

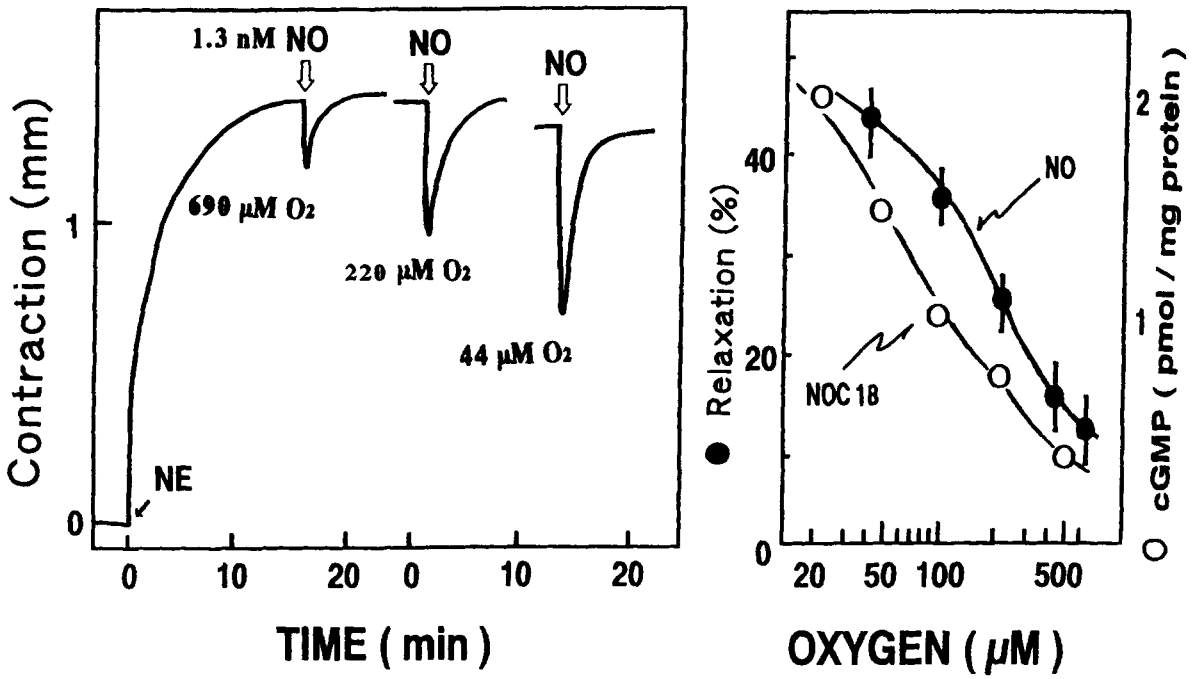

FIGURE 1 Effect of oxygen and NO on arterial relaxation and cGMP generation. Endothelium-denuded aortic specimens were incubated in Krebs-Henselite solution (KRP) for 5 min at 37°C and varying oxygen concentrations. After contraction by norepinephrine, aortic specimens were relaxed by adding 1.3 nM NO (left). After incubation for 1 min with 10 μM NOC18, arterial levels of cGMP were determined (right). NO-dependent relaxation and cGMP production strongly increased under low oxygen tensions

EFFECT OF NO AND OXYGEN ON THE CIRCULATION

The infusion of high concentrations of oxygen in a medium has been used to maintain ATP levels in excised tissues and, hence, experimental conditions of cells and tissues *in vitro* are generally hyperoxic. For example, when exposed to 100% oxygen at 37°C, the oxygen concentration in a medium reaches levels as high as 690 μM. Under such conditions, low concentrations of NO induce arterial relaxation only slightly (**Fig. 1**). When saturated with air at 37°C, the oxygen concentration in a medium decreases to 220 μM. Under such conditions, the same dose of NO induces arterial relaxation more strongly than when saturated with pure oxygen. The extent of relaxation further increases by decreasing oxygen concentrations to physiologically low levels. Thus, the activity of NO in terms of inducing the relaxation of resistance arteries strictly depends on the oxygen tension in and around the vascular walls.

Because the activity of NO relating to induce vascular relaxation is enhanced by hypoxia, NO-dependent metabolism in arterial walls may also be affected by local oxygen tension. In fact, NO-dependent generation of cGMP in arterial specimens increased by lowering oxygen concentrations. Lowering oxygen tension increases cGMP levels in arteries and enhances their relaxation. Because NO also reacts rapidly with the superoxide radical and looses its depressor action, arterial resistance may principally be determined by the cross-talk of NO, superoxide and molecular oxygen in and around vascular

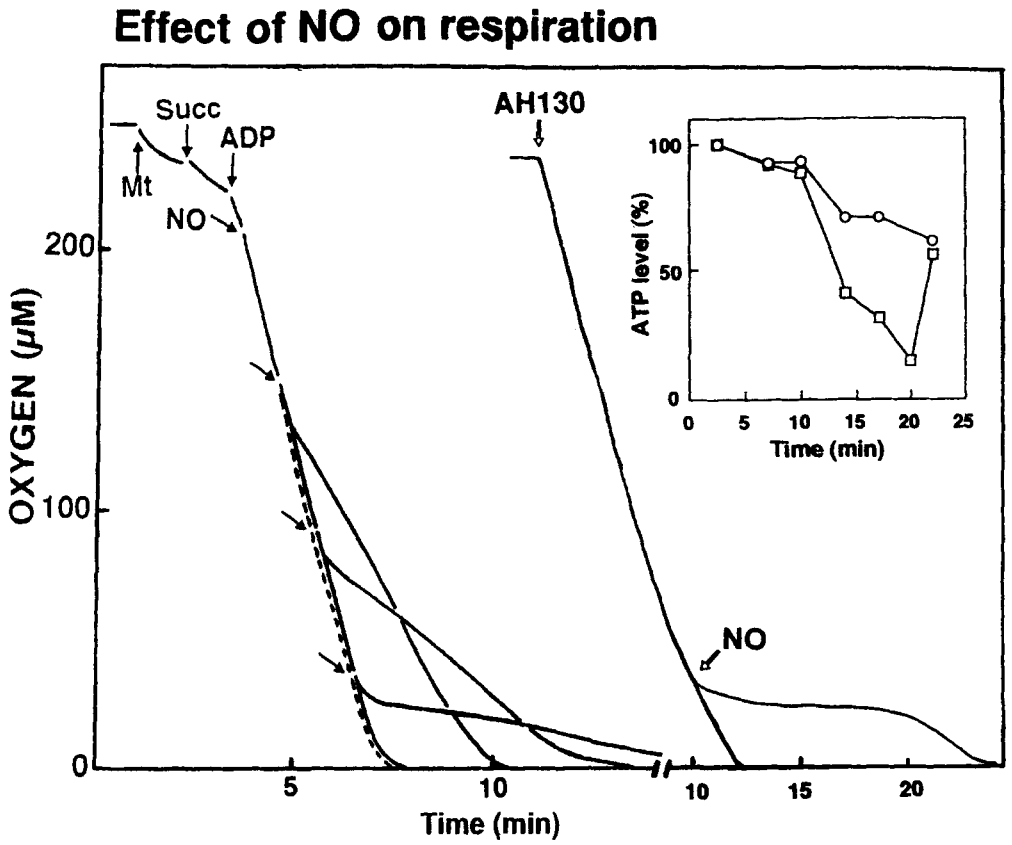

FIGURE 2 Effect of NO and oxygen on respiration and ATP synthesis in mitochondria and cells. Respiration of mitochondria and AH-130 hepatoma cells (5×10^6/ml) was measured polarographically using a Clark-type oxygen at 25°C. At the indicated times (arrows), NO was added to give final concentrations of 0.8 (for mitochondria) and 2 μM (for cells). During the time when cellular respiration was inhibited by NO, ATP level strictly decreased in a reversible manner (inset)

walls. The enhanced action of NO is partly due to an increase in the lifetime of NO under low oxygen tensions [32,33]. The enhancement of EDRF action of NO in hypoxic area might reflect the compensatory adaptation of arteries to increase oxygen delivery to their peripheral tissues for ATP synthesis.

EFFECT OF NO ON ENERGY METABOLISM OF MITOCHONDRIA AND CELLS

Because NO has an extremely high affinity for hemeproteins, it also reacts with proteins other than guanylate cyclase, such as hemoglobin [34], mitochondrial electron transfer complexes [21–23] and cytochrome P450 [24,25]. In the presence of a respiratory substrate and inorganic phosphate, ADP initiates the state-3 respiration of mitochondria by a mechanism which is reversibly inhibited by NO (**Fig. 2**). The inhibitory effect of NO increases with a parallel decrease in oxygen tension. Because a similar inhibition is also observed with other substrates, such as α-ketoglutarate and ascorbate, the inhibition occurred principally at the level of cytochrome c oxidase.

When electron transfer reaction(s) are inhibited by NO, mitochondrial complexes are satu-

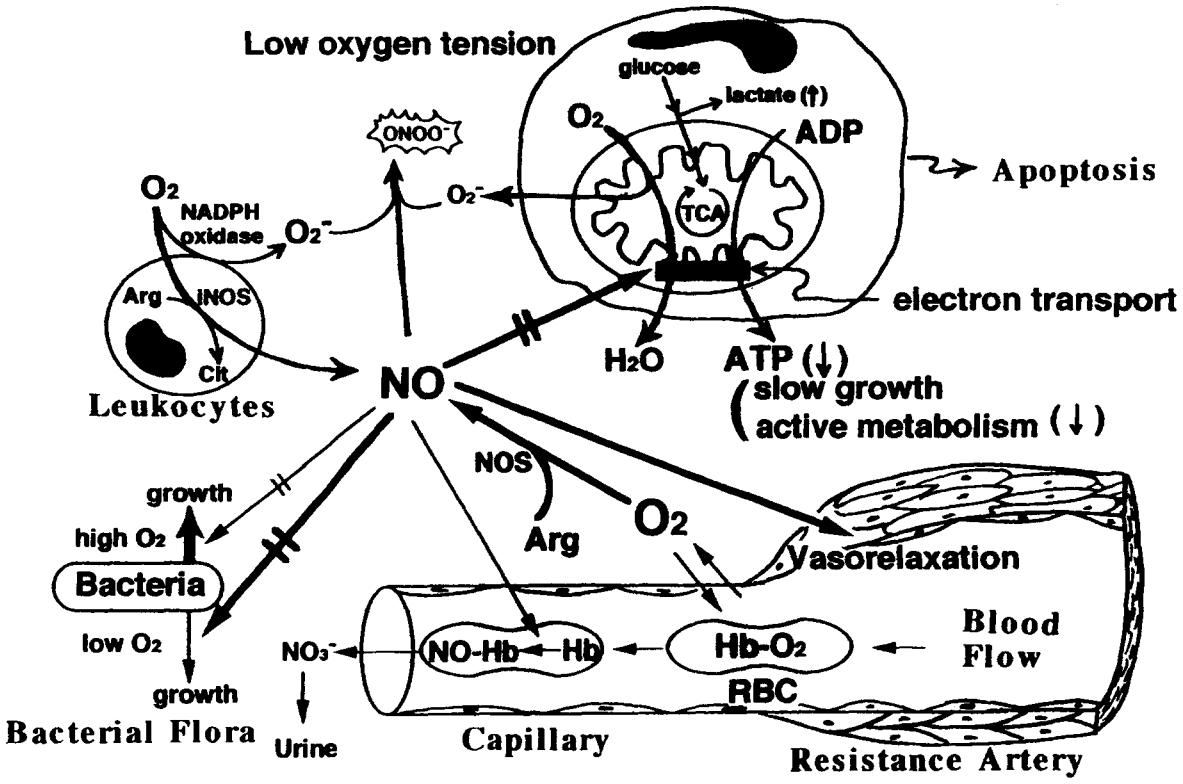

FIGURE 3 Regulation of circulatory status and ATP synthesis by NO, superoxide and molecular oxygen. Nitric oxide induces the relaxation of resistance arteries thereby enhancing oxygen delivery to peripheral tissues for ATP synthesis, while NO suppresses ATP synthesis by mitochondria. Both effects of NO are inhibited by superoxide radical and enhanced by lowering oxygen tension. Such a pivotal action of NO might play an important role in the regulation of energy metabolism in tissues in which oxygen tension changes strongly

rated with electron (reductive stress) and, hence, it easily escapes from mitochondria and reduces molecular oxygen to generate the superoxide radical. Because the superoxide radical rapidly reacts with NO, the interaction of the two radicals rapidly eliminated the inhibitory effect of NO, thus forming feed-back mechanism for the regulation of mitochondrial energy production. Although a physiologically low level of NO has negligible effect on the respiration of cells under hyperoxic conditions, it strongly inhibits respiration particularly when the oxygen tension is low [34,35]. Similar effects of NO are also observed with a wide variety of cells, such as gastrointestinal epithelial cells [36–38]. Thus, the cellular generation of ATP might be regulated pivotally by the coordination of NO action in arterial wall cells (positive effect) and mitochondria (negative effect) in peripheral tissues depending on the local concentrations of oxygen and superoxide radicals (**Fig. 3**). Thus, the cross-talk of the three types of oxyradicals, molecular oxygen, superoxide and nitric oxide, regulates the circulatory status and energy metabolism in mammals [39].

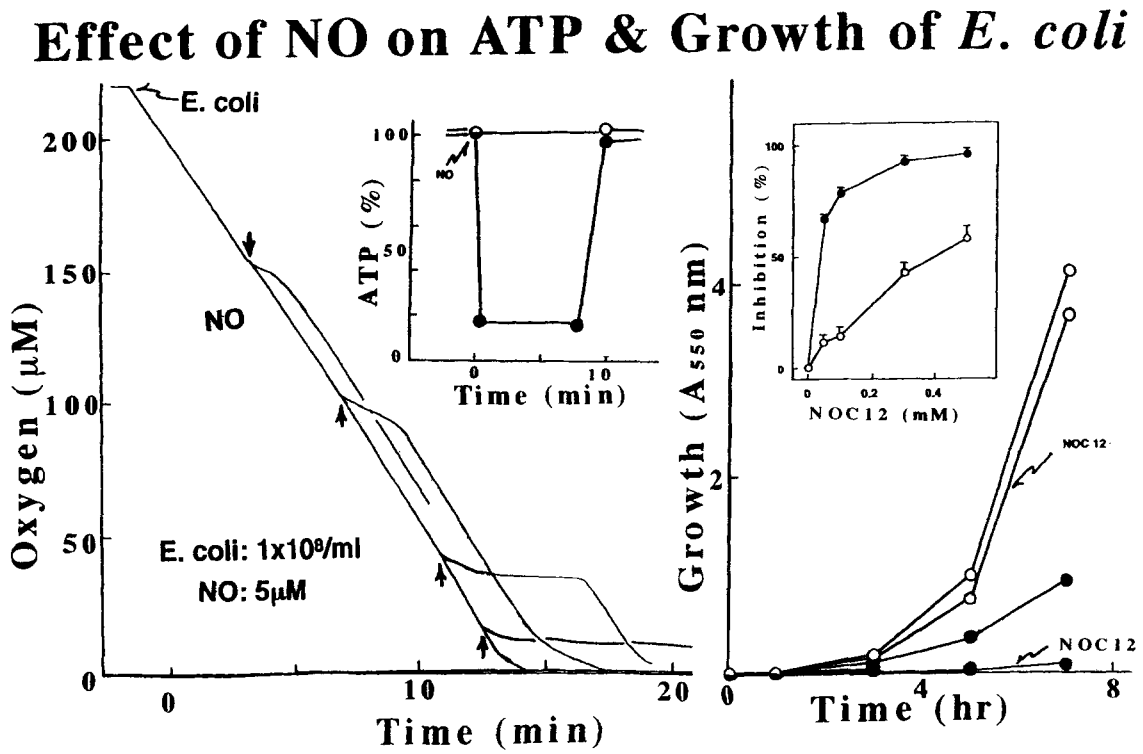

FIGURE 4 Effect of NO on the respiration and growth of *E. coli*. *E. coli*(10^8 cells/ml) were incubated in 2 ml of Hepes-Krebs Ringer buffer (pH 7.4) and their respiration was measured at 37°C. At the indicated times (*arrows*), NO was added to the reaction mixture to give a final concentration of 5 μM. Effects of NO on cellular ATP levels (inset) and the growth of *E. coli* (right) were also analyzed

EFFECT OF NO AND REACTIVE OXYGEN SPECIES ON ENTERIC BACTERIA

Although effects of NO on various bacteria have been studied extensively [15–18], most experiments were carried out *in vitro* under air atmospheric conditions in which oxygen tension is unphysiologically high. Because the intestinal lumen is anaerobic, *in vivo* effects of NO on the metabolism in 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 on the electron transfer reaction and growth of *E. coli*. The respiration of *E. coli* was reversibly inhibited by NO; the inhibitory effect increased with concomitant decrease in oxygen tension (**Fig. 4**) as observed with mitochondria and intact cells. Furthermore, cellular levels of ATP rapidly decreased immediately after inhibition of their respiration and returned to their initial levels when the inhibitory effect of NO disappeared. The growth of *E. coli* was also inhibited by NO particularly under low oxygen tensions. Thus, energy metabolism and the fate of *E. coli* is strictly affected by the lumenal concentrations of NO and molecular oxygen.

NO is generated in the oral cavity and gastric juice from nitrite and nitrate contained in saliva and foods. It should be noted that the oxygen tension in gastric juice changes markedly depending on the ingested solutions, such as tap

water (hyperoxic solution) and fresh beer (anoxic solution). Thus, in addition to the strong acidity of gastric juice, the bactericidal action of NO might also play important roles in the defense mechanism against gastric bacteria. Thus, the cross-talk of molecular oxygen, nitric oxide and superoxide radicals appears to constitute a supersystem for the regulation of the circulatory status, energy metabolism and the defense mechanism against pathogens [39].

Interestingly, a fairly small amount of erythrocytes (0.1 % hematcrit) completely abolished the inhibitory effect of NO. The inhibitory effect of NO was also suppressed by an equimolar amount of hemoglobin. These observations suggest that the rate of ATP synthesis and the growth of *E. coli* would be enhanced by gastrointestinal bleeding. It seems reasonable to administer antibiotics to patients with gastrointestinal bleeding to minimize pathologic growth of *E. coli* and other enteric bacteria.

EFFECT OF NO AND OXIDATIVE STRESS ON *H. PYLORI*

It should be noted that some pathogens often escape the bactericidal action of gastric NO. *Helicobacter pylori*, a Gram-negative microaerophilic bacterium, is one of such example that resides in the mucus layer of the stomach. Partly because of the high activity of urease on the membranes of *H. pylori*, they are able to survive, even in the acidic environment of the stomach. However, the mechanism by which *H. pylori* escapes from toxic effect of gastric NO is not known. We found that, although NO also inhibited the respiration of *H. pylori*, its effect was substantially smaller than that observed with *E. coli*(**Fig. 5**)[40]. In contrast, the respiration of both *E. coli* and *H. pylori* was inhibited irreversibly by peroxynitrite, a reaction product of NO and superoxide. This observation led us to speculate that *H. pylori* generated superoxide, thereby scavenging NO, while the resulting peroxynitrite irreversibly inhibited their respiration. In fact, *H. pylori* but not *E. coli* generated substantial amounts of superoxide radicals, as determined by luminol chemiluminescence, by an SOD-inhibitable mechanism. Thus, NO reacted rapidly with endogenously generated superoxide radicals and the resulting peroxynitrite weakly but irreversibly inhibited their respiration [40]. Thus, a supersystem driven by the cross-talk of NO, superoxide and molecular oxygen might underlie the mechanism for the regulation of energy metabolism and determine the survival of aerobic as well as microaerophilic prokaryotes.

Given the fact that *H. pylori* is localized in or near the mucosal layer of the stomach and triggered the activation and infiltration of leukocytes, constituents of gastric mucosal cells might also be affected by reactive oxygen species derived from *H. pylori* and/or activated leukocytes. Consistent with this hypothesis, the amount of 8-hydroxyguanine in gastric mucosa was shown to be increased in patients who had been infected with *H. pylori*, and this increase was reversed by eradication of the pathogen [41].

MOLECULAR MECHANISM OF H. PYLORI TRANSFORMATION

Reactive oxygen species interact with a wide variety of cellular constituents, including lipids, proteins, and DNA [42–44]. When exposed to reactive oxygen species, some amino acid residues in proteins form reactive carbonyl groups by Michael addition-type reactions. Immunoblot analysis using 2,4-dinitrophenyl (DNP) hydrazine and anti-DNP antibodies revealed an increase in the level of immunoreactive proteins with molecular sizes of 68, 50, and 45 kDa [45]. *H. pylori.* is known to undergo a transformation from a bacillary to an intermediate to coccoid forms particularly when exposed to various stresses. The amounts of immunoreactive proteins increased during the transformation from the bacillary to the coccoid forms. Oxidative

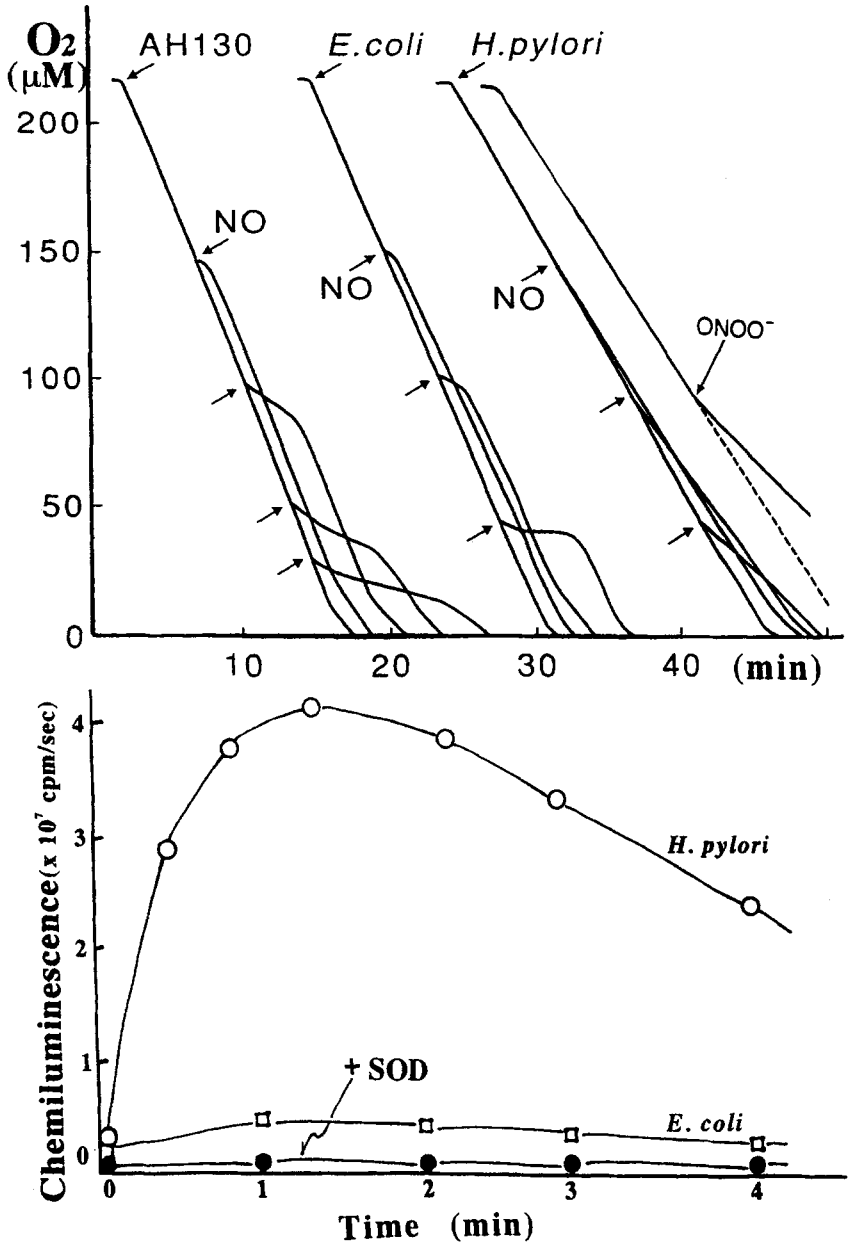

FIGURE 5 *H. pylori* generate superoxide radicals. Respiration of *H. pylori* is less sensitive to NO than that of *E. coli*. Under identical conditions, *H. pylori* (circles) but not *E. coli* (squares) generated significant amounts of superoxide radicals as analyzed by MCLA chemiluminescence. The presence of Mn-SOD (100 U / ml) completely inhibitid the chemiluminescence of *H. pylori*

modification of proteins preferentially occurs through a metal-catalyzed reaction [46]. Given the fact that the hydroxyl radical can be generated from hydrogen peroxide by the metal-catalyzed Fenton reaction [47], the amount of the catalytically active form of a transition metal

might increase during the transformation of *H. pylori*. Although *H. pylori* accumulates relatively large amounts of iron [48], the total amount of iron in the coccoid form was slightly lower than that in the bacillary form. It should be noted that, although most transition metals are present in cells in catalytically regulated protein-bound forms, they sometimes catalyze a Fenton-like reaction by a mechanism known as the "caged reaction" which is enhanced by the denaturation of metalloproteins [49]. Immunoblot analysis of protein carbonyl groups revealed that the transformation of *H. pylori* was associated with a marked increase in the abundance of oxidatively modified proteins. Thus, substantial amounts of cellular proteins, which likely include metalloproteins, undergo denaturation during the transformation of *H. pylori*. Thus, it is possible that metalloproteins contribute to the generation of protein carbonyl groups in *H. pylori*. *H. pylori* contains a large amount of urease, the large subunit of which contains six nickel atoms [50,51]. Thus, the enzyme might participate in the generation of reactive oxygen species and in the metal-catalyzed oxidation of amino acid residues in cellular proteins. Because the hydroxyl radical is highly reactive with cellular components at or near the site of its generation, metalloproteins that generate hydroxyl radicals represent the primary targets for oxidative modification by this reactive species.

Immunoblot and enzymatic analysis revealed that urease undergoes inactivation and aggregation during the transformation of *H. pylori* from the bacillary to the intermediate and to the coccoid forms (**Fig. 6**). Given that certain metalloenzymes are oxidatively denatured by reactive oxygen species, urease-associated nickel ions might become exposed on the surface or released from the enzyme during the transformation of *H. pylori*. Because the specific activity of the enzyme decreased progressively, the free form or catalytically active nickel ions seem to increase in the coccoid form of *H. pylori*. This hypothesis is consistent with the findings that cellular activity for generating the hydroxyl radical and the amount of cross-linked and inactive form of urease increased substantially during the transformation of *H. pylori*. Thus, the oxidative modification of urease might increase the availability of catalytically active forms of nickel, and thereby enhance the generation of hydroxyl radicals and the consequent inactivation and aggregation of the enzyme. Immunoblot analysis revealed that the amount of the heavy subunit and its cross-linked form of the enzyme increased progressively during the transformation from the bacillary to the coccoid form (45).

It has been well documented that both SOD and catalase play important roles in protecting organisms against oxidative stress. Thus, we also found that the specific activities of the two enzymes decreased progressively during the transformation of *H. pylori*. Because both SOD and catalase play important roles in the suppression of metal-catalyzed Fenton reactions, hydroxyl radicals would be generated in and around cells that generate the superoxide radical but have a low level of activity of the two enzymes. Thus, the coccoid form of *H. pylori* might be more susceptible to oxidative stress than the bacillary form. Consistent with this hypothesis are the findings that, although the amount of superoxide radicals generated by the coccoid form was smaller than that formed by the bacillary form of *H. pylori*, the rate of hydroxyl radical generation markedly increased during the transformation [45]. Thus, substantial increase of the aggregated form of urease in the coccoid form of *H. pylori* might reflect the increase in the generation of the hydroxyl radical by catalytically active forms of transition metals, such as nickels.

CHANGES IN GENOMIC DIVERSITY DURING TRANSFORMATION

DNA is also a potential target for reactive oxygen species and often shows oxidative damage

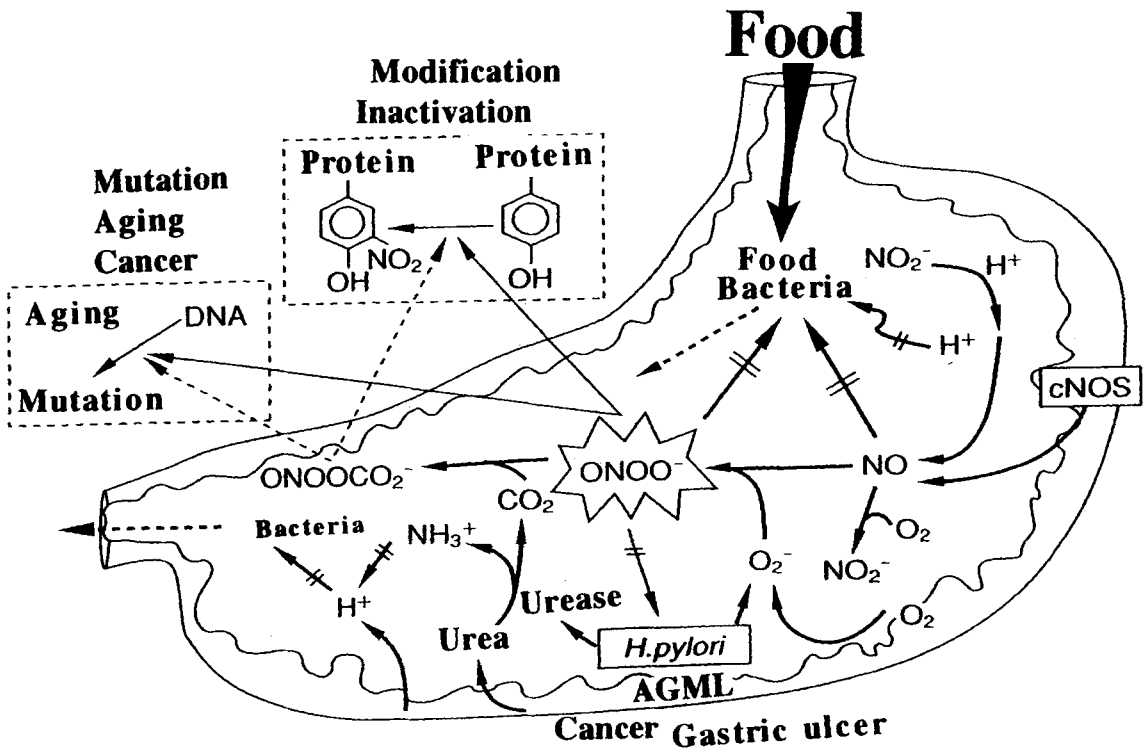

FIGURE 6 Metabolism of gastric NO and the mechanism for the survival of *H. pylori* in the stomach. *H. pylori* detoxify the bactericidal action of H^+ and NO by generating NH_4^+ and superoxide radical. The toxic effect of peroxynitrite, a reaction product of NO and superoxide, can be scavenged by carbon dioxide generated by urease. Based on such scavenging activity, *H. pylori* can survive as a successfull pathogen in gastric mucosal layer. However, reactive oxygen species generated by *H. pylori* might also serve as oxidative stress, thus resulting oxidative modification of proteins and DNA in and around cells including gastric constituent cells. Such oxidative stress might underlie the mechanism for the large genomic diversity of *H. pylori* and for the pathogenesis of gastric inflammation and cancer

and strand breakage [52–54]. It is well known that *H. pylori* shows unusually large genomic diversity [55]. Although the genomic diversity of *H. pylori* has been postulated to contribute to the success of this species as a ubiquitous pathogen, molecular mechanism for its occurrence is not known. Because peroxynitrite interacts with transition metals and forms the hazardous hydroxyl radical, it might irreversibly modify membranes, proteins and DNA. Thus, the mutagenic properties of peroxynitrite and related metabolite(s) may underlie the mechanism for increasing the genomic diversity of *H. pylori* and the etiology of gastric inflammation, ulceration and carcinogenesis. In fact, we found that the genomic DNA of *H. pylori* is affected by endogenously generated reactive oxygen species during the transformation of cells. Biochemical analysis revealed that the amount of extractable DNA from *H. pylori* decreased markedly while the amount of 8-hydroxyguanine in the cellular DNA increased progressively during the transformation from the bacillary to the intermediate to the coccoid forms [45]. The amount of frag-

mented DNA also increased during the transformation from the intermediate form to the coccoid form.

8-Hydroxyguanine is generated by oxidative modification of guanine residue [56], which results in gene mutation through A-T/G-C transversion [57]. On the basis of the marked decrease in the ability to generate O_2^- as well as the decrease in the specific activities of urease, SOD and catalase, certain components of the coccoid form of *H. pylori* appear to be oxidatively damaged in an irreversible manner. Although the amount of 8-hydroxyguanine in the intermediate form of *H. pylori* was higher than that in the bacillary form, the extent of DNA fragmentation did not differ substantially between the two forms [45]. Thus, the oxidative modification of *H. pylori* DNA by endogenously generated reactive oxygen species may be limited and not affect cellular viability during the transformation from the bacillary to the intermediate form.

To elucidate whether reactive oxygen species which are generated by *H. pylori* accelerate their transformation, we also tested the effect of high oxygen tension during cell culture. Under the standard biaerobic culture conditions, it generally requires about one week for *H. pylori* to undergo transformation from the bacillary to the coccoid form. However, about 80% of the *H. pylori* cultured under ambient atmospheric conditions underwent transformation within 24 hours. The activity of the bacillary form of *H. pylori* to generate O_2^- was significantly higher under ambient atmospheric conditions than under biaerobic conditions. Thus, oxidative stress might be one of the important factors that accelerate the rate of transformation of *H. pylori*. Consistent with previous report [58], a short-term culture of *H. pylori* under aerobic conditions markedly increased the rate of transformation as judged from the formation of protein carbonyl groups, aggregation and inactivation of urease, and the generation of 8-hydroxyguanine. Under such conditions, the heterogeneity of *H. pylori* with respect to urease activity was also increased. Thus, the generation of reactive oxygen species by *H. pylori* and the mutagenic properties of these compounds might underlie the large genomic diversity of this pathogen [59–61].

Reactive oxygen species are also toxic to most pathogens and cause genetic mutations. Because the mutation of a critical gene is unfavorable for the survival of organisms, most of them will die, presumably as the result of their inability to adapt to environmental conditions. This occurs frequently with higher organisms, such as mammals. However, if a small fraction of microorganisms underwent a mutation the phenotype of which were suitable for survival in their hazardous environment, they would rapidly grow as a successful organisms within a short duration. Because reactive oxygen species is one of the major factors that accerelates the mutation of genes, the stomach, intestinal tracts and inflammatory tissues would be the potential sites for the evolution of living organisms including bacteria and viruses (**Fig. 7**).

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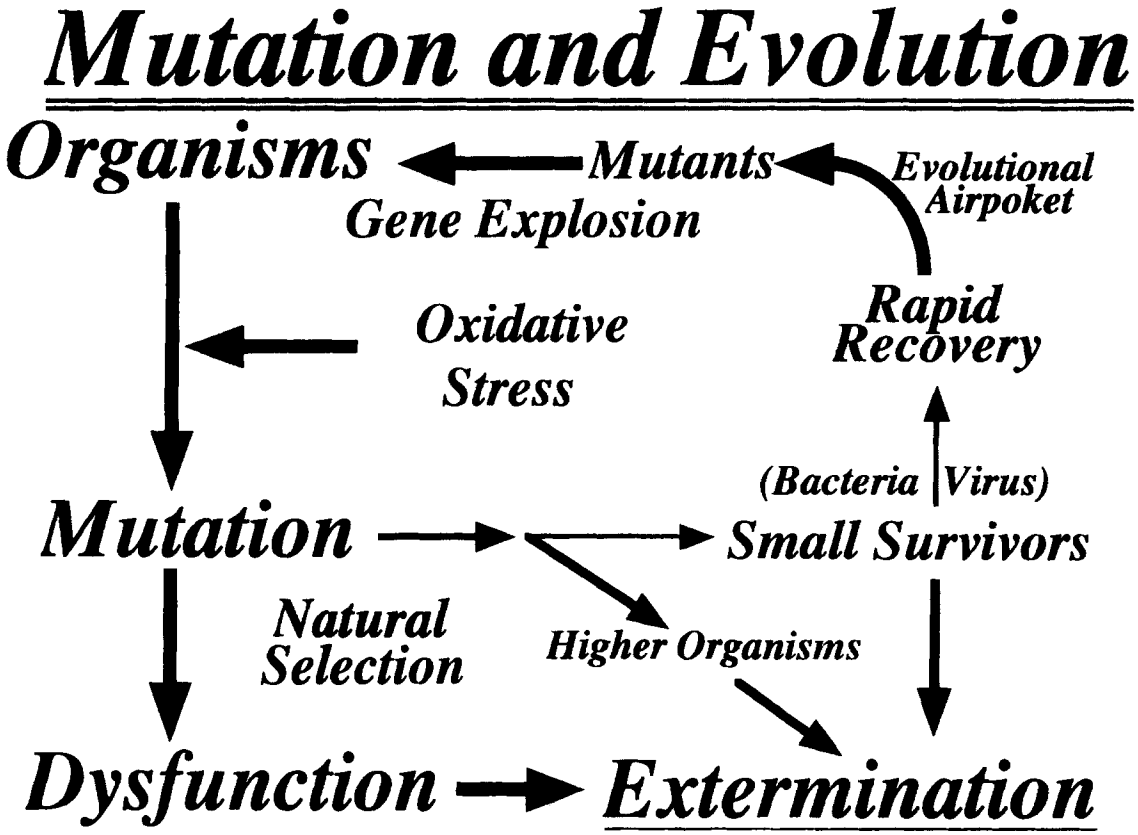

FIGURE 7 The mechanism by which oxidative stress causes mutation and enhances evolution of organisms. Reactive oxygen species and NO metabolites cause mutation of organisms. Most mutations are unfavorable for the survival of organisms, resulting in the extermination of a wide variety of species particularly under a hazardous environment. However, a small fraction of mutants, particularly those of microorganisms, may survive under hazardous conditions and become a major population. Such a mechanism, driven by oxidative stress, might operate in the process of evolution under conditions in which DNA is readily modified oxidatively. Thus, inflammation and the biaerobic conditions which are associated with oxidative stress, such as in the stomach, may serve a potential sites for facilitating the evolution of microorganisms

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