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Pronounced Enhancement of •NO-Dependent Antimicrobial Action by an •NO-Oxidizing Agent, Imidazolineoxyl *N*-Oxide

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The antimicrobial action of •NO against *Cryptococcus neoformans* was investigated by using imidazolineoxyl *N*-oxide, which we recently reported removes •NO via oxidation (T. Akaike, M. Yoshida, Y. Miyamoto, K. Sato, M. Kohno, K. Sasamoto, K. Miyazaki, S. Ueda, and H. Maeda, Biochemistry 32:827–832, 1993). No appreciable fungicidal activity was observed in neutral •NO solutions. Imidazolineoxyl *N*-oxide induced or enhanced fungicidal action in neutral or acidic •NO solutions, respectively. Our results provide convincing evidence that •NO is not a microbicidal molecular species.

Nitric oxide (•NO) has been implicated as an antitumor or antimicrobial molecule that is generated by activated macrophages (23). A number of studies have clearly demonstrated the cytostatic action of •NO on tumor cells and cryptococci through various metabolic inhibitions in the cells (3, 7, 8a, 9, 10, 15, 22). However, •NO is not as reactive and toxic as its derivatives (5, 8, 13, 14, 21, 23), and the cytocidal (cytolytic) mechanism of •NO remains obscure (8a, 16). Therefore, investigating the biological activities of •NO in more detail, focusing on its antimicrobial activity, is worthwhile. To identify the biological significance of •NO, it is essential to examine the effects of specific inhibitors of •NO and their antimicrobial actions. Until now, however, very few compounds with specific inhibitory action against •NO have been available. Only L-arginine analogs, such as N^{ω}-monomethyl-L-arginine, are widely used as inhibitors of •NO synthase to block •NO generation and analyze the effect of •NO, but L-arginine analogs do not antagonize •NO directly. In contrast, we have found that the imidazolineoxyl *N*-oxide derivative 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO) acts as an oxygen donor for •NO, forming $•NO_2$, and thus scavenges •NO (Fig. 1) (2). Therefore, it now becomes possible to investigate the role of •NO as a cytotoxic molecular species that is known to be generated by activated macrophages.

PTIO was synthesized and purified as reported recently (2). 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI) was prepared by deoxidation of PTIO with •NO and was purified by silica gel column chromatography (2). PTIO is a stable radical compound, reacts with •NO more than 10-fold faster than does molecular oxygen, and yields $•NO_2$ and PTI in a stoichiometric manner in solutions with a rate constant of $\sim 10^4\ M^{-1}\ s^{-1}$. We also demonstrated that PTIO strongly inhibited the vasorelaxation induced by endothelium-derived relaxing factor. The inhibitory potential of PTIO is comparable to that of the •NO synthesis inhibitor N^{ω}-monomethyl-L-arginine (2).

Cryptococcus neoformans (serotype A or D), originally obtained from clinical isolates, was used for the assays. The serotype A strain (TIMM1855) was a gift from K. Uchida (Research Center for Medical Mycology, Teikyo University, Tokyo, Japan), and the serotype D strain was a gift from T. Shinoda (Department of Microbiology, Meiji College of Pharmacy, Tokyo, Japan). Yeast cells were grown on Sabouraud agar plates, harvested in log-phase growth, and washed with 0.45% saline three times before use. Cells were then added to the reaction mixtures at 10^6 CFU/ml (final concentration).

Washed cells of *C. neoformans* serotype D were added to the reaction mixtures, which contained 1 mM $NaNO_2$ in 80 mM succinate buffer (pH 4.0) and generated •NO chemically (3, 20), in the presence or absence of PTIO (Fig. 2). The number of viable cells in each reaction mixture was then quantitated by colony formation on the Sabouraud agar plates after incubation for indicated periods at room temperature. $NaNO_2$, succinate buffer, and PTIO did not exhibit any fungicidal action at the concentrations used. The •NO-generating mixture showed fungicidal action (open circles). PTIO enhanced the cytocidal action in a dose-dependent manner: in the presence of 200 μM PTIO (closed circles), a 10^5-fold increase in cytocidal activity was observed during a 3-h incubation period.

An •NO-saturated solution was prepared by bubbling •NO gas (99% purity; Nippon Sanso Co., Ltd., Tokyo, Japan) into pure water, which had been degassed and bubbled with helium gas. The content of •NO was quantitated by electron spin resonance spectroscopy as described recently (2). An aliquot of •NO-saturated aqueous solution was added to the suspension of *C. neoformans* in 200 mM sodium phosphate buffer (pH 7.0) or in 200 mM succinate buffer (pH 4.0). •NO (380 μM) without PTIO did not kill *C. neoformans* within a 24-h incubation period at pH 7.0. At pH 7.0, in the presence of PTIO, •NO (130 μM) did not induce fungicidal action within 8 min (Fig. 3), but after 3 h of incubation with 130 μM •NO and 200 μM PTIO, 92% of the cells were killed (data not shown). The viability of *C. neoformans* was not affected by either PTI (200 μM) or PTIO (500 μM) alone. At pH 4.0, •NO (130 μM) without PTIO did not induce cytotoxicity against *C. neoformans* within 8 min (Fig. 3), but after 3 h of incubation, 99% of the cells were killed (data not shown). In contrast, as demonstrated in Fig. 3, PTIO significantly enhanced fungicidal action under acidic conditions. Under these cytotoxic conditions generated by •NO plus PTIO, almost all •NO was converted to $•NO_2$, as judged by electron

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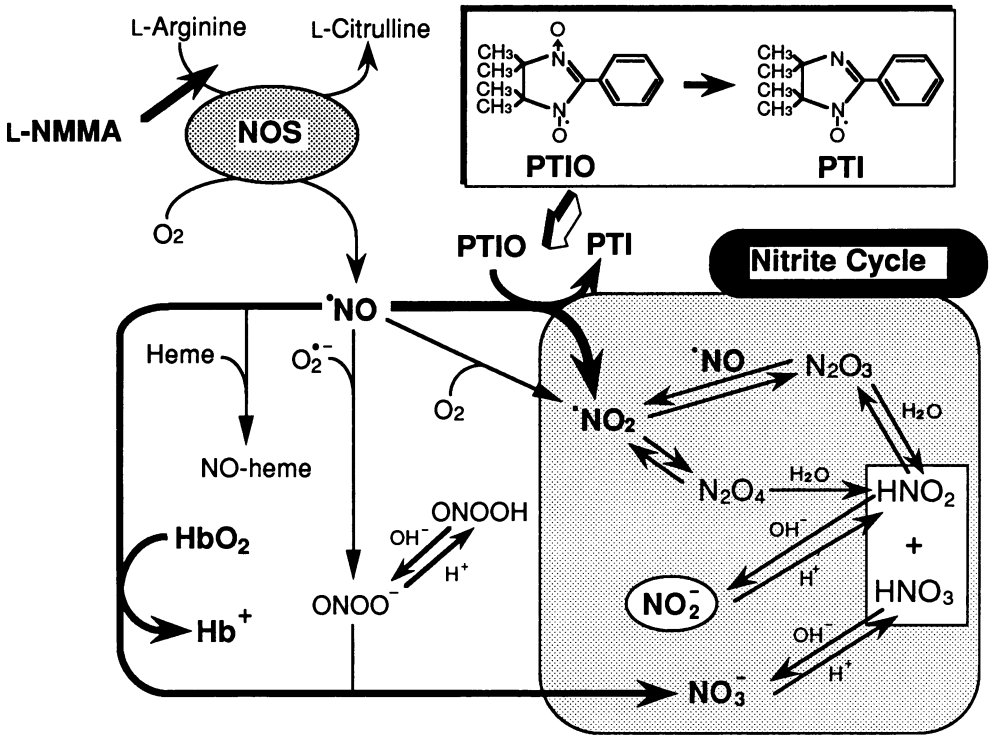

FIG. 1. L-Arginine–nitrite–nitrate flow (11) and the use of PTIO to determine the biological significance of ·NO. Until now, it has been impossible to distinguish the biological significance of ·NO from that of its reactive derivatives by conventional reagents or methods (e.g., the nitric oxide synthase [NOS] inhibitor N^{ω}-monomethyl-L-arginine [L-NMMA], the ·NO scavenger HbO_2 or its photometric assay, and measurements of nitrate and nitrite or L-citrulline). However, we did so by using PTIO. The pH-dependent nitrite cycle (20) was modified according to recent reports (5, 8, 13, 14, 17, 19).

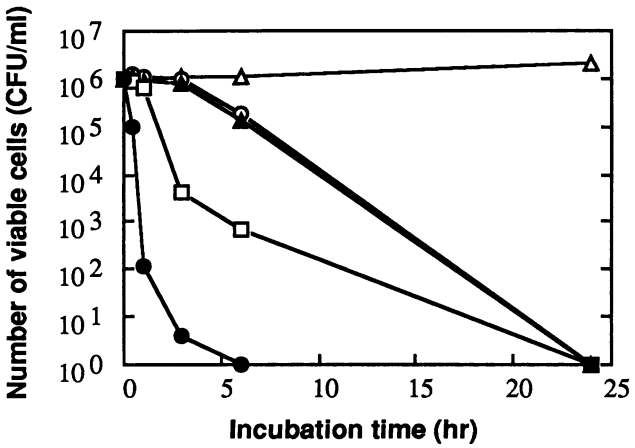

FIG. 2. Fungicidal action against *C. neoformans* of a system chemically generating ·NO. *C. neoformans*, serotype D, was added to either the reaction mixture containing 1 mM $NaNO_2$ in 80 mM succinate buffer (pH 4.0) with various concentrations of PTIO or to the succinate buffer alone. The number of viable cells after the indicated incubation periods was measured by the colony-forming assay. Each datum point represents the mean value for three experiments. Symbols: ○, 0 μM PTIO; ▲, 2 μM PTIO; □, 20 μM PTIO; ●, 200 μM PTIO; △, succinate buffer only.

spin resonance spectroscopy by our recently reported method (2) (data not shown).

The cytotoxic effects of various concentrations of ·NO and PTIO were examined with different yeast strains at pH 4.0 (Fig. 4). Dose-dependent fungicidal action was observed with both ·NO and PTIO.

The effects of ·NO and PTIO on the viability of *Staphylococcus aureus* (ATCC 25923) were also examined, and a strong enhancement of ·NO-dependent cytotoxicity similar to the enhancement of fungicidal action of PTIO (data not shown) was observed.

We compared the actions of PTIO with those of oxyhemoglobin (HbO_2), which entraps and oxidizes ·NO as follows: $\cdot NO + HbO_2 \rightarrow Hb^+ + NO_3^-$ (11). HbO_2 was purified from healthy human erythrocytes as described previously (1). ·NO (130 μM) killed 99% of the cells after incubation for 3 h at pH 4.0, and HbO_2 (100 or 300 μM) reduced the fungicidal action to 89 or 26%, respectively. These results can be interpreted as HbO_2 converting ·NO to the stable compound, nitrate, thus bypassing the nitrite cycle, so that the fungicidal effects of toxic nitrogen oxide derivatives (Fig. 1) are no longer exerted.

The antimicrobial effect of the ·NO-generating system has been known for years (3, 4, 6, 20). The mechanisms for this effect have been studied extensively, originally because of the worldwide use of nitrite in meat curing and now as a model of antimicrobial action of reactive nitrogen oxides within activated macrophages (3, 6). A pH-dependent nitrite cycle had been proposed, and the reactive nitrogen species responsible for antimicrobial action was suggested to be ·NO, $\cdot NO_2$, N_2O_3, N_2O_4, or HNO_2 (Fig. 1) (4, 6, 17, 20).

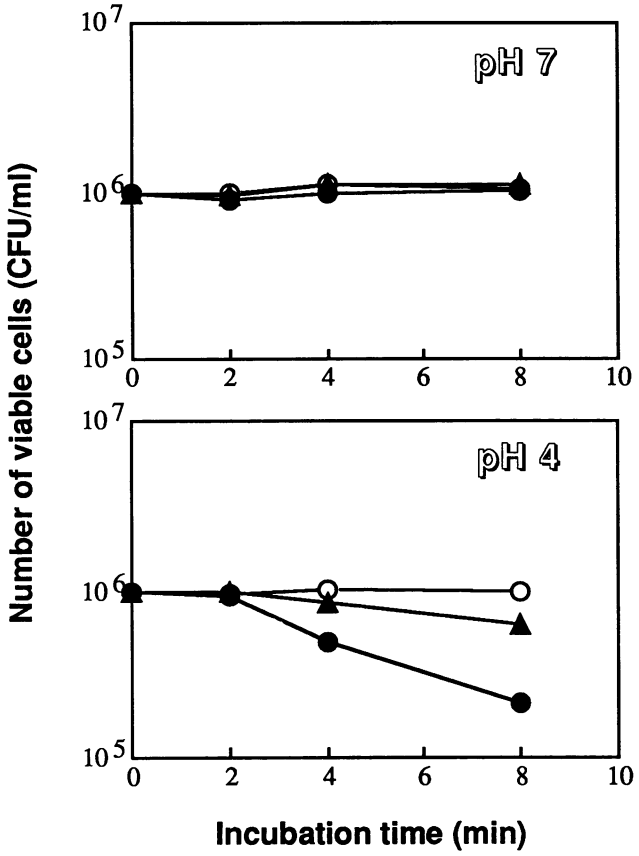

FIG. 3. Fungicidal action of authentic •NO against *C. neoformans* in buffer (pH 7.0 or 4.0). •NO-saturated solution was added to the suspension of *C. neoformans* in 200 mM sodium phosphate buffer (pH 7.0) or in 200 mM succinate buffer (pH 4.0) to yield 130 μM •NO. The number of viable cells was measured by the colony-forming assay after the indicated incubation periods at room temperature. Symbols: ○, 0 μM PTIO; ▲, 200 μM PTIO; ●, 500 μM PTIO. Each value shown represents the mean value for two experiments.

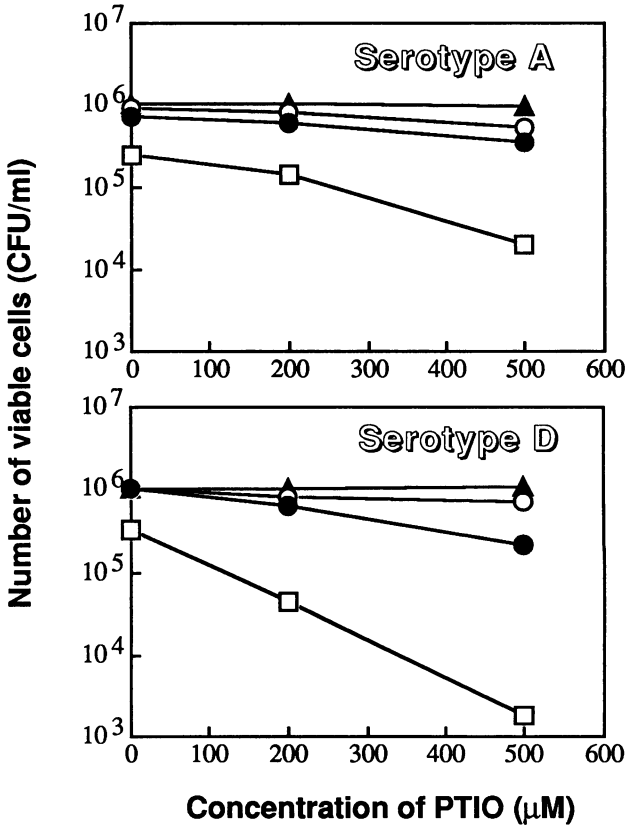

FIG. 4. Dose-dependent fungicidal action of PTIO and •NO. *C. neoformans*, serotype A or D, was incubated in 200 mM succinate buffer (pH 4.0) at room temperature with various concentrations of PTIO and/or •NO, and the number of viable cells was measured by the colony-forming assay after 8-min incubation periods at room temperature. Each value shown represents the mean values for two experiments. Symbols: ▲, 0 μM •NO; ○, 60 μM •NO; ●, 120 μM •NO; □, 240 μM •NO.

Recently, Stamler et al. (21) proposed the possible involvement of various redox-activated forms of nitrogen monoxides such as nitrosonium cation (NO^+) and nitroxyl anion (NO^-) in a number of pathophysiological phenomena induced by •NO. N_2O_3 and N_2O_4 are known to possess potent chemical reactivities equivalent to that of NO^+, which confers nitration of various nucleophiles (21). However, a good tool for distinguishing the biological reactivity of each nitrogen oxide involved in this cycle has not been available (4–6, 17, 20).

In this study, we used PTIO to identify the antimicrobial action of •NO, and our results provide convincing evidence that (i) •NO is not a fungicidal molecule and (ii) the •NO-dependent fungicidal action was enhanced by PTIO. Therefore, it had been suggested that one or more of the nitrogen oxides (such as $•NO_2$, N_2O_4, HNO_2, and N_2O_3) generated in the nitrite cycle would be cytocidal against various microbes, whereas we have demonstrated unequivocally that •NO has little cytocidal potency (Fig. 1). This conclusion is consistent with the observations of other researchers who have studied the cytotoxic activities of •NO for mammalian cells and cryptococci (3, 8–10, 20–22).

Previously, Kosaka et al. (13, 14) reported that mutagenic action of NO_2, N_2O_4, and N_2O_3 against both *Salmonella typhimurium* and *Escherichia coli* was more potent than that of •NO; •NO had only negligible action against these bacteria. Although mutagenesis may not necessarily be related to direct cytocidal action of nitrogen oxides, these observations seem to be consistent with our finding that $•NO_2$, N_2O_4, and N_2O_3 would be the principal cytotoxic agents rather than •NO. Recently, Rockett et al. (19) reported that a saturated solution of •NO was not cytotoxic against *Plasmodium falciparum*. This finding also seems to be compatible with our results, except that they did not test the toxic effects of $•NO_2$, N_2O_4, and/or N_2O_3.

The cytotoxic potential of peroxynitrite ($ONOO^-$), which is formed with •NO and $O_2^{\cdot-}$, has been another current focus in research on •NO-dependent bactericidal action (12, 18, 23). However, as revealed in this study, it has become apparent that strong fungicidal activity could be exhibited through the •NO oxidation process without the involvement of $ONOO^-$ generation. Therefore, it is crucially important to clarify the involvement of toxic nitrogen oxides ($•NO_2$, N_2O_4, N_2O_3, and HNO_2) other than •NO and $ONOO^-$ (ONOOH) in the antimicrobial activity of activated macrophages.

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