Antagonistic Action of Imidazolineoxyl *N*-Oxides against Endothelium-Derived Relaxing Factor/•NO through a Radical Reaction†

Takaaki Akaike,‡ Masaki Yoshida,§ Yoichi Miyamoto,‡ Keizo Sato,‡ Masahiro Kohno,∥ Kazumi Sasamoto,⊥ Kiminori Miyazaki,⊥ Shoichi Ueda,§ and Hiroshi Maeda*,‡

Departments of Microbiology and Urology, Kumamoto University School of Medicine, Kumamoto 860, Japan, ESR Application Laboratory, Analytical Instruments Division, JEOL, Akishima 196, Japan, and Dojindo Laboratories, Kumamoto 861-22, Japan

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ABSTRACT: A labile inorganic free radical, nitric oxide (•NO), is produced by nitric oxide synthase from the substrate L-arginine in various cells and tissues. It acts as an endothelium-derived relaxing factor (EDRF) or as a neurotransmitter in vivo. We investigated the reactivity of stable radical compounds, imidazolineoxyl *N*-oxides such as 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), carboxy-PTIO, and carboxymethoxy-PTIO against •NO/EDRF in both chemical and biological systems. By using electron spin resonance (ESR) spectroscopy, imidazolineoxyl *N*-oxides were found to react with •NO in a stoichiometric manner (PTIO/•NO = 1.0) in a neutral solution (sodium phosphate buffer, pH 7.4) with rate constants of $\sim 10^4$ M^{-1} s^{-1}, resulting in the generation of NO_2^-/NO_3^- and imidazolineoxyls such as 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI), carboxy-PTI, or carboxymethoxy-PTI. Furthermore, the effects of imidazolineoxyl *N*-oxides on acetylcholine- or ATP-induced relaxation of the smooth muscle of rabbit aorta were tested. The vasorelaxations were inhibited by all three imidazolineoxyl *N*-oxides markedly. The inhibitory effects of carboxy-PTIO was almost 2-fold stronger than those of •NO synthesis inhibitors, N^ω-nitro-L-arginine and N^ω-monomethyl-L-arginine. Generation of EDRF/•NO was identified by reacting the PTIO in aortic strips and quantitating the reaction product with ESR spectroscopy. Thus, it was clarified that imidazolineoxyl *N*-oxide antagonize EDRF/•NO via a unique radical–radical reaction with •NO.

Nitric oxide (•NO)[1] is a labile free radical and is produced by nitric oxide synthase (•NO synthase) from the substrate L-arginine in various cells and tissues. It acts as an endothelium-derived relaxing factor (EDRF) (Furchgott, 1988; Ignarro et al., 1987; Palmer et al., 1987) or as a neurotransmitter in vivo (Shibuki & Okada, 1991; Bredt et al., 1991; Schuman & Madison, 1991). •NO is a simple, small, hydrophobic molecule and possesses one extra electron, which renders it membrane permeable and chemically reactive. A major molecular event in the signal transduction by •NO seems to be activation of guanylate cyclase by formation of an •NO–heme iron complex, thus resulting in enhanced production of cyclic GMP, which is linked with a series of physiological phenomena (Craven & DeRubertis, 1978; Ignarro, 1991).

Until recently, few substances were known to interact directly with •NO and effectively antagonize its physiological action except for a few endogenous compounds, e.g., iron-(heme-) containing proteins such as hemoglobin (Drapier et al., 1991; Corbett et al., 1991; Kosaka et al., 1992) and $O_2^{\bullet-}$ (Blough & Zafiriou, 1985; Gryglewski et al., 1986; Saran et al., 1990). To detail the pathophysiological significance of •NO, it seems critically important to identify a specific reagent to neutralize •NO directly in biological systems. We investigated the mechanism of the reaction of EDRF/•NO with imidazolineoxyl *N*-oxide derivatives by using various procedures, including electron spin resonance (ESR) spectroscopy. A potent antagonistic action of these imidazolineoxyl *N*-oxides against EDRF/•NO through a unique radical reaction is demonstrated in this paper.

EXPERIMENTAL PROCEDURES

Synthesis of Imidazolineoxyl N-Oxide Derivatives. 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (Figure 1A, **2a**) was synthesized as reported previously (Osiecki & Ullman, 1968). 1,3-Dihydroxy-4,4,5,5-tetramethyl-2-(4-carboxyphenyl)tetrahydroimidazole (Figure 1A, **1b**) was synthesized as follows: 2,3-bis(hydroxyamino)-2,3-dimethylbutyl sulfate in water was neutralized at 0 °C with aqueous $KHCO_3$, followed by the addition of 4-formylbenzoic acid. The mixture was stirred overnight at 22 °C, and precipitates were collected and dried in vacuo to give **1b**: ^{1}H NMR (DMSO-d_6) δ 7.73 (dd, 4 H, Ar-H), 4.57 (s, 1 H, CH), 4.10 (br s, 2 H, NOH), 1.05 (s, 6 H, CH_3), 1.09 (s, 6 H, CH_3); FAB-MS, m/z 279 $(M - H)^-$. The potassium salt of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) (Figure 1A, **2b**) was synthesized as follows: to **1b** in *N*,*N*-dimethylformamide was added PbO_2. After stirring for 3 h at 22 °C, PbO_2 was filtered off and the filtrate was concentrated to give a residue that was dissolved in water.

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* Address correspondence to this author.

‡ Department of Microbiology, Kumamoto University School of Medicine.

§ Department of Urology, Kumamoto University School of Medicine.

∥ Analytical Instruments Division, JEOL.

⊥ Dojindo Laboratories.

[1] Abbreviations: EDRF, endothelium-derived relaxing factor; •NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion radical; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; PTI, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; carboxymethoxy-PTIO, 2-[4-(carboxymethoxy)phenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; NNA, N^ω-nitro-L-arginine; NMMA, N^ω-monomethyl-L-arginine; PE, phenylephrine; Tempol, 2,2,6,6-tetramethyl-4-hydroxypiperidine; SOD, superoxide dismutase; ESR, electron spin resonance; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment mass spectroscopy; IR, infrared.

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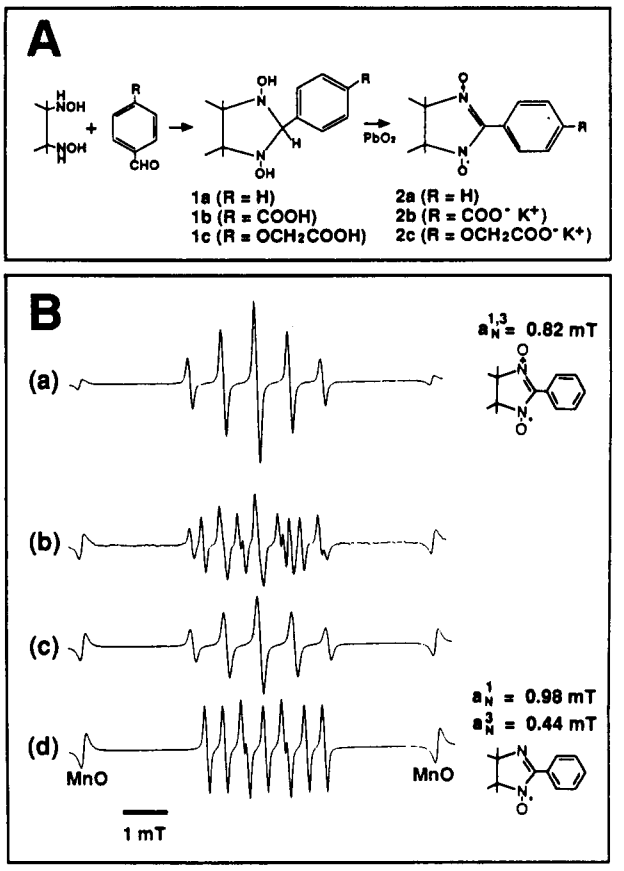

FIGURE 1: (A) Schematic diagram of synthesis for three imidazolineoxyl *N*-oxide derivatives. See text for details. (B) ESR spectra of PTIO and those obtained for the reaction mixture of PTIO plus •NO. (a) An experimental spectrum of PTIO. A well-resolved and very stable ESR spectrum of PTIO was obtained in 0.25 M sodium phosphate buffer (pH 7.4) at 25 °C. (b) An experimental spectrum of the reaction mixture of •NO and PTIO. •NO-saturated aqueous solution (20 μL) was added to a 30 μM PTIO solution (180 μL) in 0.25 M sodium phosphate buffer, pH 7.4. Three minutes after addition of the •NO solution, the ESR spectrum was recorded at room temperature. Just two components were identified in the experimental spectrum (b) by computer simulation, as resolved in (c) and (d). The simulated ESR signal (c) is identical with the signal in (a) (PTIO); the simulated signal (d) was identified as purified phenyltetramethylimidazoline-*N*-oxyl (PTI). See text for details of ESR measurement.

After the pH was adjusted to 8.0, the solution was lyophilized to give carboxy-PTIO: IR (KBr disk) 1360 cm^{-1} (N–O); FAB-MS, m/z 276 $(M - H)^-$. The potassium salt of 2-[4-(carboxymethoxy)phenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxymethoxy-PTIO) (Figure 1A, **2c**) was prepared from 4-formylphenoxyacetic acid in a manner similar to that for carboxy-PTIO: IR (KBr disk) 1360 cm^{-1} (N–O); FAB-MS, m/z 306 $(M - H)^-$. PTIO is lipophilic, while both carboxy- and carboxymethoxy-PTIO were less lipophilic and were water soluble, with solubilities more than 100-fold that of PTIO. These three imidazolineoxyl *N*-oxides, however, showed similar reactivity with •NO.

Other Reagents. N^{ω}-Nitro-L-arginine methyl ester (NNA), phenylephrine, acetylcholine chloride, ATP, indomethacin, and 2,2,6,6-tetramethyl-4-hydroxypiperidine (Tempol) were purchased from Sigma Chemical Industry Co., St. Louis, MO. N^{ω}-Monomethyl-L-arginine (NMMA) was obtained from Calbiochem, La Jolla, CA. Human recombinant Cu,Zn-superoxide dismutase (SOD) was a kind gift from Nippon Kayaku Co., Ltd., Tokyo, Japan. Hemoglobin was isolated and purified from human red blood cells as described recently (Akaike et al., 1992). Thin-layer chromatography (TLC) plates (silica gel 60) were purchased from Merck, Darmstadt, Germany. •NO gas with 99% purity was obtained from Nippon Sanso Co., Ltd., Japan. To prepare the •NO-saturated solution, •NO gas (99% purity) was bubbled (500 mL/min for 3 min) into pure water, which had been evacuated and flushed with helium gas. All other reagents were of the highest analytical grade commercially available.

ESR Spectroscopy. The reaction of PTIO with •NO in solution was examined by using ESR spectroscopy (JES-RE1X, JEOL, Tokyo, Japan). The ESR signal of imidazolineoxyl *N*-oxides in the reaction with •NO was measured with a quartz flat cell (inner size, 60 × 10 × 0.31 mm) at 25 °C. The reaction of PTIO with •NO was initiated by adding •NO-saturated aqueous solution to imidazolineoxyl *N*-oxide in 0.25 M sodium phosphate buffer (pH 7.4) at 25 °C.

Conditions for ESR measurements were as follows: modulation frequency, 100 kHz; modulation amplitude, 0.05 mT; scanning field, 336.0 ± 5 mT; response time, 0.03 s; sweep time, 1 min; microwave power, 5 mW; microwave frequency, 9.421 GHz. Absolute concentrations of these imidazoline radicals were determined by double integration of the ESR spectra after normalization against the standard signal intensity of manganese oxide, in which a 1.0 μM solution of Tempol was used as a primary standard for the ESR spectra (Akaike et al., 1992).

Kinetic Analysis of the Reaction of •NO and PTIO by the Stopped-Flow Method in Neutral Solution. Various concentrations of •NO solution (1 mL) and an equal volume of 1 mM PTIO in 0.25 M sodium phosphate buffer solution (pH 7.4) were injected rapidly into a quartz flat cell in an ESR cavity, and the peak height of the ESR signal of PTIO was recorded continuously. The amount of PTIO was quantitated by double integration of each spectrum. From the reaction velocity during the initial 1-s reaction period, a rate constant was determined.

Bioassay System for •NO/EDRF Generation. Female New Zealand White rabbits, weighing 2.5–3 kg, were anesthetized with sodium pentobarbitone and exanguinated. The chest was opened for removal of the thoracic aorta. After excess fat and connective tissue were removed, the aorta was cut into rings 5 mm wide. The rings were mounted vertically in 20-mL organ baths filled with Krebs solution, and isometric tension development was recorded with an ink-writing recorder. The medium was maintained at 37 °C and bubbled with a 95% O_2/5% CO_2 gas mixture. Tissues were precontracted with 0.15 μM phenylephrine, after which acetylcholine- or ATP-induced relaxation was measured, as were the effects of a series of imidazolineoxyl *N*-oxides and imidazoline-*N*-oxyl derivatives, NNA, and NMMA on this smooth muscle relaxation induced by acetylcholine or ATP. The vascular tone of aortic rings was studied in the presence of 3.0 μM indomethacin after phenylephrine-induced contraction of the smooth muscle.

Quantitation of Oxygen Concentration. After addition of various amounts of •NO (0.5 mL of pure water) to 1.5 mL of phosphate buffer solution (0.25 M sodium phosphate buffer, pH 7.4) in the presence or absence of imidazolineoxyl *N*-oxides, oxygen concentration consumed was measured by using a Clark-type electrode (biological oxygen monitor, Rank Brothers, Cambridge, England) at 25 °C. An equilibrium concentration of O_2 was assumed to be 253 μM for the air-saturated solution at 25 °C.

TLC of PTIO and a Reaction Product of PTIO/•NO. Various amounts of •NO-saturated solution were added to the 0.5 mM PTIO in 0.25 M sodium phosphate buffer (pH

7.4). Each aliquot (40 μL) of the reaction mixture was applied to the TLC plate; it was developed with n-hexane/chloroform/methanol (10:5:1, v/v). The spot of PTIO and its reaction product were easily detected under UV radiation, and the homogeneity of each compound was confirmed.

Identification and Quantitation of a Reaction Product of PTIO with EDRF/•NO Generated in Aortic Rings. Aortic specimens 5 cm long from rabbits were cut into pieces of rings 5 mm wide and were incubated with 500 μM PTIO and Cu,-Zn-SOD (500 units/mL) in the presence or absence of 10 μM acetylcholine or 1 mM NMMA in Krebs solution (1.5 mL) for 30 min at 37 °C. During this reaction, some PTIO and PTI may possibly be incorporated into the cell membrane of the aorta due to their lipophilicity, as described above. Therefore, 6 mL of ethanol was added to 1.5 mL of the reaction mixture after incubation of the aortic rings, followed by extraction for 1 min at room temperature, in order to quantitate the amount of PTI formed in the vascular tissue accurately. After the aortic rings were removed, the reaction solution [80% (v/v) solution] was then centrifuged at 1500*g* for 20 min at 4 °C to remove the tissue debris. The resulting supernatant was concentrated to 1.0 mL in vacuo at 45 °C for a few minutes in the presence of potassium ferricyanide (1 mM) to nullify the nonspecific reduction of PTIO. A reaction product of PTIO with EDRF/•NO in the solution was isolated on the TLC plate as just described and was subjected to quantitation by ESR spectroscopy.

RESULTS

Three types of imidazolineoxyl N-oxides, phenyltetramethylimidazolineoxyl N-oxide (PTIO), carboxy-PTIO, and carboxymethoxy-PTIO, were synthesized (Figure 1A), and their reactions with •NO were examined. Figure 1B shows the ESR spectra of PTIO and that of the reaction mixture of PTIO and •NO. The computer simulation of the experimental spectrum (a) showed the hyperfine splitting constant (hfc) for PTIO, $a_N^{1,3}$ = 0.82 mT, which is almost consistent with that reported previously (Ullman et al., 1972). Carboxy-PTIO and carboxymethoxy-PTIO gave ESR signals similar to that of PTIO: $a_N^{1,3}$ = 0.81 mT for carboxy-PTIO and $a_N^{1,3}$ = 0.82 mT for carboxymethoxy PTIO (spectra not shown).

The spectrum obtained for the reaction mixture of PTIO and •NO [Figure 1B (b)] was found by computer simulation to be composed of just two components, one of which was identical with that of PTIO, as shown in (c). Furthermore, the other signal [Figure 1B (d)], with an hfc of a_N^1 = 0.98 mT and a_N^3 = 0.44 mT, was revealed to originate from an imidazoline-N-oxyl compound, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI), by using TLC, as shown in Figure 2A, and by using NMR spectroscopy and FAB-MS. Namely, a reaction product of PTIO was extracted with chloroform. The organic layer was washed with water, concentrated in vacuo, and chromatographed on silica gel with n-hexane/chloroform/methanol (10:5:1, v/v). A reddish syrupy product, which showed a single spot on TLC, was isolated and identified as PTI; ^{1}H NMR ($CDCl_3$), δ 1.09 (d, 4-CH_3); FAB-MS, *m/z* 218 (M + H)$^+$. No reaction product other than PTI was identified as derived from PTIO, as described below in detail.

The decrease in PTIO corresponds to that of PTI generated in the reaction of PTIO with •NO (Figure 2B), in which the amount of PTIO plus PTI, quantitated by ESR, remained unity. This result clearly showed stoichiometric conversion of PTIO to PTI. Both carboxy-PTIO and carboxymethoxy-PTIO also showed the same reactivity with •NO as did PTIO (Figure 2C). The amount of oxygen consumed was exactly

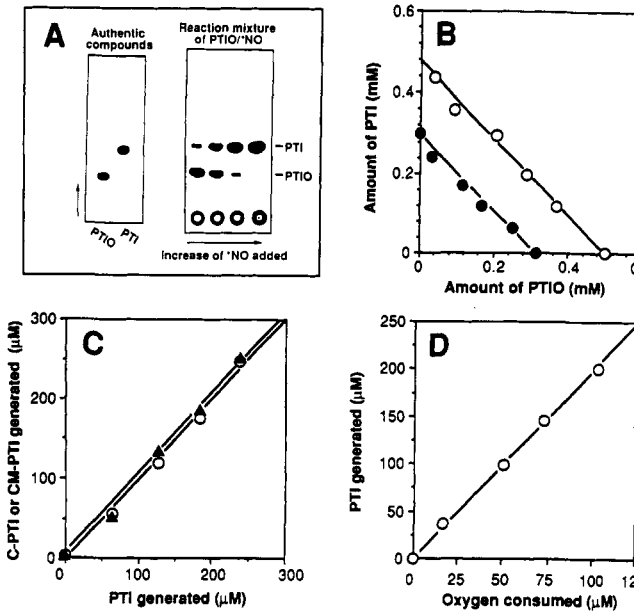

FIGURE 2: (A) TLC of PTIO and PTI and of the reaction mixture of •NO and PTIO. Various amounts of •NO-saturated solution were added to PTIO (0.5 mM) in 0.25 M sodium phosphate buffer, pH 7.4. Each aliquot (40 μL) of the mixture was analyzed by TLC. (B) Correlation between the amount of PTIO and the amount of PTI generated in the reaction of PTIO and •NO. The reaction of PTIO and •NO was performed in the same manner as in (A). Imidazoline radicals in the original reaction mixture (○) were quantitated by ESR spectroscopy. After isolation and extraction of either PTIO or PTI from the reaction mixture by TLC, each imidazoline radical was also quantitated by ESR spectroscopy (●). (C) The amounts of the reaction products, i.e., imidazoline-N-oxyls such as PTI, carboxy-PTI (C-PTI), and carboxymethoxy-PTI (CM-PTI), in the reaction of PTIO, carboxy-PTIO (C-PTIO) (○) or carboxymethoxy-PTIO (CM-PTIO) (▲) with •NO were quite similar. The reaction of PTIOs and •NO was performed in the same manner as in (A). The amount of imidazoline-N-oxyls generated was quantitated by ESR. (D) Correlation between oxygen consumption and PTI generation in the reaction of PTIO with •NO. After addition of various amounts of •NO to 0.25 M sodium phosphate buffer (pH 7.4) with or without PTIO, oxygen consumption was measured with a Clark-type oxygen electrode. The amount of PTI generated in the system was quantitated by ESR.

50 mol % of PTI generated (Figure 2D). It is known that •NO reacts with molecular oxygen dissolved in solution as follows:

$$2\,{}^{\bullet}NO + O_2 \rightarrow 2\,{}^{\bullet}NO_2 \qquad (1)$$

PTIOs would react similarly with •NO, by second-order kinetics:

$$^{\bullet}NO + PTIO \rightarrow {}^{\bullet}NO_2 + PTI \qquad (2)$$

These results indicate that PTIOs mediate conversion of •NO to $^{\bullet}NO_2$ via a radical–radical reaction in a completely stoichiometric manner, followed by generation of nitrite and nitrate by the reaction of $^{\bullet}NO_2$ ($2N_2O_4$) and H_2O as follows:

$$2\,{}^{\bullet}NO_2 \rightleftarrows N_2O_4 \xrightarrow{H_2O} NO_2^- + NO_3^- + 2H^+ \qquad (3)$$

Reactions subsequent to eq 2, i.e., eq 3, were confirmed by detection and quantitation of NO_2^- and NO_3^- in this system by high-pressure liquid chromatography according to the method of Okada (1988). The ESR spectra and signal intensities of PTIOs were not affected at all by mixing $^{\bullet}NO_2$ gas with PTIOs in water (pH 7.4), even in the presence of an excess of $^{\bullet}NO_2$ to PTIOs (up to 10 M excess) (data not shown). This indicates that there is no involvement of $^{\bullet}NO_2$ in the reaction of PTIOs with •NO.

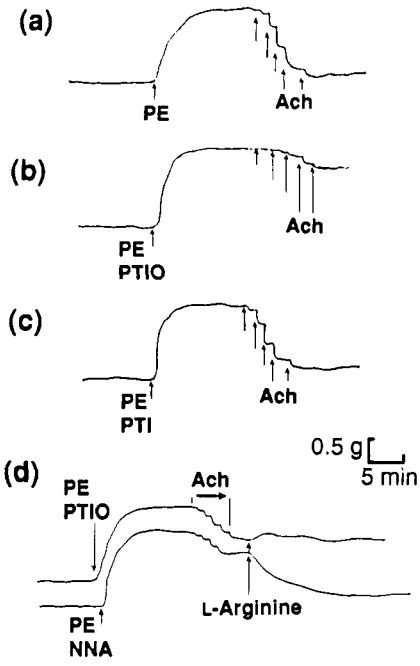

FIGURE 3: Effects of imidazoline radicals on acetylcholine-induced vasorelaxation of rabbit thoracic aorta rings. (a) Control, without imidazoline compounds. (b and c) Effect of PTIO (300 μM) or PTI (300 μM) on vasorelaxation, respectively. (d) Effect of L-arginine (5 mM) on inhibition by PTIO (100 μM) (upper tracing) or NNA (100 μM) (lower tracing) of acetylcholine- (Ach-) induced vasorelaxation. Tissues were precontracted with 0.15 μM phenylephrine (PE), after which acetylcholine was added serially at concentrations of 0.01, 0.03, 0.1, 0.3, and 1.0 μM as shown by arrows in (a), (c), and (d). In (b), vasorelaxation was induced in the same manner as in (a), (c), and (d), except that acetylcholine was administered serially at 0.01, 0.1, 0.3, and 3.0 μM as shown by arrows. See text for details.

The reaction rates of PTIOs and •NO were measured by using ESR spectroscopy. From the velocity obtained during the initial 1-s reaction period in the reaction systems of PTIOs/•NO, the rate constants were determined to be 5.15×10^3 M^{-1} s^{-1} for PTIO/•NO, 5.27×10^3 M^{-1} s^{-1} for carboxymethoxy-PTIO/•NO, and 1.01×10^4 M^{-1} s^{-1} for carboxy-PTIO/•NO.

We further investigated whether PTIOs can react with •NO and inhibit the biological function of •NO as an EDRF of aortic smooth muscle from rabbits. Stimulation by acetylcholine or ATP is known to mediate generation of EDRF, i.e., •NO, during this vasorelaxation. All PTIOs inhibited this •NO-related vasorelaxation induced by acetylcholine in a dose-dependent manner (Figures 3 and 4). Similarly, vasorelaxation induced with ATP was also markedly inhibited by all PTIOs (data not shown). In contrast, virtually no inhibitory action was observed against vasorelaxation by PTI, a reaction product of PTIO and •NO (Figures 3 and 4). The inhibition of smooth muscle relaxation by PTIOs was not abolished by the addition of L-arginine, the precursor of •NO biosynthesis, whereas L-arginine significantly suppressed the inhibition of vasorelaxation by NNA (Figure 3). This result indicates that the inhibition of vascular relaxation by PTIOs was not due to direct inhibition of •NO synthase. More specifically, L-arginine analogues such as NNA and NMMA are competitive inhibitors of •NO synthase and thus L-arginine can reverse these inhibitory effects (Kilbourn et al., 1992). In contrast, PTIO seems to react directly with EDRF/•NO generated by •NO synthase in the endothelium. EDRF-dependent relaxation of the smooth muscle could be reinduced when acetylcholine was administered again to aortic tissues that had been treated with imidazoline compounds for up to 1 h after stimulation by acetylcholine or ATP, followed by

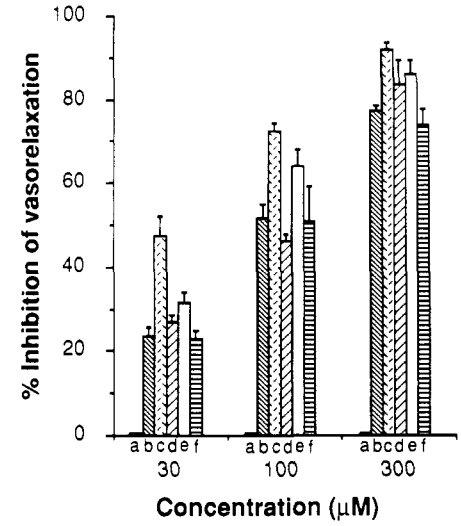

FIGURE 4: Effects of concentrations of various compounds on vascular relaxation of rabbit thoracic aorta. Bioassay conditions were the same as those in Figure 3, and the inhibitory activities of various compounds against acetylcholine- (3.0 μM) induced vasorelaxation were determined: a, PTI; b, PTIO; c, carboxy-PTIO; d, carboxymethoxy-PTIO; e, NNA; f, NMMA. Data are shown as means ± SE (n = 3–4). See text for details in the experiments.

washing out of these reagents. Thus, it is suggested that the inhibitory effect of imidazolineoxyl *N*-oxide radicals on vasorelaxation did not result from cytotoxicity against endothelial cells of the aorta.

We compared the inhibitory potential of the analogues of L-arginine with that of PTIOs against EDRF-dependent muscle relaxation. As shown in Figure 4, the inhibitory action of PTIOs was similar to or greater than that of NNA or NMMA. Among three PTIOs tested, carboxy-PTIO was the most potent inhibitor and showed ~2-fold greater EDRF inhibitory activity than NNA after standardization of the activity vs concentration (Figure 4). This higher inhibitory potency of carboxy-PTIO agrees with its higher rate constant.

The generation of •NO by acetylcholine-induced stimulation of aorta and reaction of PTIO with •NO were substantiated by identification of a product of the reaction of PTIO with •NO in aortic smooth muscle specimens. More specifically, we demonstrated the generation of PTI during acetylcholine-induced relaxation of the smooth muscle, which was strongly suppressed in the presence of NMMA, by using TLC combined with ESR (Figure 5). This result indicates that conversion of PTIO to PTI depends mostly on EDRF/•NO biosynthesis in the endothelium.

DISCUSSION

In this experiment, we elucidated a novel and potent inhibitory action of imidazolineoxyl *N*-oxide derivatives (PTIOs) against EDRF/•NO via a radical–radical reaction, as shown in eq 2. A possible reaction of PTIO and •NO was preliminarily described by Ullman et al. (1970). Furthermore, PTIO was applied to measure the concentration of •NO in the atmosphere, based on its gas-phase reaction with •NO (Nadeau & Boocock, 1977). However, these reports did not clarify the detailed reaction mechanism of PTIOs with •NO in water and in the ex vivo setting.

To date few substances except for hemoglobin and iron-containing compounds appear to be available for direct detection of •NO generated in biological systems, e.g., in activated macrophages or in endothelium (Drapier et al., 1991; Corbett et al., 1991; Kosaka et al., 1992). The •NO-

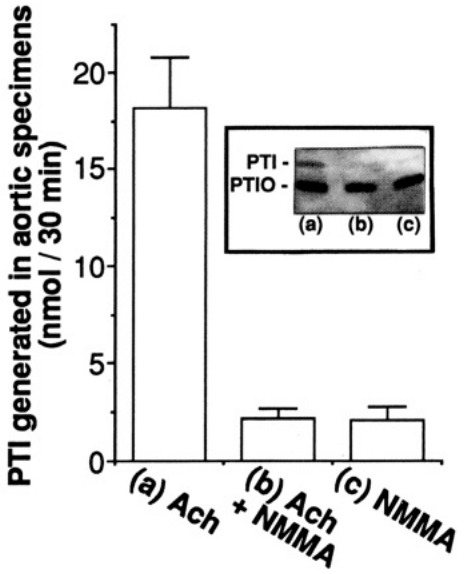

FIGURE 5: Conversion of PTIO to PTI during vasorelaxation induced by acetylcholine. Aortic specimens 5 cm long from rabbits were incubated with 0.5 mM PTIO and Cu,Zn-SOD (500 units/mL) in the presence or absence of 10 μM acetylcholine (Ach) or 1 mM NMMA in Krebs solution (1.5 mL) for 30 min at 37 °C. Imidazoline compounds in the reaction mixtures were isolated on TLC plates as shown in the inset. PTI from homogeneous spots on the TLC plate, as shown in the inset, was extracted and was subjected to quantitation by ESR spectroscopy. See text for details. Data are shown as means ± SD (n = 3–4).

hemoglobin adduct, however, is relatively unstable under aerobic conditions, and ESR measurement at 77 K or below is necessary for accurate detection of these iron–nitrosyl complexes (Drapier et al., 1991; Corbett et al., 1991; Kosaka et al., 1992). Thus, it is difficult to determine the precise amount of •NO produced in biological systems by measuring the ESR signal of NO-hemoglobin. On the basis of our data presented here, we conclude that PTIOs not only would be potent inhibitors of EDRF/•NO but also would be useful for quantitation of •NO generated during various physiological phenomena.

EDRF/•NO is a free-radical species, having a biological half-life of 4–50 s under bioassay conditions (Förstermann et al., 1984; Griffith et al., 1984; Rubanyi et al., 1985; Cocks et al., 1985; Furchgott & Vanhoutte, 1989), and it exerts its biological effects against vascular smooth muscle via activation of cytosolic guanylate cyclase by forming a nitrosyl–iron complex with the prosthetic heme group of this enzyme (Craven & DeRubertis, 1978; Ignarro, 1991). It was also reported that •NO generated in chemical and biological systems could react with sulfhydryl compounds (Stamler et al., 1992), resulting in the generation of nitrosothiols, and it did with molecular oxygen as in eq 1.

The kinetics of the reaction of EDRF/•NO with various substances occurring in vivo, however, remains unclear. We estimated the rate of the reaction of •NO with a sulfhydryl compound (L-cysteine), bovine serum albumin, molecular oxygen, or hemoglobin by means of a competition assay with PTIO in a neutral solution by using ESR spectroscopy (Mitsuta et al., 1990) (data not shown). The results showed that the relative reaction rate with •NO decreased in the following order: hemoglobin > carboxy-PTIO > PTIO = carboxymethoxy-PTIO = L-cysteine > albumin > molecular oxygen. Hemoglobin (heme irons) reacted with •NO ~3-fold faster than did carboxy-PTIO, and albumin and molecular oxygen reacted more slowly (albumin, 3-fold slower; molecular oxygen, at least 0.1-fold slower) than did PTIO. In addition, based on the rate constant of the reaction of PTIOs with •NO ($\sim 10^4$ M^{-1} s^{-1}), the half-life of •NO in the presence of 30–300 μM PTIOs was expected to be 2.3–0.23 s, shorter than that under physiological conditions in the bioassay system as described above. Because PTIOs showed significant inhibition of vasorelaxation at 30 μM or higher (Figure 4), the kinetic characteristics of the reaction of •NO and imidazolineoxyl N-oxide radicals in cell-free chemical systems seem to correlate well with the reaction of the imidazoline radicals with EDRF/•NO in this ex vivo bioassay system with aortic smooth muscle (Figures 3 and 4).

These results indicate that imidazolineoxyl N-oxide derivatives effectively antagonize •NO produced in biological systems such as vascular endothelium via a unique and novel mechanism involving a radical–radical reaction. This is the first evidence showing a potent antagonistic action of these stable organic radicals, imidazolineoxyl N-oxides, against signal transduction of •NO in a vascular system. Moreover, in a preliminary experiment, we found significant inhibitory effects of the imidazolineoxyl N-oxides on EDRF/•NO-mediated hypotension in rats and rabbits (unpublished observation). Thus, the antagonistic actions of this type of compound against •NO could be useful to analyze the pathophysiological roles of •NO in vivo.

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