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Oxygen-Dependent Inhibition of Neutrophil Respiratory Burst by Nitric Oxide

SHIGEMICHI IHA[a], KUNZO ORITA[a], TOMOKO KANNO[b,†], TOSHIHIKO UTSUMI[c], EISUKE F. SATO[d], MASAYASU INOUE[d] and KOZO UTSUMI[b,*]

[a]*The First Department of Surgery, Okayama University Medical School, Okayama 700, Japan,* [b]*Institute of Medical Science, Center for Adult Diseases, Kurashiki, Kurashiki 710, Japan,* [c]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan and* [d]*Department of Biochemistry, Osaka City University Medical School, Abeno-ku, Osaka 545, Japan*

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Effect of nitric oxide (NO) on the respiratory burst of neutrophils was examined under different oxygen tensions. Phorbol myristate acetate (PMA) stimulated oxygen consumption and superoxide (O_2^-) generation in neutrophils by a mechanism which was inhibited reversibly by NO. The inhibitory effect of NO increased significantly with a decrease in oxygen tension in the medium. The inhibitory effect of NO was suppressed in medium containing oxyhemoglobin (HbO_2), a NO scavenging agent. In contrast, 3-morpholinosydnonimine (SIN-1), a compound that rapidly generates peroxynitrite ($ONOO^-$) from the released NO and O_2^-, slightly stimulated the PMA-induced respiratory burst. These results suggested that NO, but not $ONOO^-$, might reversibly inhibit superoxide generation by neutrophils especially at physiologically low oxygen tensions thereby decreasing oxygen toxicity particularly in and around hypoxic tissues.

Keywords: Neutrophils, nitric oxide, superoxide, respiratory burst, oxygen tension, peroxynitrite

Abbreviations: CLA, 2-methyl-6-phenyl-3,7-dihydroimidazol[1,2-α]pyrazine-3-one; Cyt.c, ferricytochrome c; KRP, KrebsRinger-phosphate; NO, MCLA, 2-methyl-6-[ρ-methoxyphenol]-3,7-dihydroimidazol [1,2-α]pyrazine-3-one; nitric oxide; O_2^-, superoxide; $ONOO^-$, peroxynitrite; OZ, opsonized zymosan; PMA, phorbol myristate acetate; RPMN, rat peritoneal neutrophils; SOD, superoxide dismutase; SIN-1, 3-morpholinosydnonimine

INTRODUCTION

The respiratory burst coupled with O_2^- generation is one of the major responses of activated neutrophils. Generation of O_2^- is catalyzed by NADPH oxidase, a membrane-bound flavoprotein-b type cytochrome complex.[1–3] The recent discovery of NO as a metabolic modulator has initiated a swiftly developing area of research. NO plays important roles in various processes, such as neurotransmission, vasodilatation and the inhibition of platelet aggregation.[4–6]

It has been reported that some enzymes, such as protein kinase C, calcium-dependent proteinase (calpain), aconitase and some members of the respiratory chain are inactivated by

*Corresponding author.
†On leave from Doonan Institute of Medical Science, 4I-9, Isikawa-cho, Hakodate 041, Japan.

NO.[7–9] Recent studies revealed that the membrane potential, electron transport and ATP synthesis in mitochondria were reversibly inhibited by NO in an oxygen concentration-dependent manner.[10–12]

NO has been shown to inhibit O_2^- production by neutrophils as measured by a Cyt c reduction method.[13,14] The formation of a nitrosyl compound with NADPH oxidase has been postulated for the mechanism of inhibition.[11,15,16] However, NO rapidly reacts with Cyt c and forms nitrosyl-Cyt c,[17] which makes it difficult to use this hemeprotein for the assay of superoxide. Thus, it is inadequate to use the Cyt c reduction method for analyzing the effect of NO on O_2^- generation by neutrophils. Since the stimulated-neutrophils continuously generate O_2^- which rapidly reacts with NO and forms $ONOO^-$, the resulting $ONOO^-$ may also be responsible for the NO-induced changes in neutrophil metabolism.[18,19] Thus, the mechanism by which NO inhibits the respiratory burst of neutrophils remains to be elucidated. The present study shows the effect of NO on the oxygen metabolism of stimulated neutrophils at varying oxygen tensions.

MATERIALS AND METHODS

Chemicals

Arachidonic acid, ferricytochrome c (Cyt c), PMA, hypoxanthine and xanthine oxidase were purchased from Sigma Co. (St. Louis, MO). NO gas (99%) and SIN-1 were obtained from Nippon Sanso Co. Ltd. (Tokyo) and Dojindo Laboratories (Kumamoto), respectively. All other chemicals were of analytical grade and were obtained from Nacalai Tesque Co. (Kyoto).

Preparation of NO

NO solution was prepared with Krebs-Ringer-phosphate (pH 7.4) containing 20 mM phosphate buffer, pH 7.4, after removing oxygen by bubbling with argon gas for 10 min and then with NO gas which was passed through a KOH column (2 × 2.5 cm) to remove nitrogen dioxide (NO_2) for 30 min at 25°C. Aliquots of the NO-saturated solution (1.9 mM) were added to neutrophils in KRP medium using a stainless-steel needle on a gas-tight syringe.[12]

Preparation of Neutrophils

Rat peritoneal neutrophils (RPMN) were obtained from the rat 16 h after intraperitoneal injection of 2% nutrose by washing twice with calcium-free KRP as described previously.[20] Isolated neutrophils were resuspended in KRP at a concentration of 1×10^8 cells/ml. Viability of cells was determined by the trypan blue exclusion method.

Analysis

Oxygen consumption was measured in a closed system by means of a Clark type oxygen electrode at 37°C and recorded continuously. Superoxide production was assayed by the superoxide dismutase (SOD)-inhibitable reduction of Cyt c in a spectrophotometer equipped with a water-jacketed cell holder and magnetic stirrer at 37°C.[21] Briefly, the reaction was started by adding neutrophils ($1 - 5 \times 10^6$ cells/ml) in KRP medium containing 10 mM glucose, 40 – 60 μM Cyt c and 1 mM $CaCl_2$ in the presence or absence of various ligands. The change in absorbance at 550–540 nm ($A_{550-540}$) was monitored continuously using a dual-beam spectrophotometer (Shimadzu, UV-3000 spectrophotometer.[21]

RESULTS AND DISCUSSION

Effect of NO on Superoxide Generation by Neutrophils

Upon stimulation by various ligands, neutrophils generate O_2^- and related metabolites.[20] Recent

studies using the Cyt c reduction method reported that NO inhibited the O_2^- generation by activated neutrophils from guinea pigs.[13,14] Figure 1 shows that NO also showed apparent inhibition of Cyt c reduction by PMA-stimulated neutrophils. The inhibition occurred transiently in a concentration-dependent manner. After recovery from the inhibited state, the rate of Cyt c reduction was identical with that of untreated cells. Similar effects of NO were also observed with opsonized zymosan (OZ) and arachidonate (data not shown). These results suggested that NO reversibly inhibited the oxygen

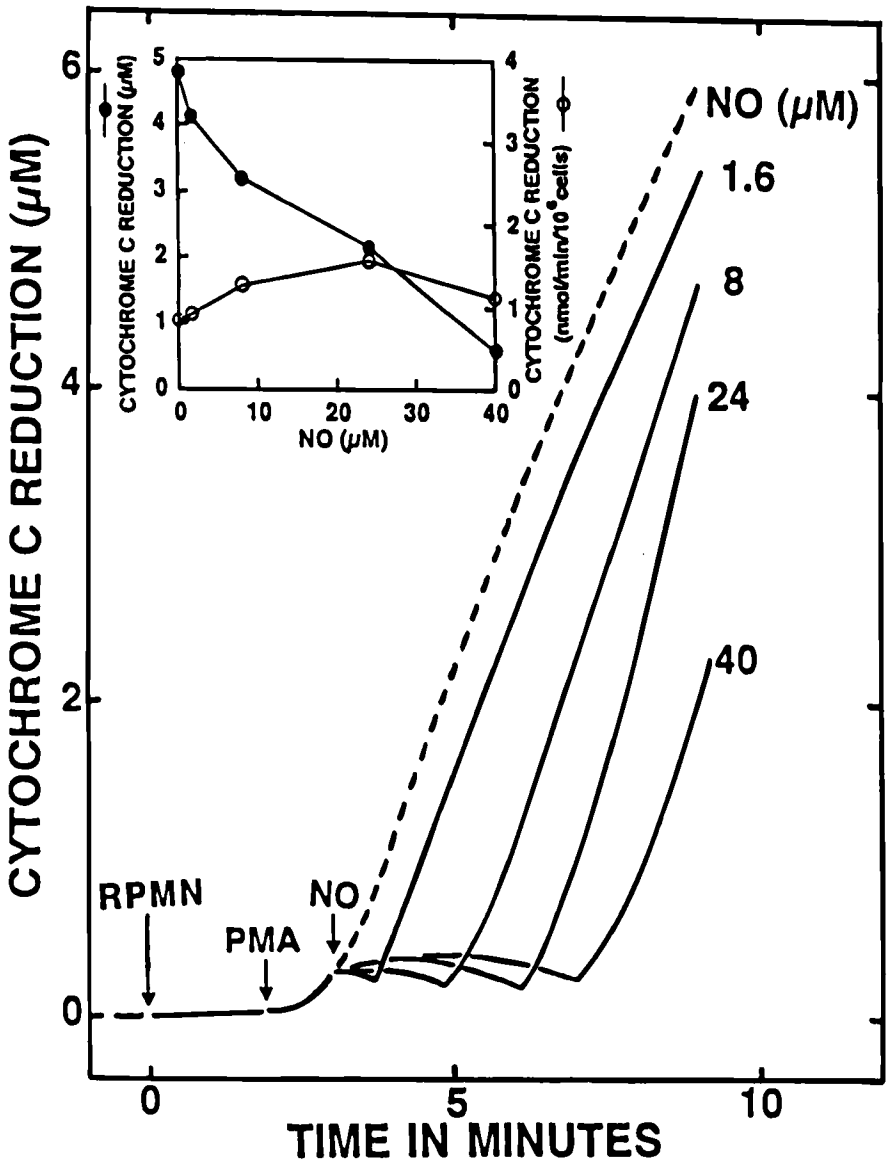

FIGURE 1 Effect of NO on Cyt c reduction by PMA-stimulated neutrophils. Rat peritoneal neutrophils (RPMN) (1×10^6 cells/ml) were incubated at 37°C in Krebs-Ringer-phosphate (KRP, pH 7.4) containing 10 mM glucose, 1 mM $CaCl_2$ and 40 μM Cyt c. PMA (2 nM) and various concentrations of NO were added at the indicated times. Dotted line shows the control experiment in the absence of NO. Insert shows the degree of Cyt c reduction (closed circle) and the rate of Cyt c reduction (open circle) 5 min after adding NO.

burst without causing irreversible damage of the cellular machinery especially at concentrations lower than 40 μM. These results are quite different from those observed with experiments using fairly high concentrations of NO.[13]

Effect of NO on Cyt c Reduction by Xanthine Oxidase-Generated Superoxide

To study the inhibitory mechanism of NO, xanthine oxidase was used as a source for O_2^-. The xanthine oxidase-dependent reduction of Cyt c was also inhibited by NO in a concentration-dependent manner (Figure 2a). Again, the rate of Cyt c reduction fully recovered at certain times after adding NO. It should be noted that, when NO was added in the reaction mixture prior to hypoxanthine, the Cyt c reduction was not inhibited as measured by uric acid production (Figure 2b). These results suggested that the oxidase might not be affected directly by NO. Thus, the Cyt c reduction method for monitor-

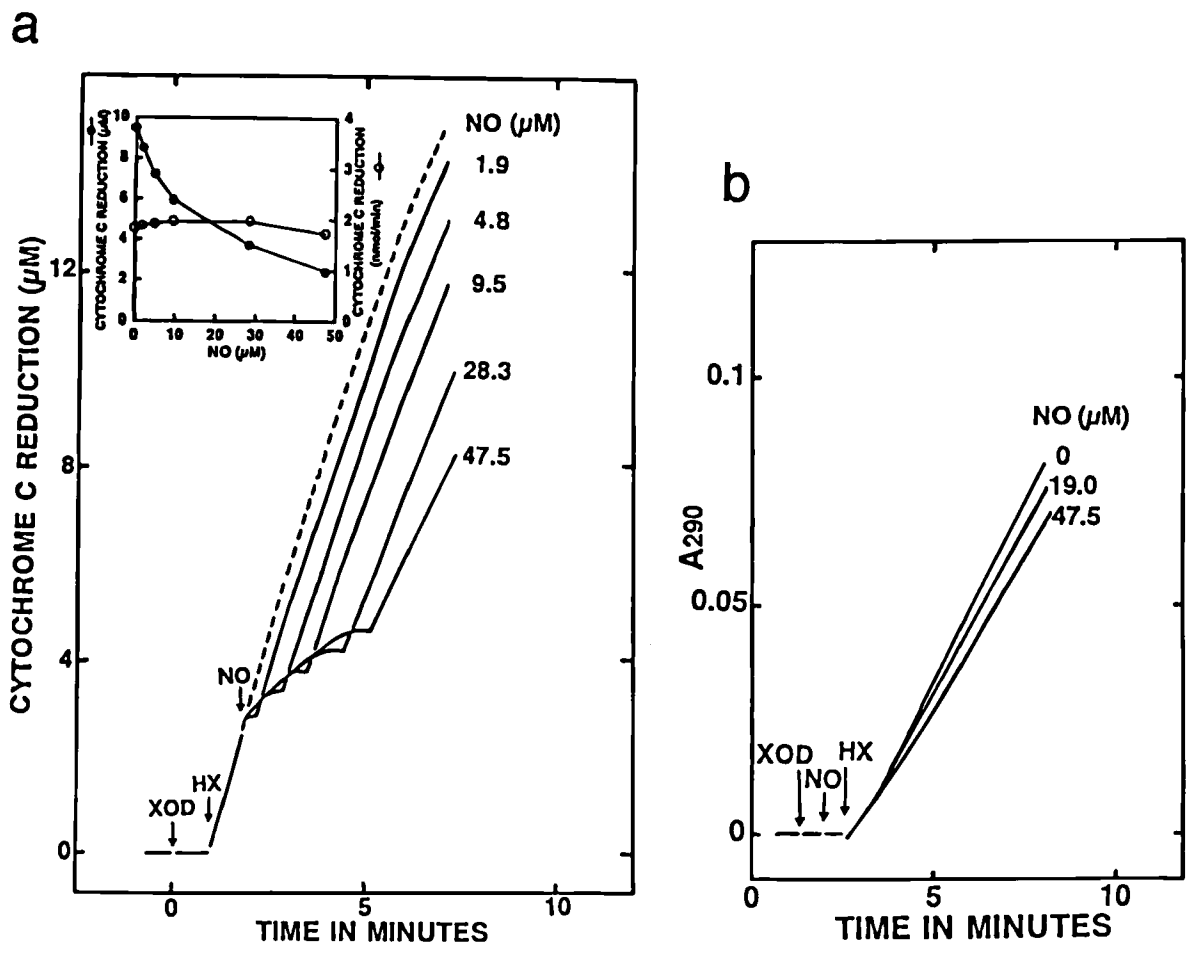

FIGURE 2 Effect of NO on Cyt c reduction and uric acid generation by the xanthine oxidase. a. Xanthine oxidase (5 mU/ml) was incubated in KRP containing 60 μM Cyt c at 37°C. Various concentrations of NO were added 2 min after adding 50 μM hypoxanthine and O_2^- generation was monitored by Cyt c reduction at 550 nm. The dotted line shows the control experiment without added NO. Insert shows the degree of Cyt c reduction (closed circle) and the rate of Cyt c reduction (open circle) 5 min after adding NO. b. Xanthine oxidase (5 mU/ml) was incubated in KRP at 37°C. One min after incubation with various concentrations of NO, the reaction was started by adding 100 μM hypoxanthine. Uric acid production was monitored by the increase in the absorbance at 290 nm.

ing the O_2^- generation by neutrophils might not be adequate for assessing the biological effect of NO. Using 2-methyl-6-[p-methoxy-phenyl]-3,7-dihydroimidazol[1,2-α]pyrazin-3-one (MCLA), an O_2^--specific chemiluminescence probe,[22] Fukahori *et al.*[23] revealed that NO (18 – 67 nM) reversibly suppressed the activity of xanthine oxidase. They found an initial and abrupt decrease in chemiluminescence by direct quenching of O_2^- by NO (67 nM) and its prolonged attenuation which lasted beyond the half-life of NO (6 sec). In contrast, fairly high concentrations of NO (49–203 μM) were required for suppressing the production of uric acid by xanthine oxidase (46 nM). Thus the NO concentrations required for the inhibition of uric acid production and O_2^- generation differ significantly from each other. This suggested that the mechanism underlying the apparent inhibition of xanthine oxidase activity might be different from that for the inhibition of Cyt c reduction.

Effect of NO on the Absorption Spectrum of Cyt c

Since NO reacts with Cyt c and forms nitrosyl complex,[17] the differential absorption spectrum of Cyt c in the presence and absence of NO was obtained by using a double-beam spectrophotometer. Immediately after adding 19 μM NO to the KRP solution containing 40 μM Cyt c, strong absorption was observed at 529 and 563 nm. This absorption spectrum coincided with that reported for nitrosyl-Cyt c.[17] The spectrum decreased time-dependently and completely disappeared 5 min after adding NO (data not shown). Similar changes in the absorption spectrum were also observed after adding NO to the cell suspension containing 40 μM Cyt c (Figure 3a). However, after complete decrease in the NO-dependent absorption at 563 nm, the absorption at 550 nm responsible for the reduced form of Cyt c increased in the presence of PMA (Figure 3b). The rate of Cyt c reduction was identical to that with untreated neutrophils. These results also indicated that the Cyt c reduction method might be inadequate for testing the effect of NO on O_2^- generation by neutrophils especially during the early period after adding NO.

Effect of NO on the Oxygen Consumption by Neutrophils

To investigate the inhibitory mechanism of NO without being influenced by the assay method for determining O_2^-, its effect on the oxygen consumption by neutrophils was observed. PMA enhanced the oxygen consumption of RPMN with concomitant generation of O_2^- (Figure 4). Immediately after adding NO, the oxygen concentration instantaneously decreased. After a short lag period, the rate of oxygen consumption was inhibited transiently for about 3 min. Similar effects of NO on the oxygen consumption were also observed with xanthine oxidase (data not shown). These results suggested that the inhibitory effect of NO on O_2^- generation by neutrophils was very weak particularly in medium containing air saturated oxygen.

Effect of NO on the Respiratory Burst at Different Oxygen Tension

We previously reported that the effect of NO on mitochondrial respiration continued significantly longer at physiologically low oxygen concentrations than in medium saturated with air oxygen.[12] Thus, the effect of NO on the oxygen consumption by RPMN was also examined at various oxygen tensions. The inhibitory effect of NO gradually increased with the decrease in oxygen concentrations. It should be noted that, at different oxygen tensions, the extent of the instantaneous decrease in oxygen concentration after adding NO remained constant. The rate of oxygen uptake recovered to the control level at any oxygen concentrations examined (Figure 5). These results suggested that the initial drop of

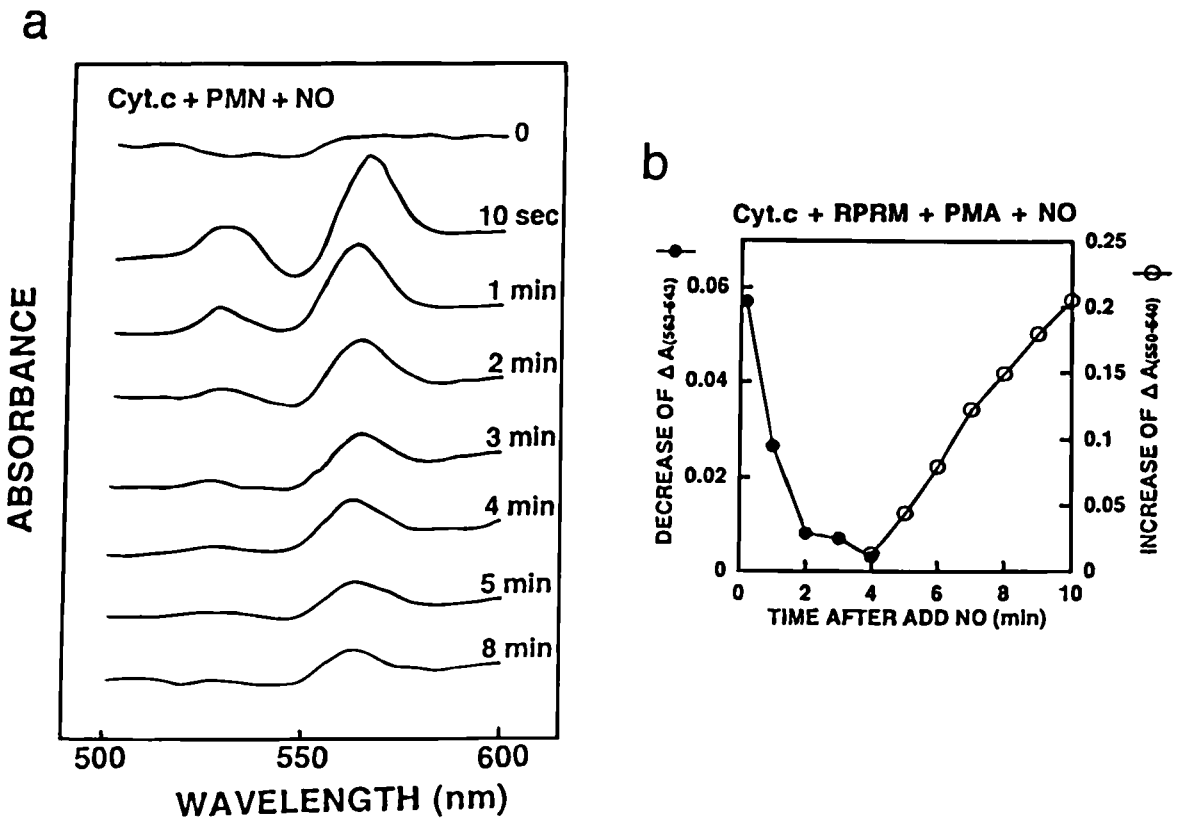

FIGURE 3 Time dependent changes in the absorption spectrum of Cyt c. RPMN (1×10^6 cells/ml) were incubated in KRP containing 40 μM Cyt c in the presence or absence of 2 nM PMA. a. Changes in absorption spectrum of Cyt c after adding 30 μM NO in the absence of PMA. b. Changes in absorption of Cyt c at 562 nm and 550 nm after adding NO in the presence of PMA.

oxygen concentration might be due to direct reaction of NO with O_2^- while the transient inhibition might reflect cellular effects whose lifetime increased significantly at low oxygen tensions.[12]

Effect of NO- and Neutrophil-Concentrations on the Oxygen Burst

The extent of the initial decrease in oxygen concentration and the time required for the recovery of the inhibition of respiratory burst depended on the concentration of NO (Figure 6). In the presence of 2×10^6 cells/ml, maximum inhibition was observed with 30 μM NO. The inhibition also depended on the concentration of neutrophils. At low concentrations of RPMN (1×10^6 cells/ml), the inhibition was strong and sustained for a fairly long period. On the contrary, the inhibitory effect was decreased by increasing the concentration of neutrophils (~5×10^6 cells/ml) (data not shown). Thus, the efficiency of inhibition seems to depend on the ratio of NO to neutrophil concentrations. Similar phenomena were also observed with isolated mitochondria.[12] The steady-state levels of NO in and around neutrophils have been postulated to be about 8 μM.[24] Thus, endogenously generated NO by neutrophils might inhibit the O_2^- generation particularly under physiologically low oxygen tensions (~50 μM). Thus, the inhibitory effect of NO may play important roles in the inflammatory locus where the infiltrated neutrophils generate O_2^-.[25]

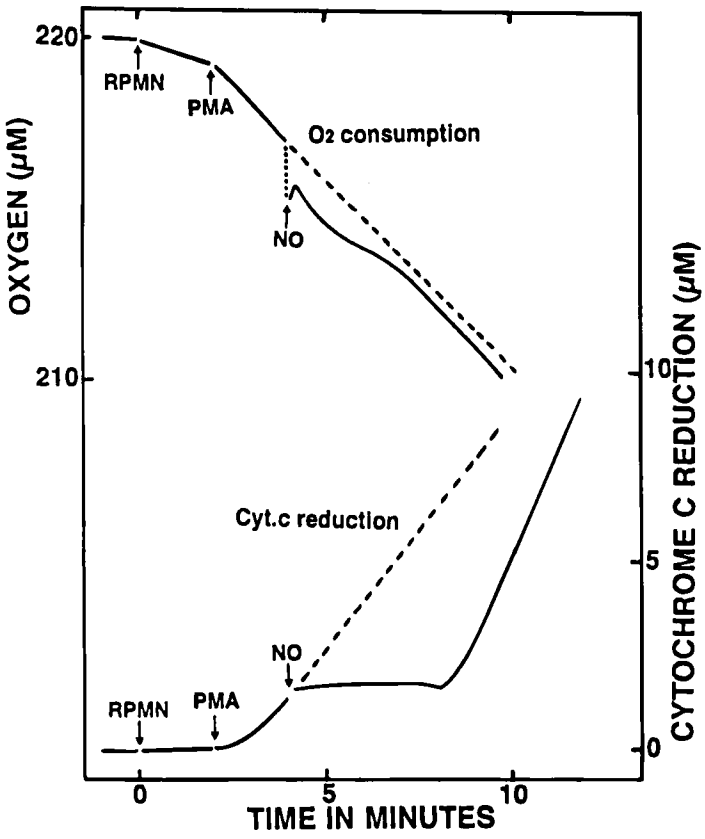

FIGURE 4 Effect of NO on the absorption of Cyt c and oxygen consumption. RPMN (1×10^6 cells/ml) were incubated as in FIGURE 1 and stimulated by 2 nM PMA. Two min after adding PMA, 30 μM NO was added to the mixture. For the measurement of the oxygen burst, Cyt c was not included in the incubation mixture. Oxygen consumption and Cyt c reduction (change in absorption at 550 nm) were recorded simultaneously. Dotted lines show the control experiments without added NO.

Effect of Oxyhemoglobin on the Inhibitory Effect of NO

Since oxyhemoglobin (HbO_2) stoichiometrically reacts with NO,[22] the protein has been used for the determination of NO. To elucidate whether the inhibition was caused by NO and/or its metabolites, the inhibitory effect on neutrophil respiration was also examined in the presence or absence of HbO_2. In the presence of a fairly low concentration of HbO_2, the inhibitory effect of NO was markedly suppressed without affecting the initial drop in oxygen concentration (Figure 7). The suppressive effect of HbO_2 depended on its concentration (data not shown). These results suggested that NO rather than its metabolites might directly inhibits the machinery requires for the generation of O_2^- by neutrophils.

Effect of Peroxynitrite on the Oxygen Burst

Since the stimulated neutrophils continuously generate O_2^- which rapidly reacts with NO, the resulting $ONOO^-$ might induce cellular dysfunction including enzyme inactivation[18,19] and apoptosis.[31] To test the possible involvement of $ONOO^-$ in the NO-induced inhibition, the effect of SIN-1, a $ONOO^-$ generating agent,[26] on the respiratory burst of neutrophils was examined. A fairly high concentration of SIN-1 did not inhibit the oxygen burst but slightly enhanced the rate of oxygen consumption (data not

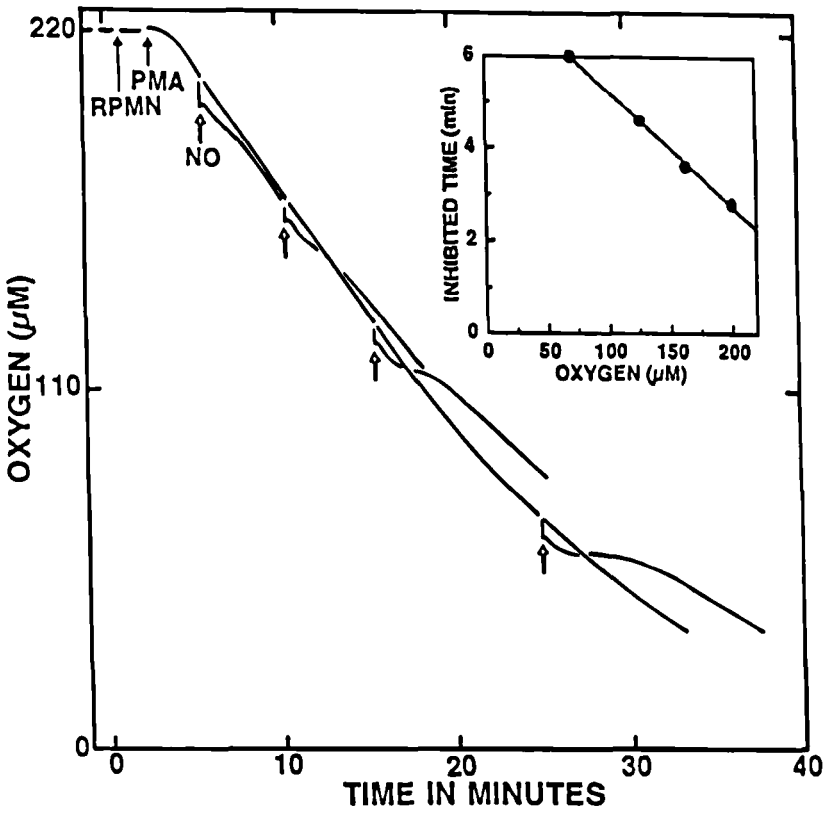

FIGURE 5 Effect of oxygen tensions on the NO-dependent inhibition. RPMN (3×10^6 cells/ml) were incubated in KRP containing 10 mM glucose and 1 mM $CaCl_2$. At the indicated times, 20 μM NO was added to each mixture. Insert shows the inhibited time by NO at different oxygen tension of the medium.

shown). These results indicate that NO but not $ONOO^-$ might be responsible for the inhibition of both respiratory burst and $O_2^{\cdot-}$ generation by neutrophils.

The present work demonstrates that NO rapidly reacts with Cyt c and forms nitrosyl-Cyt c and, hence, the Cyt c reduction method is inadequate for the analysis of NO-induced changes in $O_2^{\cdot-}$ generation by neutrophils. Therefore, alternative methods such as, chemiluminescent techniques using 2-methyl-6-[ρ-methoxyphenol]-3,7-dihydroimidazol[1,2-α]pyrazine-3-one(MCLA) or 2-methyl-6-phenyl-3,7-dihydroimidazol[1,2-α]pyrazine-3-one (CLA) luminescence, would be used for $O_2^{\cdot-}$-detection. Unfortunately, preliminary experiments using MCLA showed that $O_2^{\cdot-}$-dependent chemiluminescence of MCLA was also quenched by NO. Thus, further studies using other reagents such as CLA, should be performed for the detection of $O_2^{\cdot-}$ generation by neutrophils.

It was also found that the inhibitory effect of NO was low in air-saturated medium (220 μM O_2 at 37°C) and required a fairly high concentration of NO while it was strong at physiologically low oxygen tensions (~50 μM). Although $ONOO^-$ has been postulated to inhibit NADPH oxidase and related systems,[19,24,27] the experiments with HbO_2 revealed that NO rather than its reactive metabolite(s) directly inhibited the $O_2^{\cdot-}$ generation in neutrophils. Recent studies revealed that nanomolar concentrations of NO reversibly inhibited the respiration of mitochondria by interacting with cytochrome c oxidase.[12,28,29]

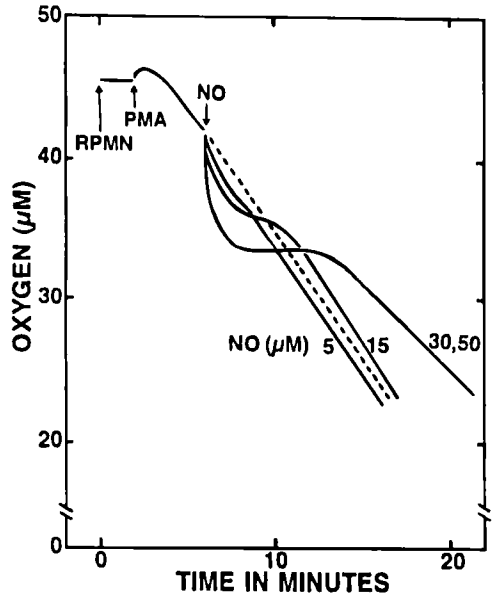

FIGURE 6 Dose-dependent inhibition of PMA-stimulated oxygen uptake. RPMN (2×10^6 cells/ml) were incubated in KRP containing 10 mM glucose and 1 mM $CaCl_2$. Various concentrations of NO (0, 5, 15, 30 and 50 μM) were added 2 min after stimulation with 2 nM PMA. Dotted line shows the control in the absence of NO.

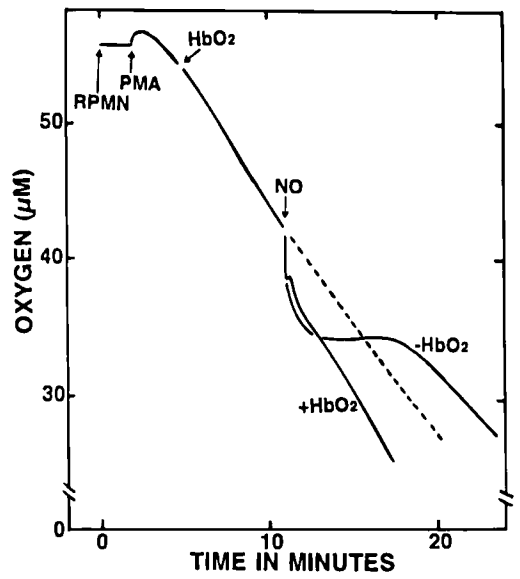

FIGURE 7 Effect of HbO_2 on the inhibitiory effect of NO. Experimental conditions were as described in FIGURE 6. PMA-stimulated O_2 uptake of neutrophils was inhibited by adding 30 μM NO 7 min after stimulation with 2 nM PMA. HbO_2 (15 μM) was added 2 min after stimulation with PMA. Dotted line shows the control in the absence of NO.

Moreover, immunocytochemical study revealed that NO synthase was also localized in mitochondria.[30] Thus, cytochrome c oxidase has been postulated to be one of the main targets of NO. Since vascular endothelial cells[24] and stimulated neutrophils generate a large amount of NO and O_2^-, respectively, peroxynitrite and/or hydroxyl radical would be generated in and around the sites of neutrophil infiltration, which may result in irreversible damage of cellular constituents.[27,31] This possibility should be studied further.

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