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Effect of Aromatic Nitroso-Compounds on Superoxide-Generating Activity in Neutrophils[1]

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Aromatic nitroso-compounds such as nitrosobenzene inhibited the respiratory burst of intact neutrophils induced by various stimulants, including phorbol 12-myristate 13-acetate and a chemotactic peptide. The compounds also inhibited NADPH-dependent oxygen consumption by cell-free preparations of neutrophils. This indicates that nitroso-compounds act directly on the NADPH-oxidase system. The inhibitory effects induced by several nitroso-compounds, 2-nitrosotoluene, nitrosobenzene, 4-nitrosophenol, and 1-nitrosopyrrolidine, were examined and their inhibition constants, the concentrations causing 50% reduction of oxygen consumption, were found to be 0.043, 0.173, 0.672, and 32.1 mM, respectively. These values correlated well with the hydrophobicity of the compounds: a more hydrophobic compound was a more potent inhibitor against NADPH oxidase, suggesting that the oxidase has a hydrophobic site(s) for interaction with the inhibitors.

Key words: human and porcine neutrophils, inhibition, nitroso-compounds, respiratory burst.

When activated by a variety of soluble or particulate stimulants, neutrophils exhibit a dramatic increase in oxygen consumption and concomitant generation of the superoxide anion (O_2^-) (*1*) (for reviews, see Refs. *2-5*). This highly reactive molecule is involved in bacterial killing either directly or *via* metabolic intermediates such as hydrogen peroxide and hydroxyl radicals. This phenomenon, known as the "respiratory burst," is due to activation of a membrane-bound NADPH-oxidase system.

Much effort has been directed into elucidation of the nature of NADPH oxidase, and several lines of evidence show that the oxidase is comprised of a membrane-bound cytochrome b_{558} and several cytosolic proteins: the former binds the redox components, FAD and heme (*6, 7*), for the electron transfer from NADPH to molecular oxygen; the latter are involved in the transformation of the cytochrome from the dormant to the activated states (*8-12*).

Iizuka *et al.* reported that heterocyclic nitrogenous bases such as pyridine and imidazole reversibly inhibited the respiratory burst and simultaneously induced a spectral change in cytochrome b_{558} of intact neutrophils (*13*). The inhibition constant, the concentration causing 50% reduction of oxygen consumption, coincided with the concentration that gave a half-maximum change in the spectrum of cytochrome b_{558}. This suggests that the replacement of the internal ligand with the externally added ligand causes inhibition of the respiratory burst. However, similar inhibition was not observed in the reconstituted system with purified cytochrome b_{558} (*14*), indicating that the reaction site was not the heme of cytochrome.

Here we show that nitroso-compounds such as nitrosobenzene (NB) and nitrosotoluene (NT), which are known to form complexes with heme proteins (*15-19*), inhibit the respiratory burst more effectively than do pyridine and imidazole. The inhibitory effects are correlated with the hydrophobicity of the nitroso-compounds.

MATERIALS AND METHODS

Reagents—Phorbol 12-myristate 13-acetate (PMA), zymosan, digitonin, *N*-formylmethionylleucylphenylalanine (fMLP), NADPH, and heparin were purchased from Sigma. Sodium oleate, Dextran T-500, and Ficoll-Conray solution were obtained from Nacalai Chem. Pharmacia and Immuno. Biol. Lab., respectively. Nitroso-compounds, NB, NT, 4-nitrosophenol (NP), and 1-nitrosopyrrolidine (NPY), whose structural formulas are shown in Fig. 1, were from Aldrich Chemical. NB and NT were dissolved in ethanol, and NP in 0.1 N NaOH. These solutions were freshly prepared daily and diluted to the appropriate concentration before the assay. All other reagents were commercially available and of reagent grade.

Preparation of Neutrophils—Human and porcine whole blood anticoagulated with heparin (final 10 units/ml) were diluted with phosphate-buffered saline (PBS), mixed with

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Abbreviations: fMLP, *N*-formylmethionylleucylphenylalanine; HBSS, Hanks' balanced salt solution; NB, nitrosobenzene; NP, 4-nitrosophenol; NPY 1-nitrosopyrrolidine; NT, 2-nitrosotoluene; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.

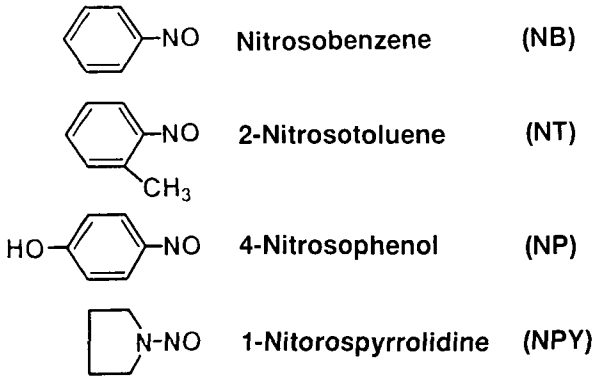

Fig. 1. **Structural formulas of the nitroso-compounds.**

Dextran T-500 (final 1.5%), and allowed to stand at room temperature for 20-30 min. The leukocyte-rich plasma was removed and centrifuged at 200 $\times g$ for 10 min. The pellet was resuspended in PBS, then centrifuged at 400 $\times g$ for 30 min over Ficoll-Conray solution to remove platelets and mononuclear cells (*20*). The supernatant was decanted and contaminating red cells were removed by hypo-osmotic shock in distilled water for 30 s. The suspended granulocytes were pelleted by centrifugation at 200 $\times g$ for 10 min and washed twice with Hanks' balanced salt solution (HBSS) buffered at pH 7.3 with 25 mM HEPES. More than 90% of the cells were identified to be neutrophils.

Measurement of Oxygen Consumption by Intact Human Neutrophils—The neutrophil suspension of 1×10^7 cells/ml in HBSS was incubated at 28°C in the chamber (0.59 ml) of a Clark oxygen electrode (Yellow Springs Instrument). After 5 min of incubation, nitroso-compounds were added at a desired concentration in a volume that did not exceed 10 μl. After 2 min of incubation, the cells were stimulated and the change in oxygen consumption was monitored. The rate of oxygen consumption was measured graphically from the chart and expressed as percentage of control activity. The potency of the inhibitors to prevent oxygen consumption was shown as an inhibition constant, IC_{50}, which represents the concentration causing 50% inhibition.

Measurement of NADPH-Dependent Oxygen Consumption by Cell-Free Preparations of Porcine Neutrophils—Porcine neutrophils were disrupted by freezing and thawing, then incubated at 28°C in the chamber of a Clark oxygen electrode in the presence of 1 mM EGTA and 1.5 mM NADPH in HBSS without Ca^{2+} (Ca^{2+}-free HBSS). The concentration of cell-free preparations was equivalent to 5×10^7 cells/ml. The suspension was treated with a desired concentration of NB for 2 min, then stimulated with 0.25 mM sodium oleate. To compare the inhibitory effect of NB in cell-free preparations with that in intact cells, similar experiments were performed with intact porcine neutrophils. In this case, NADPH was omitted from the cell suspension and the concentration of neutrophils was 3×10^7 cells/ml.

RESULTS

Effect of NB on the Respiratory Burst by Human Neutrophils—The respiratory burst of PMA-stimulated human neutrophils monitored by use of an oxygen electrode

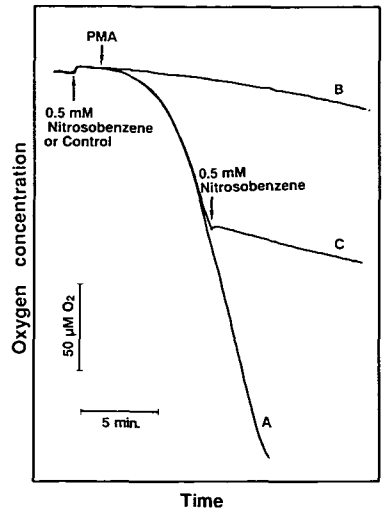

Fig. 2. **Inhibitory effect of nitrosobenzene (NB) on oxygen consumption by intact human neutrophils.** Human neutrophils (10^7 cells/ml) were suspended in HBSS (pH 7.4) and incubated at 28°C in the chamber of a Clark oxygen electrode. After 2 min of incubation, the reaction was started by the addition of PMA (0.2 μg/ml) and oxygen consumption was recorded. (A) Cells were incubated in the presence of ethanol (0.3%) as a control, (B) NB at 0.5 mM was added before PMA-stimulation. (C) Cells were exposed to 0.5 mM NB during the reaction. Maximum rate of oxygen consumption in the control experiment was 16.6 nmol O_2/min/10^7 cells.

TABLE I. **Effect of nitrosobenzene (NB) on the respiratory burst in human neutrophils induced by various stimulants.** Neutrophils at 10^7 cells/ml in HBSS were incubated with various concentrations of NB for 2 min, then a stimulant was added at its optimum concentration (shown in parentheses). The control activities for each stimulant were 16.6, 30.5, 9.0, 2.2, 6.6 nmol O_2/min/10^7 cells, respectively.

Stimulant	IC_{50} (mM)
PMA (0.2 μg/ml)	0.173
Sodium oleate (0.25 mM)	0.174
Opsonized zymosan (0.5 mg/ml)	0.093
Digitonin (0.8 μg/ml)	0.076
fMLP (2 μM)	0.043

(Fig. 2A) was immediately inhibited by the addition of NB either before (Fig. 2B) or after the activation (Fig. 2C). This result suggests that inhibition by NB was due to its action directly on the NADPH oxidase, not on the activation process including the signal transduction and the assembly of the activated components.

NB similarly inhibited the respiratory burst of neutrophils exposed to other soluble stimulants, sodium oleate, digitonin, and fMLP, or to a particulate stimulant. The inhibition constants, IC_{50}, are listed in Table I. NB was effective against all five stimulants tested in this study, indicating that it acts at the most distal step of neutrophil activation, probably on NADPH oxidase.

The integrity of NB-treated neutrophils was confirmed by the cell viability tests, a dye-exclusion test with trypan blue, and a membrane permeability test by the measurement of lactate dehydrogenase activity as a cytosol marker enzyme (data not shown).

Effect of NB on NADPH-Dependent Oxygen Consumption in Cell-Free Preparations of Porcine Neutrophils—We

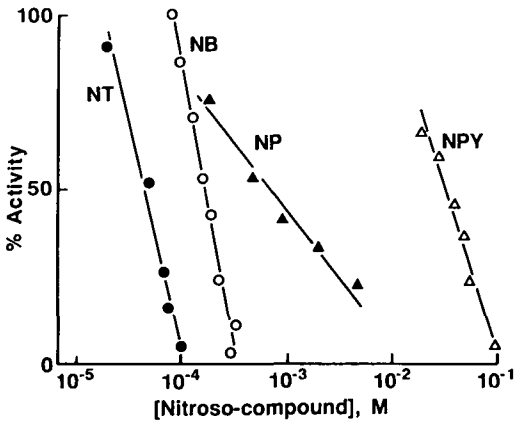

Fig. 3. **Residual activity of PMA-induced oxygen consumption in human neutrophils treated with nitroso-compounds.** Human neutrophils at 10^7 cells/ml in HBSS (pH 7.4) were incubated at 28°C for 2 min with increasing concentration of nitrosobenzene (NB, ○), 2-nitrosotoluene (NT, ●), 4-nitrosophenol (NP, ▲), and 1-nitrosopyrrolidine (NPY, △). NB and NT were dissolved in ethanol and added in 2-μl aliquots. NP was dissolved in 0.1 N NaOH and added in 10-μl aliquots. NPY was added directly. The cells were stimulated by 0.2 μg/ml of PMA, and the rate of oxygen consumption at each concentration of nitroso-compound was converted to percentage of control activity.

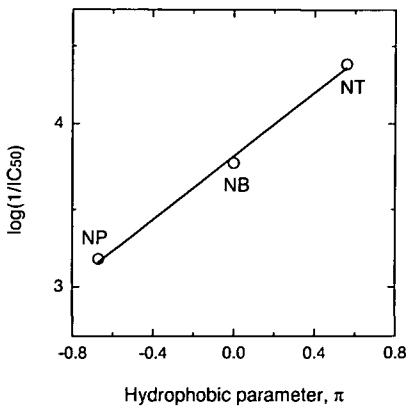

Fig. 4. **Correlation of the inhibition constants, IC_{50}, with the hydrophobicity for various nitroso-compounds.** The IC_{50} values of nitroso-compounds in human neutrophils were taken from Table II. The values of $\log(1/IC_{50})$ were plotted against π values for the compounds. The π values, which are the hydrophobic parameters obtained by Hansch *et al.*, are 0.56, 0.00, and −0.67, respectively, for -CH_3 (NT), -H (NB), and -OH (NP) (*21*).

TABLE II. **Effect of nitroso-compounds on PMA-induced oxygen consumption by human neutrophils.** Experimental conditions were the same as in Fig. 3.

Compound	IC_{50}(mM)
Nitrosobenzene (NB)	0.173
2-Nitrosotoluene (NT)	0.043
4-Nitrosophenol (NP)	0.672
1-Nitrosopyrrolidine (NPY)	32.1

TABLE III. **Reversibility of the inhibition by nitroso-compounds of PMA-induced oxygen consumption by porcine neutrophils.** Porcine neutrophils (3×10^7 cells/ml) were pre-incubated with the indicated concentration of nitroso-compound for 2 min, then washed twice with HBSS. Control cells were incubated with the same volume of ethanol, the solvent of NB and NT. The rate of oxygen consumption was measured after the stimulation with PMA (0.2 μg/ml). In the presence of nitroso-compounds at the above concentrations, PMA-induced oxygen consumption was almost completely abolished.

Compound	Concentration (mM)	Residual activity after washing (%)
Nitrosobenzene (NB)	1	64
2-Nitrosotoluene (NT)	1	5
1-Nitrosopyrrolidine (NPY)	200	111

examined the effect of NB on the respiratory burst in cell-free preparations of porcine neutrophils in the presence of 1 mM EGTA, 1.5 mM NADPH as an electron donor, and 0.25 mM sodium oleate as a stimulant. The inhibition constant in cell-free preparations was found to be 5.4 μM, 30 times higher than that in intact porcine neutrophils, 0.145 mM. Together with the results that NB inhibited the respiratory burst induced by the various stimulants in intact cells (Table I), this result confirms that NB does not block the signal transduction in the activation process, but acts directly on NADPH oxidase.

Effect of Nitroso-Compounds on the Respiratory Burst by PMA-Stimulated Human Neutrophils—As illustrated in Fig. 3, NB, NT, NP, and NPY each caused a concentration-dependent inhibition of the respiratory burst by PMA-stimulated human neutrophils. Table II shows the inhibition constants, IC_{50}, of these compounds for PMA-stimulated human neutrophils. The IC_{50} values of NB, NT, and NP ranged from 0.040 to 0.70 mM, markedly lower than those for pyridine and imidazole (about 30 mM) reported previously (*13*).

Correlation of Inhibitory Effect of Nitroso-Compounds with Their Chemical Properties—The correspondence between the solubility in aqueous solution and the order of IC_{50} values of the nitroso-compounds suggests that the hydrophobicity of these compounds is responsible for their potency to inhibit the respiratory burst. Figure 4 shows the relationship between $\log(1/IC_{50})$ and the hydrophobic parameter (π), the parameter determined by the substituent attached to the benzene skeleton of the aromatic nitroso-compounds, -CH_3 (NT), -H (NB), and -OH (NP) (Fig. 1). These hydrophobic parameters were taken from the paper of Hansch *et al.* (*21*). This plot quantitatively shows that a compound with a higher hydrophobicity has a higher inhibitory effect.

The skeleton of NPY, the weakest inhibitor of the NADPH-oxidase activity (Table II), is quite different from those of NB, NT, and NP (Fig. 1) and its π value is not available in the literature (*21*). However, taking into account of its structural formula, it is plausible that NPY is the most hydrophilic and therefore has the highest IC_{50} value of the nitroso-compounds tested in this study.

Reversibility of the Inhibition by Nitroso-Compounds of PMA-Induced Oxygen Consumption by Porcine Neutrophils—We measured the residual activity of NB-, NT-, and NPY-treated neutrophils after washing the reagent-treated cells twice with HBSS. As shown in Table III, the inhibition by the most hydrophobic compound, NT, was irreversible; that by NB, which is of intermediate hydrophobicity, was partially reversible; and that by NPY, which is thought to be the most hydrophilic reagent, was reversible. This

indicates that a more hydrophobic compound interacts with the NADPH-oxidase system more strongly and is more difficult to remove by washing. Namely, the inhibitory effect is also correlated with the degree of irreversibility of inhibition.

DISCUSSION

To elucidate the mechanism of activation of neutrophils and the concomitant production of superoxide anion, various inhibitors have been developed and effectively utilized as biological tools. Although these inhibitors of the respiratory burst result in the termination of the superoxide production, their sites of action are not necessarily the superoxide-generating system itself. For example, islet-activating protein (*22-24*) and dibutyryl-cAMP (*25*) prevented the signal transduction and therefore inhibited neutrophil activation by fMLP but not by PMA; antibiotic cerulenin (*26, 27*), which prevented the mobilization of membrane-bound Ca^{2+}, completely inhibited fMLP- or A23187-induced activity and partially inhibited PMA-induced activity. To investigate the action of the system, it is necessary to find useful specific inhibitors.

As reported previously, pyridine inhibited the NADPH-oxidase activity and induced a spectral change of cytochrome b_{558} *in vivo* (*13*). However, inhibition by pyridine was not observed in the reconstituted system with purified cytochrome b_{558} (*14*). On the other hand, CO and other respiratory inhibitors had no effect on the absorption spectra or the superoxide-generating activity of cytochrome b_{558} *in vivo* (*20*) or in the purified form (*14, 28*). Here we have shown that nitroso-compounds inhibit the activity but cause no spectral changes. These results suggest that the endogenous axial ligands of the heme of cytochrome b_{558} are too strongly bound to be replaced by these exogenous ligands, and that the sites of interaction with pyridine and the nitroso-compounds are not the heme itself.

The parallelism between the hydrophobicity and the potency of inhibition of nitroso-compounds suggests that their reaction site is a hydrophobic region of the NADPH-oxidase system, which is located in cell membrane. Hydrophobicity seems to be advantageous for inhibitors to penetrate the cell membrane, accumulate in lipid bilayers and reach the reaction site. We discuss the mechanism of the inhibitory effect in the following paper (*29*).

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