

## Nitric Oxide, an Endothelial Cell Relaxation Factor, Inhibits Neutrophil Superoxide Anion Production via a Direct Action on the NADPH Oxidase

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### Abstract

Nitric oxide provokes vasodilation and inhibits platelet aggregation. We examined the effect of nitric oxide on superoxide anion production by three sources: activated intact neutrophils, xanthine oxidase/hypoxanthine, and the NADPH oxidase. Nitric oxide significantly inhibited the generation of superoxide anion by neutrophils exposed to either FMLP ( $10^{-7}$ M) or PMA (150 ng/ml) ( $IC_{50} = 30 \mu\text{M}$ ). To determine whether the effect of nitric oxide on the respiratory burst was due to simple scavenging of  $O_2^+$ , kinetic studies that compared effects on neutrophils and the cell-free xanthine oxidase system were performed. Nitric oxide inhibited  $O_2^+$  produced by xanthine oxidase only when added simultaneously with substrate, consistent with the short half-life of NO in oxygenated solution. In contrast, the addition of nitric oxide to neutrophils 20 min before FMLP resulted in the inhibition of  $O_2^+$  production, which suggests formation of a stable intermediate. The effect of nitric oxide on the cell-free NADPH oxidase superoxide-generating system was also examined: The addition of NO before arachidonate activation ( $t = -6$  min) significantly inhibited superoxide anion production. Nitric oxide did not inhibit  $O_2^+$  when added at NADPH initiation ( $t = 0$ ). Treatment of the membrane but not cytosolic component of the oxidase was sufficient to inhibit  $O_2^+$  generation. The data suggest that nitric oxide inhibits neutrophil  $O_2^+$  production via direct effects on membrane components of the NADPH oxidase. This action must occur before the assembly of the activated complex. (*J. Clin. Invest.* 1992; 90:1116–1121.) Key words: nitric oxide • neutrophil • xanthine oxidase • NADPH oxidase

### Introduction

At sites of inflammation activated neutrophils migrate across vascular endothelium. The endothelial lining cells, in response to cytokines, undergo alterations that promote neutrophil adhesion and diapedesis (1–3). Endothelial cells also release me-

diators such as adenosine, prostacyclin, and nitric oxide (an endothelium-derived relaxation factor), which act locally to vasodilate and inhibit platelet activation (4–10). In addition, prostacyclin and adenosine serve a “defensive” function: they inhibit the activation of neutrophils exposed to chemoattractants and may thereby protect the endothelium from free radical injury (11, 12).

Whether nitric oxide similarly protects vascular endothelium from neutrophil-mediated injury is unknown. Formed from L-arginine in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction (6), nitric oxide is a diffusible molecule which activates guanylate cyclase in a variety of cell types (7–10). Diverse biological effects have been described, including smooth muscle dilation, neuronal transmission, microbial cytotoxicity, and gastrointestinal adaptive relaxation (13). In these studies we have examined the effect of nitric oxide on the production of superoxide anion by human neutrophils, xanthine oxidase/hypoxanthine, and the cell-free NADPH oxidase. The data suggest that nitric oxide inhibits superoxide anion generation via a direct action on a membrane component of the NADPH oxidase. This effect, which can be shown to be distinct from the direct free radical scavenging of superoxide anion by nitric oxide, may represent a previously unrecognized mechanism of endothelial defence against neutrophil-mediated injury.

### Methods

**Neutrophil isolation.** Human neutrophils were obtained from the heparinized venous blood of healthy volunteers by Ficoll-Hypaque centrifugation, dextran sedimentation, and hypotonic lysis (14).

**Preparation of nitric oxide.** Nitric oxide solutions were prepared after bubbling nitric oxide gas through isotonic Hepes buffer (149 mM NaCl, 5 mM KOH, 10 mM Hepes, 1.2 mM  $MgCl_2$ , 1.29 mM  $CaCl_2$ ) as previously described (15). Nitric oxide was allocated into cell suspensions using gas-tight syringes. The reactivity of nitric oxide with thiols is the basis of the thionitrobenzoic acid assay which is used to quantitate nitric oxide (15). Briefly, nitric oxide solutions were combined with 100  $\mu\text{M}$  TNB in 0.1M Tris, pH 8.1, at 37°C for 10 min. Absorbance for reduced thiol was measured with a spectrophotometer (model 1201, Milton-Roy Co., Rochester, NY). The concentration of reduced thiol was determined by using an extinction coefficient at 412 nm of 12  $\text{mM}^{-1}\text{cm}^{-1}$ .

**Lysosomal enzyme release.** For the determination of enzyme release, neutrophils ( $5 \times 10^6/\text{ml}$ ) were combined with 5  $\mu\text{g}/\text{ml}$  cytochalasin B and nitric oxide at designated concentration in a final volume of 1 ml. After stimulation with FMLP or phorbol myristate acetate (PMA) for 5 min cells were removed and aliquots of the supernatants were taken for standard determination of  $\beta$ -glucuronidase and lyso-

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zyme (16). Cell death was assessed by release of cytoplasmic lactate dehydrogenase (LDH) (17).

**Aggregation.** Neutrophils ( $1.25 \times 10^6$ /ml) were resuspended in an isotonic HEPES saline buffer and warmed to  $37^\circ\text{C}$  for 5 min in the presence or absence of nitric oxide. Aggregation was measured as previously described (15). Briefly, the change in the transmittance of light through the stirred (900 rpm) suspensions of cells was monitored in a dual chamber aggregometer (Payton Scientific, Inc., Buffalo, NY). Responses were compared by calculating the areas under the aggregation curves in the first minute using a Micro Plan II morphometric tablet (Laboratory Computer Systems, Inc., Cambridge, MA).

**Intracellular calcium (Fura-2).** The elevation of intracellular  $\text{Ca}^{2+}$  in response to FMLP stimulation was monitored using the fluorescent dye Fura-2 as described (18). Neutrophils ( $5 \times 10^7$ /ml) were incubated with  $10 \mu\text{M}$  Fura-2 (Molecular Probes, Inc., Eugene, OR) for 20 min at  $37^\circ\text{C}$ . The free Fura-2 was removed by washing and fluorescence measured at 340 and 380 nm.

**Superoxide anion generation.** Superoxide anion generation was monitored by determination of reduction of cytochrome C in the presence or absence of superoxide dismutase as previously described (16). Briefly, neutrophils ( $1.25 \times 10^6$ /ml) were combined with 0.9 mg/ml horse heart ferricytochrome C (type III) plus  $5 \mu\text{g}/\text{ml}$  cytochalasin B, and various inhibitory compounds (nitric oxide, S-nitrosothiols) at designated concentration in a final volume of 1 ml. After stimulation with FMLP or PMA (5 min) cells were spun down and the supernatants were collected. Absorption at 550 nm was determined in a spectrophotometer and the nanomoles of superoxide anion generated were calculated as previously described (16).

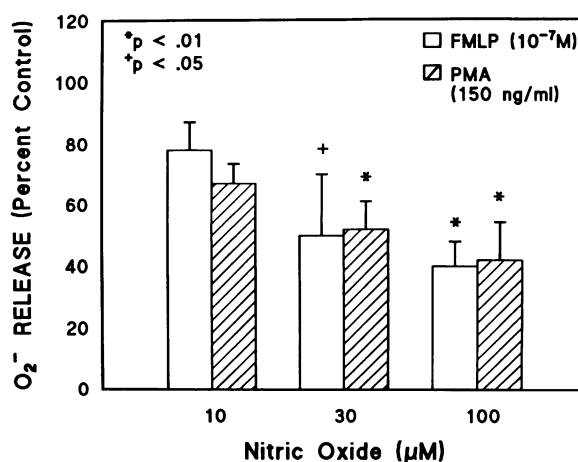
**Xanthine oxidase generation of superoxide anion.** Reaction mixture (final volume, 1 ml) contained ferricytochrome C (0.9 mg/ml), 50 mM Pi pH 7.4 and xanthine oxidase (0.05 mg/ml) to which nitric oxide was added at varied concentrations. Superoxide anion production was initiated by the addition of hypoxanthine (2 mg/100 ml). Superoxide was continuously monitored and expressed as nanomoles of cytochrome C reduced.

**Cell-free NADPH oxidase generation of superoxide anion.** All activation materials were kept on ice. Plasma membranes and cytosol were prepared from neutrophils disrupted by nitrogen cavitation (19). A standard activation mix was derived using the method of Eklund and co-workers (20). Briefly, plasma membrane, cytosol,  $\text{MgCl}_2$ ,  $\text{GTP}\gamma\text{S}$ , in the presence or absence of nitric oxide, were allocated in a total volume of 50  $\mu\text{l}$  comprising 0.34 M sucrose, 0.13 M NaCl, and 10 mM Pi, pH 7.4 (buffer A). The NADPH oxidase was activated by exposure to 0.1 mM arachidonic acid (6 min,  $22^\circ\text{C}$ ). After arachidonate activation initiation of superoxide anion generation was achieved by addition of 0.1 mM ferricytochrome C plus 0.3 mg/ml NADPH in a final volume of 0.8 ml (achieved through addition of buffer A).

## Results

### Effect of nitric oxide on neutrophil superoxide anion generation

Human neutrophils were exposed to nitric oxide (5 min,  $37^\circ\text{C}$ ) at concentrations of 10–100  $\mu\text{M}$  before addition of  $10^{-7}\text{M}$  FMLP or PMA (150 ng/ml). As shown in Fig. 1 treatment of neutrophils with 10  $\mu\text{M}$  nitric oxide reduced FMLP-dependent  $\text{O}_2^-$  production to  $78 \pm 9.1\%$  control ( $P = 0.05$ ) and PMA dependent production to  $67 \pm 6.5$  ( $P < 0.03$ ). 30  $\mu\text{M}$  nitric oxide further reduced FMLP-induced  $\text{O}_2^-$  release to  $50 \pm 20\%$  ( $P < 0.05$ ) and PMA-induced release to  $52 \pm 9.1\%$  ( $P < 0.01$ ) of control. The effects of nitric oxide were not due to cell death (LDH release  $< 5\%$ ). Sodium nitrite or sodium nitrate (breakdown products of nitric oxide) did not inhibit superoxide anion generation at concentrations up to 100  $\mu\text{M}$ . Hemoglobin (200 nM, which scavenges nitric oxide) completely reversed the capacity of nitric oxide to inhibit superoxide anion generation (data not shown).



**Figure 1.** Effect of treatment with nitric oxide on  $\text{O}_2^-$  generation by neutrophils. Neutrophils ( $1.25 \times 10^6$ /ml) were incubated in the absence (control) or presence of nitric oxide (5 min,  $37^\circ\text{C}$ ) before exposure to FMLP ( $10^{-7}\text{M}$ ) or PMA (150 ng/ml). Data represent the mean ( $\pm$  SEM) of at least three separate determinations on cells from different donors.

### Effect of nitric oxide on aggregation, degranulation, and calcium mobilization

As shown in Table I nitric oxide also inhibited the homotypic aggregation of activated neutrophils. Significant inhibition of both FMLP and PMA-induced aggregation was achieved at 30  $\mu\text{M}$  nitric oxide ( $78 \pm 12\%$  and  $70 \pm 14\%$  control, respectively;  $\text{IC}_{50} \sim 100 \mu\text{M}$ ). Nitric oxide (10–100  $\mu\text{M}$ ) consistently enhanced degranulation (lysozyme,  $\beta$ -glucuronidase release, Table I), although the difference above control did not reach statistical significance. We also examined the effect of nitric oxide (30  $\mu\text{M}$ ) on the rise of cytosolic calcium after exposure to FMLP ( $10^{-7}\text{M}$ ). Nitric oxide (5 min,  $37^\circ\text{C}$ ) did not inhibit FMLP-induced increases of cytosolic calcium as reported by the fluorescent probe Fura-2 ( $310 \pm 34$  vs.  $316 \pm 26$  nM,  $P = \text{NS}$ ).

**Table I.** Effect of Nitric Oxide on Neutrophil Aggregation and Degranulation

Condition	Neutrophil function		
	Aggregation	Lysozyme	$\beta$ -Glucuronidase
	percent control		
FMLP $10^{-7}\text{M}$			
NO 10 $\mu\text{M}$	102 $\pm$ 11	ND	ND
NO 30 $\mu\text{M}$	78 $\pm$ 12	145 $\pm$ 33	127 $\pm$ 29
NO 100 $\mu\text{M}$	54 $\pm$ 15*	120 (2)	134 (2)
PMA 150 ng/ml			
NO 10 $\mu\text{M}$	84 $\pm$ 14	ND	ND
NO 30 $\mu\text{M}$	70 $\pm$ 14	130 $\pm$ 24	128 $\pm$ 14
NO 100 $\mu\text{M}$	48 $\pm$ 8 <sup>†</sup>	115 (2)	133 (2)

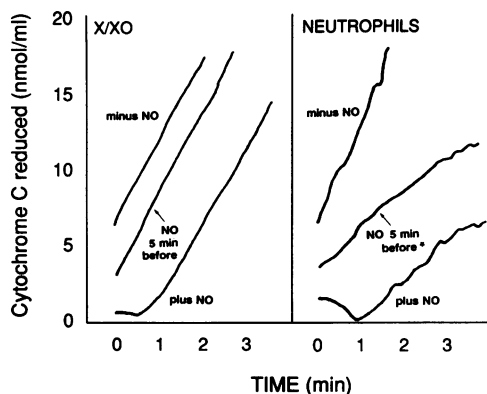
Neutrophils were incubated in the absence (control) or presence of nitric oxide (5 min,  $37^\circ\text{C}$ ) before exposure to FMLP or PMA. Results are expressed as the mean $\pm$ SEM of at least three experiments except where indicated by parentheses. \*  $P = 0.0337$ , <sup>†</sup>  $P = 0.0078$ . Abbreviation: ND, not determined.

### Kinetics of the nitric oxide effect

To determine whether the effect of nitric oxide on superoxide anion generation was due to simple free radical scavenging, kinetic studies that compared neutrophils and a cell-free xanthine oxidase source of  $O_2^-$  were performed. The preincubation interval between the addition of nitric oxide to the system and the initiation of  $O_2^-$  release was varied.

**Xanthine oxidase.** Superoxide anion production by xanthine oxidase was initiated by the addition of hypoxanthine to the reaction mixture. In the absence of nitric oxide this resulted in the immediate generation of  $O_2^-$  with a reaction rate of 94 nmol/min/mg (measured for 2 min, Fig. 2). The addition of nitric oxide (30  $\mu$ M) simultaneously with hypoxanthine initiation resulted in a 30-s lag before the detection of  $O_2^-$ . Once initiated, the reaction rate over 2 min was identical to control. In contrast, the addition of 30  $\mu$ M nitric oxide 5 min before hypoxanthine initiation had no effect on superoxide anion production. The inability of nitric oxide to inhibit the xanthine oxidase when added 5 min before hypoxanthine initiation is consistent with its short half-life ( $t_{1/2} < 15$  s) in oxygenated solution. Thus, nitric oxide transiently inhibits  $O_2^-$  produced by xanthine oxidase only when added simultaneously with substrate, an effect consistent with  $O_2^-$  scavenging alone.

**Intact neutrophils.** Fig. 2 illustrates the contrasting kinetic effects of nitric oxide on the production of superoxide anion by intact neutrophils exposed to FMLP ( $10^{-7}$  M). The addition of 30  $\mu$ M nitric oxide simultaneously with the addition of FMLP delayed the detection of superoxide anion for 30 s. Thereafter, in contrast to the cell-free xanthine oxidase system, the rate of  $O_2^-$  production was reduced from  $6.6 \pm 1.0$  to  $2.9 \pm 1.3$  nmol/min per  $10^6$  cells ( $P = 0.01$ ). Also in contrast to the cell-free system, nitric oxide inhibited the generation of superoxide anion when added 5 min before exposure to FMLP: the reaction rate was reduced to  $2.3 \pm 0.7$  nmol/min per  $10^6$  cells ( $P = 0.01$ ). Indeed, neutrophil superoxide anion generation could be shown to be inhibited when the exposure to nitric



**Figure 2.** Effect of treatment with nitric oxide on  $O_2^-$  generation by neutrophils or by xanthine oxidase. Figure is representative of three experiments performed in duplicate. Continuous time measurement used to determine the effect of treatment with nitric oxide on release of  $O_2^-$  by xanthine oxidase (*x/xo*) and by neutrophils exposed to FMLP. Time interval between addition of nitric oxide (30  $\mu$ M) and initiation of  $O_2^-$  release was varied. Superoxide anion production was initiated with the addition of hypoxanthine (0.4 mM) or FMLP ( $10^{-7}$  M) as described in Methods.  $O_2^-$  generation was analyzed in the absence of nitric oxide (*minus NO*), 5 min after the addition of nitric oxide (*NO 5 min before*), or with the addition of nitric oxide simultaneous with the initiation of  $O_2^-$  production (*plus NO*).

oxide preceded FMLP by as long as 20 min ( $22 \pm 8\%$  control,  $P < 0.01$ ). Thus, the inhibition by nitric oxide of  $O_2^-$  produced by neutrophils is sustained beyond the expected persistence of nitric oxide in solution.

### Effect of nitric oxide on the NADPH-oxidase

The prolonged effects of nitric oxide on intact neutrophils as compared to its transient effects on the cell-free xanthine oxidase system indicate that the inhibition of neutrophil superoxide anion generation is not due to free radical scavenging alone. Nitric oxide must exert effects on cellular components which act as stable intermediates. To determine whether the signaling machinery of the intact cell was required for the sustained inhibitory activity of nitric oxide we examined the cell-free arachidonate activated NADPH oxidase. To produce superoxide anion stimulated neutrophils assemble a multiprotein complex, derived from cytosolic and membrane components. Superoxide anion production by the oxidase requires an activation process during which time cytosolic components translocate to the plasma membrane. Activation (and assembly) of the oxidase is induced in the cell-free system by a variety of agents, including arachidonic acid. After the 6-min period of arachidonate activation, NADPH is added to initiate superoxide anion production. As shown in Table II the addition of nitric oxide (40 pmol/ $\mu$ g protein) at the time of NADPH initiation ( $t = 0$  min) had no effect on the rate of superoxide anion generation calculated over 2 min. In contrast, when nitric oxide was added 10 min before arachidonate activation (16 min before NADPH initiation),  $O_2^-$  release was reduced from  $608 \pm 170$  to  $344 \pm 85$  nmol/min per mg ( $P = 0.006$ ). These data indicate that nitric oxide inhibits  $O_2^-$  production by the NADPH oxidase only if added before the assembly of the multiprotein complex. However, to determine whether nitric oxide exerted a delayed effect on a component of the assembled NADPH oxidase, we added nitric oxide (40 pmol/ $\mu$ g protein) 6 min after arachidonate activation and permitted the multi-component complex to incubate for 10 min before initiation with NADPH. Nitric oxide did not inhibit superoxide anion generation under these experimental conditions ( $331 \pm 127$  vs.  $352 \pm 157$  nmol/mg per min).

We performed studies to determine whether nitric oxide affected cytosolic or plasma membrane components of the cell-free oxidase. Each fraction was exposed separately to nitric ox-

**Table II.** Effect of Nitric Oxide on the NADPH Oxidase Superoxide Anion Generating System

Condition	NADPH oxidase activity nmol/min per mg
<b>Addition before arachidonate activation</b>	
Buffer control	$608 \pm 144$
Nitric oxide	$344 \pm 85$ ( $P < 0.006$ )
<b>Addition at NADPH initiation</b>	
Buffer control	$507 \pm 130$
Nitric oxide	$567 \pm 130$

Cytosol and plasma membrane were prepared and assayed in the cell-free NADPH oxidase activation assay as described under Methods. Nitric oxide was added at a concentration of 40 pmol/ $\mu$ g protein. Results are mean  $\pm$  SE of three experiments performed in duplicate. Arachidonate activation precedes NADPH initiation by 6 min. Rate of  $O_2^-$  production is measured over 2 min after addition of NADPH.

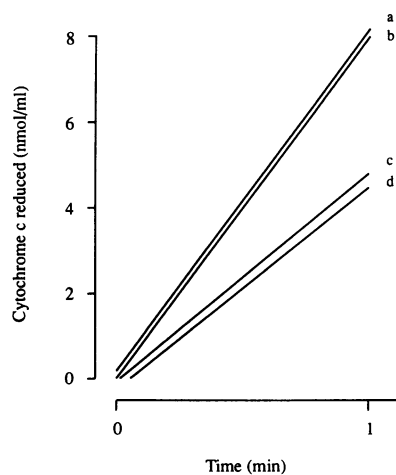
ide (10 min, 22°C) before reconstitution and arachidonate activation. Nitric oxide (40 pmol/ $\mu$ g protein) had no effect when added to the dialyzed cytosolic fraction alone prior to activation (617 $\pm$ 180 vs. 567 $\pm$ 120 nmol/min per mg,  $P = \text{NS}$ ). However, the exposure of purified plasma membranes to nitric oxide before reconstitution reduced subsequent superoxide anion generation to 266 $\pm$ 64 nmol ( $P < 0.02$ ). (Fig. 3).

## Discussion

Endothelial cells produce nitric oxide, which has been identified as an endothelium-derived relaxation factor. Nitric oxide, synthesized by endothelium via arginine oxidation by a calcium-activated NADPH oxidase (6), activates the soluble guanylate cyclase of platelets and smooth muscle cells (21). The resultant rise of cytosolic cyclic GMP mediates smooth muscle relaxation and inhibits platelet aggregation. It is now evident that cells other than vascular endothelium synthesize nitric oxide, including fibroblasts, macrophages, neutrophils, and neurons (22–27).

Our studies of human neutrophils show that nitric oxide inhibits superoxide production and homotypic aggregation, effects which may protect against tissue injury. Inhibition is due to nitric oxide, not its metabolites sodium nitrate and sodium nitrite. Furthermore, inhibition was reversed by addition of hemoglobin, a known scavenger of nitric oxide. Significant effects of nitric oxide are achieved at micromolar concentrations which approach the intraluminal rate of nitric oxide production in small blood vessels, estimated to reach 8  $\mu$ M/min (28). In vitro, stimulated macrophages, endothelial cells and neutrophils synthesize nitric oxide at a rate of 10–100 nM/5 min per  $10^6$  cells (4, 6, 24, 25, 29). It is also worth noting that nitric oxide is stable under hypoxic conditions (15), as may exist at sites of inflammation (30).

The physiological significance of the capacity of nitric oxide to inhibit superoxide anion production can be only speculative at this time. A role for nitric oxide at sites of inflammation is suggested by the capacity of key inflammatory mediators



**Figure 3.** Inhibition of cell-free NADPH oxidase activation by nitric oxide is due to direct effect on the membrane component. This figure (representative of five experiments) illustrates the continuous spectrophotometric measurement of superoxide anion produced by reconstituted NADPH oxidase after addition of NADPH at  $t = 0$ . Nitric oxide (40 pmol/ $\mu$ g) was added (10 min, 22°C) before arachidonate activation of the reconstituted system (see Methods).

(a) Control; (b) nitric oxide added to cytosol fraction alone before reconstitution and activation; (c) nitric oxide added to plasma membrane fraction alone before reconstitution and activation; (d) nitric oxide added to mixture of cytosol and plasma membrane before arachidonate activation.

(e.g., bradykinin, interleukin-1, interferon- $\gamma$ ) to induce the expression of the enzyme, nitric oxide synthase, and promote the release of nitric oxide by cultured endothelium (4, 5). It is possible, as has been proposed for prostaglandins of the E series and for adenosine (11, 12), that nitric oxide produced by activated endothelial cells at inflammatory sites functions as a defensive molecule, which protects against neutrophil mediated injury. In vivo support for such a hypothesis is provided by recent intravital microscopic studies (31) that demonstrate that endogenous nitric oxide inhibits neutrophil adhesion to the endothelium of cat mesenteric venules. In humans, there is recent evidence that nitric oxide is produced by synovial tissue in patients with inflammatory arthritis (32). The determination of whether the effect of nitric oxide in the arthritides is pro-inflammatory (owing to vasodilation and the formation of toxic peroxynitrite radicals) or anti-inflammatory (owing to inhibition of neutrophil superoxide anion production and intercellular adhesion) requires further study.

With specific regard to its effects on neutrophils our studies provide insight to the site of nitric oxide action. The capacity to inhibit PMA-induced superoxide anion generation indicates inhibition effected distal to the chemoattractant receptor coupled G protein (33–35). It is possible that nitric oxide exerts some effects via activation of a soluble guanylate cyclase as described in other cell types (8, 9). For example, the cell permeant analogue of cyclic GMP, dibutyryl cyclic GMP, potentiates neutrophil exocytosis (36), consistent with our observation that nitric oxide enhances lysozyme and  $\beta$ -glucuronidase release by FMLP-treated cells. The effect of db-cGMP on  $O_2^-$  release by activated neutrophils has been reported to be stimulus-specific (37): in one report  $O_2^-$  release by neutrophils exposed to C5a was augmented, while  $O_2^-$  release by FMLP was inhibited and  $O_2^-$  release by PMA was unchanged. In our studies nitric oxide inhibited superoxide anion release in response to both FMLP and PMA. For this, and for reasons outlined below, it is not likely that nitric oxide dependent elevations of intracellular cGMP fully explain the observations of this study.

The capacity of nitric oxide to “scavenge” superoxide anion may partially account for some of our findings, particularly with regard to effects on the cell-free xanthine oxidase system. Nitric oxide reacts directly with superoxide anion in aqueous solution and has been reported to form peroxynitrite (ONOO) (38). However, a direct reaction between two free radical species does not adequately explain the effects of nitric oxide on the intact neutrophil. First, nitric oxide also inhibits homotypic aggregation (39), functions not dependent upon superoxide anion production (40). Second, whereas the effects of a single exposure to nitric oxide on the xanthine oxidase superoxide generating system were short-lived, effects on neutrophil function were sustained for over 20 min. The xanthine oxidase studies are consistent with free radical scavenging and the brief survival of nitric oxide in aqueous solution ( $t_{1/2} < 15$  s [15]). In contrast, the prolonged effect of nitric oxide on neutrophils suggests the formation of a stable intermediate.

Studies of the cell-free NADPH oxidase superoxide anion generating system confirmed that the effects of nitric oxide are not due to either free radical scavenging alone or to activation of the guanylate cyclase. The addition of nitric oxide at the initiation (i.e., addition of NADPH) of the reaction did not inhibit the rate of superoxide anion production. An immediate inhibition, as was observed in the case of xanthine oxidase system, would be predicted in the case of free radical scaveng-

ing. In contrast, exposure of the NADPH oxidase system to nitric oxide ten minutes before arachidonate activation (16 min before NADPH initiation) significantly inhibited superoxide anion generation. This indicates that the effects of nitric oxide must be exerted prior to the assembly of the activated multiprotein complex. Indeed, the treatment of membrane components alone was sufficient to inhibit superoxide anion production by the NADPH oxidase. These experiments, which utilized purified plasma membranes reconstituted with dialyzed cytosol, cannot be explained by activation of guanylate cyclase and generation of cGMP.

The data indicate that nitric oxide inactivates a still undetermined membrane component required for the assembly and/or activation of the oxidase. The oxidase is composed of membrane bound proteins which include cytochrome  $b_{558}$  and a 45-kD flavoprotein; the cytosolic components of the oxidase include two well-characterized proteins, p47<sup>[phox]</sup> and p67<sup>[phox]</sup>, as well as a GTP-binding protein (recently identified as Rac 2) (41). This multiprotein complex is assembled within the plasma membrane after neutrophil stimulation (42–45). Wymann et al. (45) have suggested that cytoskeletal element(s) may be involved in the assembly of the NADPH oxidase, whereas Dewald et al. (43) have demonstrated that the oxidase was found in a cytoskeletal-enriched pellet of stimulated PMN. The cytosolic proteins p47<sup>[phox]</sup> and p67<sup>[phox]</sup>, which translocate from cytosol to membrane after activation, contain cytoskeletal binding sites in their primary sequence (46, 47).

There are several possible mechanisms which could account for the capacity of nitric oxide to inhibit the multiprotein complex. Some iron containing proteins such as the iron sulfur protein, aconitase, chemically react with nitric oxide to form a stable iron-nitrosyl adduct (48). Heme containing proteins such as hemoglobin also react with nitric oxide to form nitrosyl heme, a modification limited to heme proteins in which the iron is in the reduced or ferrous state (49, 50). For aconitase and hemoglobin, nitrosylation results in the inactivation of protein function (48, 50). Interestingly, nitrosyl-heme does not form with heme proteins containing iron in the oxidized or ferric state, such as mitochondrial cytochrome  $c$  oxidase (48). Two lines of evidence suggest that nitric oxide does not inactivate NADPH oxidase through a nitrosylation of heme. First, the low potential of the cytochrome suggests that the heme-iron associated with NADPH oxidase is in the oxidized state (51). Second, we report that nitric oxide, a lipid soluble gas, has no effect on activity when added after NADPH oxidase assembly. This was true even when the assembled oxidase was incubated with nitric oxide for 10 min before NADPH initiation. These data suggest that the nitric oxide effect is not mediated by inactivation of the heme group via nitrosylation which would be expected to effect the function of the assembled oxidase.

An alternative mechanism by which nitric oxide could interfere with the assembly and/or activation of the oxidase is via a reaction with sulfhydryl containing proteins. *p*-Chloromercuribenzoic acid (51), an inhibitor of thiol groups, also acts directly upon the membrane component of the oxidase to abolish superoxide anion production. Based on spectrophotometric analysis, Bellavite et al. (51) suggest that *p*-chloromercuribenzoic acid inhibits the NADPH oxidase by reaction with a thiol closely associated with the heme group of cytochrome  $b_{558}$ . Nitric oxide reacts with tissue sulfhydryls to form *S*-nitrosothiol compounds, such as *S*-nitrosocysteine (52–54); the incor-

poration of nitric oxide into an *S*-nitrosothiol enhances its stability and potency (55). Our data raise the possibility that nitric oxide, like *p*-chloromercuribenzoic acid, reacts with one or more thiol groups which are important in the function of cytochrome  $b$ . This could result from interference with the conformation of the assembled oxidase necessary for electron transfer.

In summary, our studies demonstrate that nitric oxide inhibits neutrophil superoxide anion production via a direct action on a membrane component of the NADPH oxidase. The effect of nitric oxide must be exerted before the assembly of the activated complex and may involve a reaction with sulfhydryl groups of the multiprotein complex. These observations suggest that nitric oxide, released in response to cytokines by endothelial cells or macrophages at sites of inflammation, may protect against neutrophil-dependent tissue injury.

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