Nitric Oxide Inactivates NADPH Oxidase in Pig Neutrophils by Inhibiting Its Assembling Process*

(Received for publication, July 31, 1997, and in revised form, October 8, 1997)

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 52, Issue of December 26, pp. 32773–32778, 1997
Printed in U.S.A.

The effects of nitric oxide (NO) on superoxide (O2·-) generation of the NADPH oxidase in pig neutrophils were studied. NO dose-dependently suppressed O2·- generation of both neutrophil NADPH oxidase and reconstituted NADPH oxidase. Effects of NO on NADPH-binding site and the redox centers including FAD and low spin heme in cytochrome b558 and the electron transfer rates from NADPH to heme via FAD were examined under anaerobic conditions. Both reaction rates and the Km value for NADPH were unchanged by NO. Visible and EPR spectra of cytochrome b558 showed that the structure of heme was unchanged by NO, indicating that NO does not affect the redox centers of the oxidase. In reconstituted NADPH oxidase system, NO did not inhibit O2·- generation of the oxidase when added after activation. The addition of NO to the membrane component or the cytosol component inhibited the activity by 24.0 ± 5.3% or 37.4 ± 7.1%, respectively. The addition of NO during the activation process or to the cytosol component simultaneously with myristate inhibited the activity by 74.0 ± 5.2% or 70.0 ± 8.3%, respectively, suggesting that cytosol protein(s) treated with myristate becomes susceptible to NO. Peroxynitrite did not interfere with O2·- generation.

Nitric oxide (NO) is now recognized as one of the key mediators in many physiological and pathological processes (see reviews in Refs. 1 and 2). NO is also known to be a multifunctional molecule, one function of which is to inactivate biologically important enzymes such as mitochondrial respiratory enzymes and GAPDH, which play important roles in energy production (3, 4), ribonucleotide reductase, which is the key enzyme for protein synthesis (5), and the superoxide-generating enzymes, NADPH oxidase (6) and xanthine oxidase (7). Particularly important is the effect of NO on NADPH oxidase, because under conditions such as inflammation, the accumulation of phagocytes is a common feature and the induction of NO synthase has been shown. It is plausible that increased formation of NO interferes with the activity of NADPH oxidase and reduces superoxide (O2·-) production (8, 9). Despite the importance of the NO effect on NADPH oxidase, no detailed study has been carried out since the initial report by Clancy et al. (6) in which inhibition of O2·- generation by NO was demonstrated. The underlying mechanism was suggested to be the direct interaction of NO on the membrane components of NADPH oxidase (6).

The NADPH oxidase of phagocytes is a multi-component electron transport system, in which activation requires the assembly of three cytosolic regulatory proteins (p47phox, p67phox, and Rac1/Rac2) to membrane-bound cytochrome b558 (10, 11). Cytochrome b558 is postulated to be a membrane-bound flavocytochrome with six-coordinated low spin heme and FAD as redox centers. The electrons provided by NADPH are thought to be transferred in a linear sequence, NADPH → FAD → heme (Fe3+) → O2. The heme in cytochrome b558 is assumed to be the terminal electron donor in the production of O2·- from molecular oxygen due to its unusually low redox potential, −245 mV (12). Although the most plausible site of NADPH oxidase attacked by NO was suggested to be in membrane protein(s) (6), a detailed analysis of these effects has not been performed. Considering that nitrated-lactone complex easily forms in heme-containing enzymes (3), the heme structure of cytochrome b558 and the electron flux from substrate (NADPH) to redox centers, FAD and low spin heme, in NADPH oxidase should be examined to clarify the effects of NO on its O2·-generating activity.

In the present study, we examined the effects of NO on electron fluxes in neutrophil NADPH oxidase. Under aerobic conditions the effects of NO on O2·-generating activity of NADPH oxidase (reaction 1) was examined by the cytochrome c reduction method. In this study, we also employed the solubilized NADPH oxidase obtained from stimulated cells and measured its O2·-generating activity in the presence of NO. Under anaerobic conditions the effects of NO on the electron transfer reaction in each redox center was examined: NADPH → FAD → exogenous electron acceptor, cytochrome c (reaction 2) and NADPH → FAD → cytochrome b558 (reaction 3). Under both aerobic and anaerobic conditions, the binding ability of NO to the six-coordinated low spin heme (His-Fe3+) of cytochrome b558 (reaction 4) was examined by both visible absorption and EPR spectroscopy. We also studied the effects of NO on the activation of the oxidase (reaction 5), i.e. assembly of cytosolic and membrane components using the reconstituted NADPH oxidase system.

Finally, we studied the effects of peroxynitrite (ONOO−) on NADPH oxidase to confirm that the results obtained above were caused by NO (and not by ONOO−) because addition of NO in the presence of O2·- produces ONOO− at nearly diffusion-limited rates (13) and because ONOO− is known to be a potent oxidant.

**EXPERIMENTAL PROCEDURES**

**Materials**—Myristic acid and arachidonic acid from Wako Pure Chemical (Tokyo, Japan) were dissolved in ethanol. Heptylthiogluco-side was purchased from Dojindo Laboratories (Kumamoto, Japan). NADPH was from Oriental Yeast (Tokyo, Japan). Superoxide dismutase, cytochrome c (type VI from horse heart), and phorbol myristate acetate (PMA) were purchased from Sigma. Other reagents were of commercial grade.

*This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: PMA, phorbol myristate acetate; SH3, Src homology 3.
analytical grade.

NO Solution—Saturated NO solution was prepared according to the methods published elsewhere (7, 14). Briefly, nitrogen gas was bubbled through 50 mM phosphate buffer (pH 7.0) for 20 min to remove dissolved oxygen. Then authentic NO gas (99%, Nippon Sanso Co. Ltd., Tokyo) that had passed through 1 M KOH to remove nitrogen dioxide was bubbled into the solution for 20 min. The concentration of NO was measured spectrophotometrically as previously reported (15).

Synthesis of ONOO−—Peroxynitrite was synthesized by the reaction of acidified H$_2$O$_2$ and NaNO$_2$ in a quenched flow reactor with a subsequent stabilization induced by 1.5 M NaOH as described previously (16). The purity and concentration of ONOO− was checked by its absorbance at 302 nm. The production of ONOO− from NO and O$_2$ was also confirmed by the increase in absorbance at 302 nm.

Preparation of Neutrophils—Neutrophils were obtained from pig blood as reported previously (17) by the Conray-Ficol differential density configuration method.

Isolation of Membrane and Cytosolic Components from Neutrophils—The cell pellet was frozen and thawed in the presence of phenylmethylsulfonyl fluoride at a final concentration of 1 mM and sonicated in ice-cold Krebs-Ringer phosphate buffer containing 0.34 M sucrose. Membrane vesicles and cytosol were obtained from sonicated cells by centrifugation (100,000 x g for 60 min) (18). The membrane component of the NADPH oxidase was solubilized from the membrane vesicle with heptylthioglucoside (19) and used for further purification of cytochrome b$_{558}$. The cytosol and solubilized NADPH oxidase from resting membrane vesicle were used in the reconstituted NADPH oxidase. All procedures were applied to both resting and stimulated cells. Cell stimulation was induced by myristate as reported previously (19).

Purification of Cytochrome b$_{558}$—Cytochrome b$_{558}$ was purified from the membrane component (20) with slight modifications to avoid denaturation of heme in cytochrome b$_{558}$. A buffer composed of 50 mM phosphate buffer (pH 7.0), 50 mM NaCl, 10% glycerol, and 0.6% heptylthioglucoside was used to calculate S.D. of at least three experiments. Nonlinear least squares regression methods published elsewhere (7, 14). Briefly, nitrogen gas was bubbled into the solution for 20 min. The concentration of NO was measured spectrophotometrically as previously reported (15).

Effects of NO on NADPH Oxidase The measurements were performed with 30 µM cytochrome c at 37 °C by the addition of 4 ng PMA in Me$_2$SO (as indicated by the arrow). Trace A, assay buffer (50 mM phosphate buffer, pH 7.4) without neutrophils. Trace B, neutrophils (1 × 10$^6$ cells) in the absence of NO. Trace C, 25 µM NO added 2 min before the addition of cytochrome c (3 min prior to PMA).

Results and Discussion

Effect of Nitric Oxide on the Superoxide-forming Activity of Neutrophils (Reaction 1)—Fig. 1 shows the time course of cytochrome c reduction by PMA-stimulated neutrophils in the absence (trace B) or the presence of 25 µM NO (trace C). As a control, trace A was measured in the absence of neutrophils. NO was added 2 min before the addition of cytochrome c to avoid the possible binding of NO to cytochrome c, because NO is reported to bind to a variety of hemoproteins such as hemoglobin (24) or iron-sulfur centers (25). As shown in Fig. 1A, cytochrome c reduction was not observed when NO was added to the assay buffer, indicating that there is no direct interaction of NO with cytochrome c. These results were consistent with the report that the rate of NO reaction with cytochrome c is severalfold slower than that with oxygen or proteins under aerobic conditions (26). 2 min after the addition of NO to the cell suspension, the residual concentration of NO was less than 1% of its initial concentration, which excludes the quenching of O$_2^-$ through direct reaction of NO to O$_2^-$. Neutrophils without NO produced O$_2^-$ at a rate of 85 nmol/min/10$^7$ cells (trace B), whereas O$_2^-$ generation was decreased to about 70% of that in the presence of NO. These inhibitory effects by NO on O$_2^-$ generation in neutrophils were also supported by the decrease in oxygen consumption in the presence of NO (21 $\pm$ 3.4% reduction). When neutrophils were preincubated with 25 µM nitrite or nitrate (which are end products of NO), no change in O$_2^-$ production was observed. Dose dependence of NO for the inhibition of O$_2^-$ generation is shown in Fig. 2 for neutrophils and solubilized NADPH oxidase. In this comparison, we adjusted the cell number of neutrophils and sample volume of the solubilized oxidase to equalize O$_2^-$ production, i.e. 6.1 nmol of O$_2^-$/min. The inhibitory effect of NO in solubilized NADPH oxidase was less than that in neutrophils. The plausible reason
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**Fig. 2.** Dose dependence of NO-induced inhibition of \(O_2\) generating activity of stimulated neutrophils and solubilized NADPH oxidase from stimulated cells. Neutrophils (\(C\), \(5 \times 10^6\) cells) were preincubated with NO at 0–35 \(\mu M\) for 2 min before addition of 30 \(\mu M\) cytochrome \(c\) at 37 °C and then stimulated by PMA (4 ng in MeSO). Solubilized NADPH oxidase prepared from stimulated cells (\(\lambda\), 35 \(\mu M\) of protein) was preincubated with NO at 0–35 \(\mu M\) 2 min before the addition of 30 \(\mu M\) cytochrome \(c\) at 25 °C. Superoxide generation was started by addition of 0.1 mM NADPH.

for the difference became clear through further experiments to investigate the mechanisms with which NO inactivates the \(O_2\)-generating activity of NADPH oxidase.

First, we examined whether NO impairs the binding ability of NADPH to oxidase (reaction 1). \(K_m\) values for NADPH of the solubilized NADPH oxidase were determined in the presence or the absence of NO. As shown in Table I, the \(K_m\) obtained with NO was almost the same as that of the oxidase in the absence of NO (35.0 ± 4.2 \(\mu M\) versus 30.6 ± 3.4 \(\mu M\), respectively), indicating that NO treatment does not affect the NADPH-binding site of the oxidase.

**Effect of NO on Electron Flux through Redox Centers (Reactions 2 and 3)—** Electron transfer reactions from NADPH to FAD (reaction 2) and from NADPH to cytochrome \(b_{558}\) (reaction 3) were studied using solubilized NADPH oxidase from stimulated neutrophils under anaerobic conditions (Table II). The cytochrome \(c\)-reducing activities of the NADPH oxidase obtained from both stimulated and resting cells were in the same range, either in the presence or the absence of 25 \(\mu M\) NO, indicating that the electron flux from NADPH to FAD is not affected by NO. The interdomain electron transfer from NADPH through FAD to heme in cytochrome \(b_{558}\) was measured by the reduction of cytochrome \(b_{558}\). The reduction rates of cytochrome \(b_{558}\) of both stimulated and resting NADPH oxidase did not differ in the presence or the absence of NO, indicating that the electron flux between FAD and the low spin heme of cytochrome \(b_{558}\) is not the site of impairment by NO.

**The Detection of a Complex between NO and the Heme of Cytochrome \(b_{558}\) (Reaction 4)—** To examine whether NO binds to the heme of cytochrome \(b_{558}\) or changes the spin state of the heme, nitrosyl-heme formation was measured by both visible absorption and EPR spectra of solubilized NADPH oxidase. Fig. 3 shows the difference spectra of cytochrome \(b_{558}\) in NADPH oxidase from resting cells obtained by subtracting the oxidized spectrum from the dithionite-reduced spectrum in the absence (Fig. 3A) and the presence (Fig. 3, B and C) of NO. Spectra were measured after incubating the oxidase with NO for 5 and 15 min at 4 °C (Fig. 3, B and C, respectively). No apparent difference was found in the \(\alpha\)-peak (558 nm) or \(\gamma\)-peak (427 nm) in the presence of NO. The same results were obtained again, when solubilized NADPH oxidase from stimulated cells was examined, suggesting that the structure of heme was not affected by NO. However, a very high and unphysiological concentration of NO (1 mM) changed the cytochrome \(b_{558}\) conformation slightly; the spectral intensity was reduced by about 10%, and the \(\gamma\)-peak was shifted about 0.6 nm.

To investigate whether NO is trapped at the heme site, nitrosyl-iron complex formation was examined by EPR for purified cytochrome \(b_{558}\) reduced with dithionite in the presence of NO at 77–100 K. As shown in Fig. 4A, signals characteristic of nitrosyl-iron complex were not observed. On the other hand, when five-coordinated high spin cytochrome \(b_{558}\) i.e. denatured cytochrome \(b_{558}\) was incubated with NO, a triplet hyper-

### Table I

| NO (\(\mu M\)) | \(K_m\) (\(\mu M\)) | \(V_{max}\) nmol/min/mg protein |
|---------------|---------------------|-----------------------------|
| -NO           | 30.6 ± 3.4          | 315.5 ± 27.4                |
| +NO           | 35.0 ± 4.2          | 223.8 ± 18.5                |

### Table II

| Cytochrome \(c\) reduction | Cytochrome \(b_{558}\) reduction |
|-----------------------------|---------------------------------|
| Stomated                    | Resting                        |
| -NO                         | 55.0 ± 8.2                     | 0.20 ± 0.03                  | 0.21 ± 0.05                |
| +NO                         | 52.4 ± 5.4                     | 43.3 ± 3.5                   | 0.19 ± 0.03                |

**Fig. 3.** Effects of NO on the difference absorption spectra of cytochrome \(b_{558}\). Spectrum A, difference absorption spectrum of cytochrome \(b_{558}\). The NADPH oxidase (5.3 mg protein/ml) solubilized from resting cells was reduced with a minimum amount of dithionite. Spectra B and C, spectra of reduced NADPH oxidase incubated with 25 \(\mu M\) NO for 5 (B) or 15 min (C).
The Effects of NO on the Activation and Assembly Processes of the Reconstituted NADPH Oxidase System (Reaction 5)—The effects of NO on the activation and assembly processes were examined in reconstituted NADPH oxidase (Fig. 5). When the membrane and cytosol components were mixed with myristate for 5 min in the absence of NO, the $O_2^-$-generating activity was 87 mol/s/mol of cytochrome $b_{558}$. When either the membrane or cytosol component was pretreated with NO for 5 min before myristate activation, the $O_2^-$-generating activity was decreased to 76.0 ± 5.3 and 62.6 ± 7.1% of the control, respectively (A and B in Fig. 5). When a mixture of membrane and cytosol components was activated with myristate simultaneously with NO, the $O_2^-$-forming activity was decreased to 26.0 ± 5.3% of control (C in Fig. 5). In contrast, when NO was added after the activation, its inhibitory effect was much weaker (D and E in Fig. 5). These results suggest that the main site of NO-induced inhibition is the impairment of activation, such as the assembly of cytosol protein(s) and the membrane. The inhibition of C in Fig. 5, which was more pronounced than the sum of inhibitions of each component (A and B in Fig. 5), suggests that myristate is the important factor for NO-induced inhibition in addition to the impairment of each component. To further clarify the role of myristate, the cytosol was pretreated with NO in the presence of myristate before mixing with membrane components (D in Fig. 6). The $O_2^-$-generating activity was markedly inhibited to the level when myristate and NO were simultaneously added to membrane and cytosol components (E in Fig. 6). Membrane components were pretreated with NO in a similar way as cytosol, but the inhibitory effect of NO was not changed in the presence of myristate (A and B in Fig. 6). From the results in Table II and Figs. 3 and 4, it becomes clear that NO does not impair redox centers in NADPH oxidase, suggesting that sulfhydryl groups in proteins are candidates to be attacked by NO, because NO is known to react with tissue sulfhydryls to form $S$-nitrosothiol compounds, such as $S$-nitrosocysteine (28).

Some insight for an explanation of the effect of myristate and plausible impaired site appears to be shown by a recent report on the role of Src homology 3 (SH3) domain in p47phox during activation (29). It demonstrated that specifically SH3 domain is folded, masked, and localized at its C-terminal region in dormant cells or in the resting cytosol but opens up upon activation and then binds to the membrane protein(s), mainly cytochrome $b_{558}$. There is one cysteine residue (Cys-196) in the SH3 domain that is masked in the resting state of the oxidase, but once the SH3 domain opens up upon activation by myristate, the thiol group of Cys-196 may become accessible to NO. Because there are three other cysteine residues in the SH3 domain of p47phox and 8 cysteine residues in the cytosolic p67phox protein, NO-induced oxidation of these sulfhydryl groups may be attributable to the 30–35% inhibition seen in the treatment of cytosol with NO (B in Fig. 5). Similarly the 20–25% inhibition upon treatment of the membrane component with NO may also be a result of sulfhydryl group oxidation (A in Fig. 5). This answered the observation shown in Fig. 2 in which the inhib-

![Fig. 4](image1.png)

**Fig. 4.** EPR spectra of intact (A) and denatured (B) reduced cytochrome $b_{558}$ incubated with NO. A, partially purified intact cytochrome $b_{558}$ was reduced with a minimum amount of dithionite and then incubated with 100 μM NO for 15 min at 4 °C. B, high spin cytochrome $b_{558}$ was prepared by heating low spin cytochrome $b_{558}$ at 40 °C for 120 min, followed by reduction with a minimum amount of dithionite. Reduced cytochrome $b_{558}$ with 75% high spin heme was incubated with 100 μM NO for 15 min. EPR spectra were recorded at 77 K.

![Fig. 5](image2.png)

**Fig. 5.** Effect of NO on $O_2^-$ generation in reconstituted NADPH oxidase system. Step A, neutrophil membrane (15 μg) incubated with 25 μM NO for 5 min at 0 °C before activation. Step B, cytosol (135 μg) incubated with NO. Step C, mixture of membrane and cytosol incubated with NO and myristate. Step D, NO added with cytochrome $c$ (cyt $c$) before activation and then incubated with NO for 5 min before the addition of NADPH. Step E, NO was added with 0.1 mM NADPH.

![Fig. 6](image3.png)

**Fig. 6.** Effect of NO on myristate pretreated cytosol or membrane in reconstituted NADPH oxidase system. The amounts of cytosol, membrane, myristate, and NO used in this experiment were the same as in Fig. 5. A, membrane was incubated with NO without pretreatment with myristate. B, membrane was pretreated with myristate and then incubated with NO for 5 min. C, cytosol was incubated with NO without pretreatment with myristate. D, cytosol was pretreated with myristate and then incubated with NO for 5 min and E, same as C in Fig. 5.
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FIG. 7. Contribution of peroxynitrite on $O_2^-$ generation by the solubilized NADPH oxidase from stimulated cells. A–D, after reduction of cytochrome $c$ by $O_2^-$ was observed for 3 min, NO was added. The reaction mixture contained NADPH oxidase (30 μg of protein) and 30 μM cytochrome $c$ in 500 μl of phosphate buffer (50 mM, pH 7.4). $O_2^-$ generation was started by the addition of 0.1 mM NADPH. The concentrations of NO used were 5 (A), 15 (B), 20 (C), and 35 μM (D). Superoxide dismutase (100 units/ml) was added to scavenge $O_2^-$ E, $O_2^-$ generation was measured as shown in A–D, except that 50 μM peroxynitrite was added as shown by the large arrow. F, same as E, but the NADPH oxidase was preincubated with 50 μM peroxynitrite for 2 min, and then $O_2^-$ generation was measured.

Peroxynitrite on Superoxide Generation by the NADPH Oxidase—Because NO was added to the $O_2^-$-generating system under aerobic conditions (leading to the formation of ONOO$^-$ through the reaction of NO and $O_2^-$), we examined the contributions of ONOO$^-$. The production of ONOO$^-$ was measured by the increase in absorbance at 302 nm. Upon addition of 25 μM NO to a solubilized NADPH oxidase preparation forming 310 nmoI $O_2^-$min/mg of protein, the increase in absorbance at 302 nm was 0.03, which corresponds to the generation of 18 nmoI of ONOO$^-$. Fig. 7 shows the NADPH-dependent reduction of cytochrome $c$ with $O_2^-$ produced by solubilized NADPH oxidase obtained from stimulated cells. When NO was added to an oxidase preparation generating $O_2^-$, the cytochrome $c$ reduction suddenly stopped and then restarted after a lag time. As seen in Fig. 7, this lag time increased with increasing NO concentration. There are at least three explanations for the abrupt cessation of the cytochrome $c$ reduction: (i) The $O_2^-$-generating activity of the NADPH oxidase is completely inhibited by ONOO$^-$ from the reaction of endogenously generated $O_2^-$ and the added NO, (ii) ONOO$^-$ oxidized the reduced cytochrome $c$ by $O_2^-$, (iii) The generated $O_2^-$ was quenched by NO much faster than it reacted with cytochrome $c$. The first explanation is unlikely, because the addition of ONOO$^-$ (50 μM) to the reaction mixture or pretreatment of the NADPH oxidase with ONOO$^-$ (50 μM) did not inhibit $O_2^-$ generation in the oxidase (E and F in Fig. 7, respectively). The second explanation is also unlikely, because the oxidation of reduced cytochrome $c$ by ONOO$^-$ is as slow as that with hydrogen peroxide (30). Therefore, we conclude that the temporary lag in cytochrome $c$ reduction is caused by the “quenching” of $O_2^-$ by NO. Actually the reaction of $O_2^-$ with NO is much faster than the reduction reaction of cytochrome $c$ by $O_2^-$ Note that after the lag time the reduction rate of cytochrome $c$ returned to nearly the initial value. The slight decrease in the reduction rate of cytochrome $c$ after NO addition is consistent with the slight decrease in the $O_2^-$ generation shown in the reconstituted NADPH oxidase (D and E in Fig. 5). This decrease in the slopes in Fig. 7 was also concentration-dependent. These results suggest that ONOO$^-$ formed by the addition of NO to $O_2^-$ is not a major factor in the inhibition of the $O_2^-$ generation in NADPH oxidase. This conclusion was also supported by findings in neutrophils treated with ONOO$^-$ (data now shown).

In summary, we have found that NO suppresses the $O_2^-$-generating activity of neutrophil NADPH oxidase. The suppression is more pronounced when NO attacks the oxidase during the activation process. The suppression is trivial after the activation (assembly) is completed. The affected site is a protein itself, either membrane or cytosol proteins, and the binding site of NADPH and redox centers are not impaired by NO. The results are of significant importance in various inflammatory conditions because a large amount of NO can be produced by inducible NO synthase and also a large amount of NO$^-$ is produced by infiltrating phagocytes. Furthermore, the evidence that NO inhibited the $O_2^-$-generating activity of the NADPH oxidase suggests a new mechanism for modulating the $O_2^-$-generating activity of neutrophils by endogenous NO produced by constitutive NO synthase, because expression of constitutive NO synthase and NO generation by constitutive NO synthase in neutrophils have already been shown (31–33). This mechanism might protect tissues and cells from oxidative stress at inflammation sites in vivo by the attenuation of the $O_2^-$ concentration by NO. Peroxynitrite does not play a role in this physiological process.

Acknowledgment—We are very grateful to Prof. Lawrence J. Berliner of Ohio State University for critical reading of our manuscript.

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