Characterization of Nitric Oxide Consumption Pathways by Normal, Chronic Granulomatous Disease and Myeloperoxidase-Deficient Human Neutrophils

Stephen R. Clark, Marcus J. Coffey, Rhona M. Maclean, Peter W. Collins, Malcolm J. Lewis, Andrew R. Cross and Valerie B. O'Donnell

_J Immunol_ 2002; 169:5889-5896; doi: 10.4049/jimmunol.169.10.5889
http://www.jimmunol.org/content/169/10/5889

References

This article cites 64 articles, 43 of which you can access for free at:
http://www.jimmunol.org/content/169/10/5889.full#ref-list-1

Subscription

Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Characterization of Nitric Oxide Consumption Pathways by Normal, Chronic Granulomatous Disease and Myeloperoxidase-Deficient Human Neutrophils

Stephen R. Clark,* Marcus J. Coffey,* Rhona M. Maclean,§ Peter W. Collins,‡ Malcolm J. Lewis,† Andrew R. Cross,§ and Valerie B. O’Donnell²*

The detailed mechanisms by which acutely activated leukocytes metabolize NO and regulate its bioactivity are unknown. Therefore, healthy, chronic granulomatous disease (CGD) or myeloperoxidase (MPO)-deficient human neutrophils were examined for their ability to consume NO and attenuate its signaling. fMLP or PMA activation of healthy neutrophils caused NO consumption that was fully blocked by NADPH oxidase inhibition, and was absent in CGD neutrophils. Studies using MPO-deficient neutrophils, enzyme inhibitors, and reconstituted NADPH oxidase ruled out additional potential NO-consuming pathways, including Fenton chemistry, PGH synthase, lipoxygenase, or MPO. In particular, the inability of MPO to consume NO resulted from lack of H₂O₂ substrate since all superoxide (O₂⁻) reacted to form peroxynitrite. For healthy or MPO-deficient cells, NO consumption rates were 2- to 4-fold greater than O₂⁻ generation, significantly faster than expected from a 1:1 termination of NO with O₂⁻. Finally, fMLP or PMA-stimulated NO consumption fully blocked NO-dependent neutrophil cGMP synthesis. These data reveal NADPH oxidase as the central regulator of NO signaling in human leukocytes. In addition, they demonstrate an important functional difference between CGD and either normal or MPO-deficient human neutrophils, namely their inability to metabolize NO which will alter their ability to adhere and migrate in vivo. The Journal of Immunology, 2002, 169: 5889–5896.

Nitric oxide regulates leukocyte recruitment and attachment through suppression of adhesion molecule activity, e.g., CD11/CD18 in neutrophils (1–4). Therefore, strict control of NO is essential for regulating leukocyte trafficking in vivo. Neutrophils are the most abundant leukocyte and can consume NO in a partially superoxide dismutase (SOD)³-inhibitable manner (5). This indicates some involvement of NADPH oxidase, although the detailed mechanisms by which leukocytes control NO responses, particularly in response to physiological agonists, have not been characterized. NADPH oxidase-knockout mice show decreased parasite- or hypercholesterolemia-induced leukocyte adhesion and migration, suggesting that leukocyte superoxide (O₂⁻) controls NO responses through causing its removal in vivo (6, 7). Finally, neutrophils contain several additional oxidases that can catalytically consume NO in vivo, including lipoxygenase (LOX), myeloperoxidase (MPO), and PGH synthase (PGHS), and the ability of these to modulate NO signaling within the cells has not been examined (8–14).

Herein, the mechanisms and consequences of NO consumption by isolated neutrophils from healthy, chronic granulomatous disease (CGD) or MPO-deficient humans activated with the bacterial peptide fMLP or the protein kinase C activator PMA were studied. The results reveal a critical role for NADPH oxidase with CGD neutrophils being unable to consume NO following stimulation. Rates of NO consumption by normal or MPO-deficient cells were several-fold faster than expected based on the 1:1 reaction between NO and O₂⁻; however, there was no role for MPO due to insufficient formation of H₂O₂ substrate. Finally, NADPH oxidase-dependent NO removal effectively prevented activation of soluble guanylate cyclase (sGC). These data indicate that human neutrophil NADPH oxidase is a critical regulator of NO signaling in neutrophils, and reveal an important functional difference between CGD and either normal or MPO-deficient leukocytes that will alter their ability to overcome the inhibitory effects of NO on adhesion and migration in vivo.

Materials and Methods

Neutrophil isolation

Whole blood was obtained from healthy volunteers free from nonsteroidal anti-inflammatory drugs for over 14 days. MPO-deficient patients were identified by routine screening at University Hospital Wales (Cardiff, U.K.). Whole blood from CGD patient was a kind gift from L. Moreton (Great Ormond Street Hospital, London, U.K.). Ethical permission for all donations was obtained from the Bro Taf Local Research Ethics Committee. Human neutrophils were isolated as described previously, by dextran sedimentation and Ficoll centrifugation and resuspended in a small volume of PBS, counted, and kept on ice (15).

Neutrophil fractionation

For the preparation of subcellular fractions, neutrophils were obtained from normal subjects and CGD patients by leukapheresis (16) and purified as above with the omission of dextran sedimentation. Neutrophils were...
treated with 2.5 mM disopropyl fluorophosphate for 10 min at 4°C, disrupted in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1 mM ATP, 1.25 mM EGTA, 10 mM PIPES, pH 7.3) by N₂ cavitation, and fractionated on discontinuous Percoll gradients (17, 18). This produced cytosol and plasma membrane fractions whose full concentrations were adjusted to 9 × 10⁶ and 1.25 × 10⁷ cells equivalents (cell eq ml⁻¹), respectively. Fractions were stored at −80°C for up to 1 year without loss of activity.

**Purification, repurification, and reevaluation of flavocytochrome b₅₅₅**

Flavocytochrome b₅₅₅ was purified from human neutrophils, using the methods described previously (19, 20). The final product contained 18–22 nmol heme mg⁻¹ protein, based on a molar absorption coefficient of 21.6 mM cm⁻¹ (cytochrome heme) for the dichloro-duced minus air-oxidized absorbance band at 559 nm (21). Typical activities are 120–150 mol O₂⁻ s⁻¹ mol heme.

**Purification and assay of MPO**

MPO (specific activity = 0.49 ± 0.03 mol guaiacal min⁻¹ mol) was purified from neutrophil azurophil granule preparations made as a byproduct during neutrophil fractionation described above, as described (22). MPO activity was assayed by the H₂O₂-stimulated rate of guaiacol oxidation at 37°C using ε₃₄₀ = 26.6 mM cm⁻¹ (23, 24).

**NO consumption**

Anaerobic solutions of NO were prepared as described previously and measured electrochemically using an NO sensor (Harvard AmiNO700 or World Precision Instruments 2-mm probe with inNO meter; Sarasota, FL) (9). Where NO loss was not linear, rates are given as first order rate constants (Kₐb). For all Kₐb, the square of the Pearson product moment correlation coefficient (r) of the slope of the replotted data was >0.9, confirming that the reaction was first order. Where NO consumption was linear, NO disappearance was determined and the background rate of NO loss subtracted. For measurement of neutrophil NO consumption, NO (1.9–3.8 μM) was added to 0.5 ml PBS, 2.5 mM CaCl₂, and 1.2 mM MgCl₂ with neutrophils at 37°C with stirring. Once the electrode response had stabilized, 1 μM FMA or 1 μM IMLP was added. NO consumption by isolated neutrophil membranes was measured following addition of 160 μM NADPH to 0.5 ml relaxation buffer containing 5.4 × 10⁶ cell eq of membrane extract ml⁻¹, 1.5 × 10⁷ cell eq of cytosol ml⁻¹, 10 μM GTP-γ-S, and 100 μM SDS, in the chamber of the NO electrode at 37°C with stirring. To allow assembly of NADPH oxidase components, all constituents (except NADPH) were preincubated at 25°C for 2 min, followed by 37°C for 3 min before addition of 3.8 μM NO and/or NADPH.

**RIA of cGMP**

Neutrophils (4 × 10⁶ cells ml⁻¹) in 500 μl PBS, 2.5 mM CaCl₂, and 1.2 mM MgCl₂ were placed in the chamber of the NO electrode with stirring at 37°C. NO (1.9 μM) was added, then 1 μM IMLP with/without 3 μM oxyHb. In other experiments, 1 μg ml⁻¹ PMA was added, with the phosphodiesterase inhibitor, 3-iso-2,5,6-diacetyl-1-methyl-xanthine (IBMX; 1 μM). Samples were incubated for 5 min then aliquots removed for cGMP analysis using a commercial RIA (Amersham Pharmacia Biotec, Bucks, U.K.).

**O₂⁻ generation assay**

Neutrophil O₂⁻ generation was assayed by the SOD-inhibitable reduction of cytochrome c (cyt. c) measured at 37°C with stirring using ε₅₅₅ = 21.1 μM cm⁻¹ (25). IMLP (1 μM) was added to 0.25 × 10⁷ ml⁻¹ neutrophils (10¹⁰ ml⁻¹ for CGD neutrophils) in 2 ml PBS with 2.5 mM CaCl₂, 1.2 mM MgCl₂, and 50 μM cyt. c with/without SOD (300 U ml⁻¹; Oxiss, Portland, OR). O₂⁻ generation by isolated membranes was measured on addition of 160 μM NADPH to 0.75 ml relaxation buffer containing 5.4 × 10⁶ cell eq of membrane extract ml⁻¹, 1.5 × 10⁷ cell eq of cytosol ml⁻¹, 10 μM GTP-γ-S, 100 μM SDS, 50 μM cyt. c. To allow assembly of NADPH oxidase components, all constituents (except NADPH) were preincubated at 25°C for 2 min, followed by 37°C for 3 min before addition of NO and/or NADPH.

**O₂ consumption assay**

Human neutrophil O₂ consumption was measured electrochemically using a Clark-type O₂ electrode (Rank Brothers, Cambridge, U.K.). Calibrations were performed by addition of H₂O₂ to PBS with 34 U ml⁻¹ catalase. A total of 1 μM IMLP was added to 0.5 ml PBS with neutrophils (2 × 10⁶ cells ml⁻¹), 2.5 mM CaCl₂, 1.2 mM MgCl₂, 300 U ml⁻¹ CuZn-SOD, and 34 U ml⁻¹ catalase at 37°C with stirring. Excess SOD and catalase were routinely included during neutrophil O₂ uptake measurements to ensure full reduction of O₂ to H₂O₂ according to the following equation:

\[4\text{O}_2 + 4\text{H}^+ \rightarrow 2\text{H}_2\text{O} + 3\text{O}_2\]

Measured rates of O₂ consumption were multiplied by four to give true O₂ generation rates.

**NADPH oxidation assay**

NADPH oxidation was determined at 340 nm, following addition of 160 μM NADPH to 0.75 ml relaxation buffer with 1.5 × 10⁷ cell eq of cytosol ml⁻¹; 10 μM GTP-γ-S, 100 μM SDS, and 2.7 × 10⁶ cell eq ml⁻¹ neutrophil membranes from a normal subject, a patient with partially functional flavocytochrome b₅₅₅ designated X₉₁₁ (26), or purified flavocytochrome b₅₅₅ at 25°C. To allow assembly of NADPH oxidase components, all constituents (except NADPH) were preincubated at 25°C for 5 min, before NADPH addition. Absorbance was measured before and after addition of 345 μM 2-(N,N-diethylamino)-diazenolate-2-oxide (DeaNONOate) which releases 30 μM NO min⁻¹ (27).

**Assays of neutrophil MPO**

Western blotting was performed as described (28–30). Briefly, samples (10⁶ cell eq) were probed with rabbit polyclonal anti-human MPO Ab (Calbiochem, San Diego, CA) (1:1000) and visualized using ECL (Amersham Pharmacia Biotech). MPO activity was assayed as described for purified MPO in the absence or presence of 2 mM aminotriazole (ATZ) or 1 mM azide to enable MPO vs eosinophil peroxidase to be determined (24).

**Kinetic simulations**

To understand the chemical behavior of reactive intermediates generated by activated neutrophils, simulations were performed using the Euler method, with software written by P. Neese (Universitat Konstanz, Germany). This algorithm uses numerical methods to solve the simultaneous differential equations generated from the reactions.

**Results**

**Characterization of NO loss in the electrode system**

NO (2–4 μM) decay in aerobic buffer at 37°C followed first order kinetics, with Kₐb depending on probe (Kₐb = 2.8 ± 0.3 × 10⁻³ sec⁻¹ r = 0.99, or 7.9 ± 0.9 × 10⁻³ sec⁻¹ r = 0.99, for World Precision Instruments 2 mm or Harvard AmiNO700 probes, respectively). This indicates that NO oxidation by the electrode and diffusion into the gas phase cause NO decay in these experiments, rather than autoxidation to form NO₂ which is second order. Using these Kₐb, rates of background NO loss were subtracted from all experiments. For accurate determination of NO consumption rates by cells, bolus additions of NO were used instead of donor compounds.

**Activated human neutrophils consume NO**

In the presence of resting neutrophils, NO disappearance from aerobic buffer did not increase over background and was still first order (Kₐb = 2.8 ± 0.1 or 2.4 ± 0.5 × 10⁻³ sec⁻¹ for buffer alone, or with cells, respectively). Neutrophil activation with 1 μM IMLP caused an immediate increase in NO loss (8.7 ± 0.8 mmol min⁻¹ 10⁶ cells; Fig. 1A). Similar results were obtained using PMA (1 μg ml⁻¹) as stimulus (data not shown).

**NO removal is dependent on O₂⁻ generation by NADPH oxidase**

To determine the involvement of O₂⁻ in NO consumption, 3000 U ml⁻¹ SOD or 20 μM diphenyleneiodonium (DPI) were added. Either agent alone significantly inhibited neutrophil NO consumption, however in combination, NO removal was totally blocked (Fig. 1B). Incubation with the SOD-mimetic manganese porphyrin MnTE-2-Py5⁺ (MnP, 20 μM) also attenuated NO consumption (Fig. 1B). In further support of the central role of O₂⁻ in mediating NO consumption, neutrophils from a CGD patient did not consume
NO consumption and $O_2^\bullet -$ generation were $8.7 \pm 0.8$ and $3.4 \pm 0.8$ nmoles min$^{-1}$ 10$^6$ cells, respectively; Fig. 2A). Similar results were obtained using PMA (data not shown). All experiments were conducted in HEPES- and glucose-free PBS (supplemented with CaCl$_2$ and MgCl$_2$) to ensure that artifactual radical reactions did not cause NO consumption, although comparisons in Kreb’s Ringer buffer yielded identical data (not shown). To confirm that cyt. c reduction was an accurate measure of $O_2^\bullet -$ generation, three additional approaches were taken.

**Cell-free reconstitution system.** Intact cells contain intracellular phagolysosomal compartments that might cause underestimation of $O_2^\bullet -$ generation. Therefore, pre-mixing isolated cytosol and membrane with SDS and GTP-γ-S allows NADPH oxidase to be assayed in a detergent-containing cell-free system, where all $O_2^\bullet -$ is accessible (22). Using membranes prepared from healthy neutrophils, rates of NO consumption were significantly faster than $O_2^\bullet -$ generation ($1.97 \pm 0.08$ vs $0.95 \pm 0.14$ nmol min$^{-1}$ 10$^6$ cell eq, respectively; Fig. 2B), similar to intact cell experiments (Fig. 2A).

**O$_2$ consumption.** During $O_2^\bullet -$ generation, stoichiometric amounts of $O_2$ are consumed through reduction by NADPH oxidase. Rates of fMLP-stimulated $O_2$ consumption were not significantly different to those measured using cyt. c reduction ($13.8 \pm 1.8$ vs $15.4 \pm 0.2$ μM min$^{-1}$ for cyt. c reduction and $O_2$ consumption, respectively).

**Simulations.** To examine the fate of NO and oxidant species generated in our experiments, reactions were simulated using rate constants in Table I. This showed that under our conditions all $O_2^\bullet -$ directly reacted with cyt. c, without dismutation to H$_2$O$_2$. Although rate constants for spontaneous $O_2^\bullet -$ dismutation and the reaction of $O_2^\bullet -$ with cyt. c are similar, dismutation of $O_2^\bullet -$ is second order. Therefore, in the presence of 50 μM cyt. c, continuous removal of $O_2^\bullet -$ and stoichiometric reduction of cyt. c occurs.

Collectively, these data indicate that $O_2^\bullet -$ generation rates are accurately determined in our experiments and confirm that NADPH oxidase-dependent NO consumption is considerably faster than the simple 1:1 reaction expected from termination of NO and $O_2^\bullet -$.

**NO does not stimulate NADPH oxidase**

To determine whether the fast rates of NO consumption were due to direct stimulation of electron flux through NADPH oxidase by NO (31), NADPH oxidation was determined using the cell-free reconstitution system as above where the enzyme is already active before NO addition. In this study, NADPH oxidation by highly purified flavocytochrome b$_{558}$ was not stimulated by NO (Fig. 2C). Also, NADPH oxidation by membranes from a CGD patient with partial NADPH oxidase activity, designated X91$^+$, (26) was not stimulated by NO (Fig. 2D). This mutant enzyme cannot directly reduce $O_2$, but can transfer electrons from NADPH to the flavin center and thence to artificial electron acceptors, and along with the purified flavocytochrome b$_{558}$ was used to reveal whether NADPH oxidase could directly reduce NO via the flavin or heme cofactors. For comparison, NADPH oxidation by normal membranes is shown (Fig. 2D).

**Effect of inhibitors and scavengers on NO consumption**

Addition of 100 μM diethylthiocarbamoyl acid, 20 μM indomethacin, 2 mM ATZ, 1 mM azide, or 2 mM urate were without effect on NO consumption, ruling out a role for Fenton chemistry, PGHS, or MPO (Fig. 3). Although urate is an effective scavenger of peroxyxinitrite (ONOO$^-$), its oxidation to radicals that can potentiate secondary oxidation processes do not rule out a role for ONOO$^-$ in mediating NO consumption (32, 33).
The rate of NO consumption by MPO-deficient neutrophils is greater than the rate of $\mathrm{O}_2^-$ production.

Neutrophils were isolated from a patient with MPO activity that was 9% of healthy controls (Fig. 4A). Western blotting of these cells showed virtually undetectable MPO at 60 kDa, and the heme spectrum at 472 nm was absent (Fig. 4A, inset and data not shown). NO consumption by MPO-deficient neutrophils was considerably faster than $\mathrm{O}_2^-$ generation (14.59 ± 2.26 vs. 4.45 ± 0.50 nmol min$^{-1}$ 10$^6$ cells, respectively; Fig. 4B). These differences are even greater than for healthy neutrophils (Fig. 2A). Addition of purified MPO to MPO-deficient cells at concentrations found in healthy subjects (11.25 pmol/10$^6$ cells, calculated from guaiacol oxidation rates and heme spectra) did not further stimulate NO consumption (14.90 ± 3.67 nmol min$^{-1}$ 10$^6$ cells; Fig. 4C). However, with 100 μM H$_2$O$_2$ substrate, this concentration of MPO consumed NO at easily detectable rates (4.2 μM min$^{-1}$, which would be equivalent to 5.26 ± 0.70 nmol min$^{-1}$ 10$^6$ cells, data not shown). This indicates that unlike exogenous H$_2$O$_2$, IMLP-activated neutrophils cannot support MPO-dependent NO consumption.

Kinetic simulations of NO consumption by $\mathrm{O}_2^-$ and MPO

Reactions for NO included its diffusion-limited reaction with $\mathrm{O}_2^-$ (equations 2, 4, and 6), but the second-order autoxidation reaction has not been determined; however, this reaction is considerably faster than compound II reaction in our system. Although neutrophils contain intracellular SOD, $\mathrm{O}_2^-$ is generated extracellularly in these experiments, so simulation that occurs will be spontaneous (Table II, equation 3). Some simulations also included MPO, with NO consumption occurring via reduction of either compound I or II and using either GTP-S (10 μM) or SDS (100 μM) as described in Materials and Methods. Rates of NADPH oxidation were measured before and after addition of 345 μM DeaNONOate which gives an NO release rate of 30 μM NO min$^{-1}$ (t$\text{1/2}$ = 16 min, 22–25°C; Ref. 27). D, NADPH oxidation by isolated membranes from a CGD patient with functional NADPH oxidase flavin (X91+) was measured following addition of NADPH (160 μM) to 0.75 ml relaxation buffer with 2.7 × 10$^6$ cell eq of membrane extract ml$^{-1}$, 1.5 × 10$^7$ cell eq of cytosol ml$^{-1}$, 10 μM GTP-γ-S, and 100 μM SDS as described in Materials and Methods. NADPH oxidation was measured before and after addition of 345 μM DeaNONOate. NADPH oxidation by healthy neutrophil membranes is included for reference (n ≥ 3, mean ± SD).

### Table 1. Rate constants used for kinetic simulation of cyt. c assay (see main text for results)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate Constant</th>
<th>Ref.</th>
<th>Reactant</th>
<th>Starting Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) $\mathrm{O}_2$ → $\mathrm{O}_2^-$ (to give $\mathrm{O}_2^-$ generation rate of 3.4 μM.min$^{-1}$)</td>
<td>2.38 × 10$^{-4}$ s$^{-1}$</td>
<td></td>
<td>(1) $\mathrm{O}_2$</td>
<td>240</td>
</tr>
<tr>
<td>(2) cyt. $\mathrm{c}^{2+}$ + $\mathrm{O}_2$ → cyt. $\mathrm{c}^{3+}$ + $\mathrm{O}_2$</td>
<td>2.6 × 10$^9$ M$^{-1}$ s$^{-1}$</td>
<td>61</td>
<td>(2) cyt. $\mathrm{c}^{2+}$</td>
<td>50</td>
</tr>
<tr>
<td>(3) $2\mathrm{O}_2^-$ + 2H$^+$ → H$_2$O$_2$ + O$_2$</td>
<td>8 × 10$^9$ M$^{-1}$ s$^{-1}$</td>
<td>62</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
MPO was independent of the value of this rate constant. Therefore, our final model uses the value of equation 13 to model the complete set of NO consumption reactions by MPO.

Initial simulations using equations 1–4 (Table II) at \( O_2^\cdot \) generation rates of 1.7 \( \mu M \) min\(^{-1} \) (i.e., equivalent to 3.4 nmol min\(^{-1} \) \( \times 10^9 \) cells), where NO only reacted with \( O_2^\cdot \), revealed that NO consumption rates were identical with \( O_2^\cdot \) generation, with all \( O_2^\cdot \) forming ONOO\(^{-} \), and virtually no dismutation to \( H_2O_2 \) (Fig. 5A). Including reactions of MPO caused no change in the rate of NO decay (Fig. 5A, Table II, equations 10–13). The lack of MPO-catalyzed NO consumption results from insufficient generation of \( H_2O_2 \) substrate (<1 pm). Although ONOO\(^{-} \) could potentially stimulate MPO-dependent NO consumption, its concentration remained <100 nM (34). The complete reaction sequence incorporating equations 1–9 shows the formation and decay of NO, \( O_2^\cdot \), \( H_2O_2 \), and ONOO\(^{-} \) during NADPH oxidase-dependent NO consumption and clearly shows that at these \( O_2^\cdot \) generation rates, \( H_2O_2 \) does not form until all NO has been consumed (Fig. 5B).

**NO consumption prevents neutrophil sGC activation**

cGMP generation was determined following a 5-min incubation of neutrophils with 1.9 \( \mu M \) NO, with or without fMLP, or oxyHb as NO scavenger (Fig. 6A). Experiments were repeated with the phosphodiesterase inhibitor IBMX which inhibits cGMP hydrolysis. IBMX blocks agonist-induced neutrophil activation (via cAMP elevation); therefore, in this experiment, cells were stimulated with PMA (Fig. 6B). Following incubation with 1.9 \( \mu M \) NO, elevations in neutrophil cGMP were found; however, this was effectively inhibited by simultaneous generation of \( O_2^\cdot \) (Fig. 6). Addition of 3 \( \mu M \) oxyHb to scavenge NO also fully blocked NO activation of sGC (Fig. 6).

**Discussion**

In this study, normal or MPO-deficient, but not CGD human neutrophils consumed NO through NADPH oxidase-dependent mechanisms at rates significantly faster than corresponding \( O_2^\cdot \) generation (Figs. 1 and 2). Also, NO consumption effectively blocked cGMP synthesis in the neutrophils (Figs. 1C and 5). This indicates that the ability of activated leukocytes to attenuate the bioactivity of NO is considerably greater than expected from the simple 1:1 termination between NO and \( O_2^\cdot \), and that NADPH oxidase deficiency in CGD will prevent neutrophils from attenuating the inhibitory effects of NO in vivo.

Herein, a combination of SOD and DPI fully inhibited NO consumption (Fig. 1B). The incomplete inhibition by SOD alone results from SOD-catalyzed \( O_2^\cdot \) generation which occurs when \( H_2O_2 \) builds up in the presence of high concentrations of SOD (J. P. Crow and J. S. Beckman, unpublished observations). Similarly, DPI alone did not fully inhibit NO consumption (Fig. 1). DPI reacts with a catalytic intermediate formed during flavin turnover; therefore, some enzyme catalysis occurs before full inhibition (35). The combination of DPI and SOD was fully effective, as residual \( O_2^\cdot \) produced during DPI inhibition was scavenged by SOD (Fig. 1B). Along with the lack of NO consumption by CGD cells, these data indicate that NADPH oxidase is absolutely required for fMLP or PMA-stimulated NO consumption by acutely activated human neutrophils (Fig. 1C).

Several additional oxidases could potentially consume NO in leukocytes, for example, PGHS-1, 15-LOX, and MPO (10, 11, 12, 14). However, these did not remove NO following acute activation of neutrophils. In particular, the lack of NO consumption by MPO was intriguing since this enzyme constitutes 5% of neutrophil protein, and was recently shown to catalyze \( H_2O_2 \)-dependent NO consumption following either cellular overexpression or transcytosis.
of MPO into rat aortic endothelium (14). The inability of MPO to
catalyze NO consumption following addition of exogenous purified
enzyme to MPO-deficient cells shows that even when all MPO
is extracellular, fMLP-stimulated neutrophils cannot support
MPO-dependent NO consumption (Fig. 4C). Furthermore, azide
and ATZ which effectively block consumption of NO by purified
MPO and leukocyte-dependent nitration of tyrosine are without
effect on neutrophil NO consumption (Fig. 3; Refs. 12 and 36). NO
metabolism by purified or cellular MPO is critically dependent on
exogenously added H2O2 (12, 14). In this study, kinetic simulations
showed that all O2•− generated by agonist-activated neutro-
phils in the presence of NO forms ONOO−, with no dismutation to
H2O2 (Fig. 5B). In agreement, total inhibition of H2O2 generation
when NO is consumed, H2O2 and O2•− levels increase to micromolar levels, and
ONOO− disappears.

These observations don’t exclude a role for MPO in cata-
lyzing NO consumption when H2O2 is generated by NADPH ox-
idase-independent mechanisms. In this regard, LPS injection in vivo attenuates acetylcholine-dependent vasorelaxation in wild
type, but not MPO−/− mouse aortic rings (14). In that system, H2O2 could form independent of O2•− from diverse vascular and
reticuloendothelial sources, including xanthine oxidase catalysis or
mitochondrial leakage of electrons (38, 39).

Others have suggested that leukocyte-contained MPO may pro-
mote different reactions than MPO present in the extracellular mi-
lieu, following observations that neutrophil-associated MPO does
not nitrate phagocytosed probes or bacterial proteins, in contrast to
purified MPO (40, 41). In addition, critical differences are emerg-
ing regarding the relative rates of MPO/NO2•/H2O2-dependent

Table II. Kinetic simulation of NO consumption

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate Constant</th>
<th>Ref.</th>
<th>Reactant</th>
<th>Starting Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) O2 + e− → O2•− (to give O2•− generation rate of 3.4 nmol/min • 106 cells)</td>
<td>2.38 × 10−7 M−1 s−1</td>
<td>(1) O2</td>
<td>240 μM</td>
<td></td>
</tr>
<tr>
<td>(2) first order NO decay</td>
<td>2.87 × 10−3 s−1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) 2O2•− + 2H+ → H2O2 + O2</td>
<td>8 × 104 M−1 s−1</td>
<td>62</td>
<td>(2) NO</td>
<td>3.8 μM</td>
</tr>
<tr>
<td>(4) NO + O2•− → ONOO−</td>
<td>1.9 × 1010 M−1 s−1</td>
<td>63</td>
<td>(3) MPO</td>
<td>11.25 nM</td>
</tr>
<tr>
<td>(5) ONOO− → NO2•−</td>
<td>0.69 s−1</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6) NO + ONOO− → NO2 + NO3−</td>
<td>1.3 × 10−3 M−1 s−1</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7) NO + NO2 → N2O3</td>
<td>1.1 × 106 M−1 s−1</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8) N2O3 → NO + NO2</td>
<td>4.3 × 108 s−1</td>
<td>66, 67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9) N2O3 + H2O → 2NO2 + 2H+</td>
<td>1.6 × 109 s−1</td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10) MPO + H2O2 → Cl−</td>
<td>1.8 × 107 M−1 s−1</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(11) MPO + ONOO− → Cl−</td>
<td>6.2 × 106 M−1 s−1</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12) NO + Cl− → ClII</td>
<td>8 × 105 M−1 s−1</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(13) NO + ClII → MPO</td>
<td>8 × 105 M−1 s−1</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
reactions in different types of inflammation. For example, recent studies using MPO−/− mice showed that the contribution of MPO/NO3−/H2O2 to nitration reactions is highly disease model-dependent with no role found in leukocyte-rich acute inflammatory models (42).

In contrast to MPO, PGHS expression by freshly isolated neutrophils is low, suggesting that this pathway may not consume NO in resting neutrophils (43). Finally, while neutrophils constitutively express 5-LOX protein, its activation in response to FMLP is poor (44).

An unexpected finding was that NO consumption was considerably faster than O2− generation. To determine whether this resulted from either 1) enhanced NADPH oxidase assembly rates, or 2) stimulation of electron flux through NADPH oxidase by NO, a cell-free reconstitution system was used, where the complex pre-assembled in the absence of NO, and electron flux was directly measured by NADPH oxidation (31, 45–47). Using membranes from a normal subject, NO consumption was significantly faster than O2− generation, similar to agonist-activated cells, indicating that accelerated NO consumption does not require NO to be present during NADPH oxidase assembly (Fig. 2B). Next, NADPH oxidase was determined in the reconstitution system using either highly purified flavocytochrome b558 or membranes from a patient with partially functional oxidase (X91); Ref. 26). In this study, NO did not enhance NADPH oxidation by either enzyme preparation, demonstrating that it does not stimulate electron flux through turning-over enzyme, nor accept electrons directly from a patient with partially functional oxidase (X91); Ref. 26). In this study, NO did not enhance NADPH oxidation by either enzyme preparation, demonstrating that it does not stimulate electron flux through turning-over enzyme, nor accept electrons directly from reduced NADPH oxidase flavin (Fig. 2D). Collectively, these data indicate that the accelerated rates of NO consumption are not due to NADPH oxidase stimulation by NO.

ONOO− is a reactive species that crosses cell membranes, mediating oxidation of intracellular constituents including sugars, thiols, proteins, and lipid (48–53). Its addition to macrophages forms electron paramagnetic resonance-detectable thiol and protein tyrosyl radicals that may directly consume NO (54). Inclusion of the ONOO− scavenger urate did not significantly attenuate neutrophil NO consumption. However, urate oxidation by ONOO− forms reactive radicals that can still consume NO (33). Therefore, although this could not be conclusively proven, oxidation of biomolecules by ONOO− in activated neutrophils may cause the accelerated NO consumption observed herein.

An important finding was the profound differences in NO-metabolizing capacity of CGD vs either MPO-deficient or healthy neutrophils. CGD is a rare and devastating condition, characterized by recurrent life-threatening infections, whereas MPO deficiency is relatively common (1:2000), with only a slight increased susceptibility to Candida infection (55–57). Reasons for this difference are unclear since both CGD and MPO-deficient phagocytes show similarly impaired bacterial killing in vitro (57). In the vasculature, phagocyte adhesion and migration is suppressed through the actions of endothelial-derived NO via cGMP-dependent inhibition of CD18/CD11 activity (1, 4, 58, 59). Therefore, the inability of CGD leukocytes to metabolize NO may render them more sensitive to its inhibitory effects. In vitro studies under NO-free conditions show that chemotaxis of neutrophils from CGD patients does not differ from healthy controls (60). However, decreased leukocyte adhesion and emigration in cholesterol-fed p47phox−/− mice and delayed monocyte or T cell migration into livers of Leishmania donovani-infected gp91phox−/− mice indicate that lack of NADPH oxidase renders leukocytes less able to migrate during inflammation in vivo (6, 7). This is consistent with a critical role for NADPH oxidase in regulating leukocyte responses to NO, and would not be expected in MPO deficiency where NO consumption following agonist activation is preserved (Fig. 4).

In summary, human neutrophils consume NO at unexpectedly fast rates via NADPH oxidase turnover, effectively inhibiting NO signaling in the cells themselves (6). These findings have implications for the role of NADPH oxidase in the development of inflammatory vascular disease, and for the pathophysiology of CGD where leukocyte NO consumption following agonist activation is absent.

Acknowledgments

We thank Prof. B. A. Freeman for helpful suggestions and L. Moreton for gift of CGD neutrophils.

References

27. Cross, A. R., J. Rae, and J. T. Curnutte. 1995. Cytochrome B-245 of the neu-
tronophils superoxide-generating system contains two nonidentical hemes: poten-
microgram quantities of protein utilizing the principle of protein-dye binding. 
30. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of pro-
teins from polyacrylamide gels to nitrocellulose sheets: procedure and some ap-
cyte superoxide generation by nitric oxide and peroxynitrite. J. Biol. Chem. 
275:38965.
33. Santos, C. X., E. I. Anjos, and O. Augusto. 1999. Uric acid oxidation by per-
oxynitrite: multiple reactions, free radical formation, and amplification of lipid 
myeloperoxidase with peroxynitrite: a comparison with lactoperoxidase, horse-
the inhibitory mechanism of iodonium compounds with special reference to 
nitric oxide-derived oxidants by myeloperoxidase in monocyes: pathways for 
85:950.
Chem. 270:29350.
39. White, C. R., V. Darley-Usmar, W. R. Berrington, M. McAdams, J. Z. Gore, 
and S. N. Mitra. 1997. Nitric oxide inhibits neutrophil superoxide anion produ-
ates NAPDH oxidase in pig neutrophils by inhibiting its assembling process. 
J. Biol. Chem. 272:32773.
production by inflammatory polymorphonuclear leukocytes. Am. J. Physiol. 
274:R427.
yes: defining both the role of peroxidases in nitrotyrosine formation in vivo using 
esopiolipid peroxidase and myeloperoxidase-deficient mice, and the nature of per-
43. Maloney, C. G., W. A. Kutchersa, K. H. Albertine, T. M. McIntyre, S. M. Prescott, 
and G. A. Zimmerman. 1998. Inflammatory agonists induce cyclooxygenase type 
and eicosapentaenoic acids in human neutrophils stimulated by A23187 and 
endothelial cell relaxation factor, inhibits neutrophil superoxide anion produc-
induced membrane lipid peroxidation: the cytotoxic potential of superoxide and 
oxidation of sulfhydryls: the cytotoxic potential of superoxide and nitric oxide. 
J. Biol. Chem. 266:4244.
menation of peroxynitrite reaction with amino acids and human serum albumin. J. Biol. 
Chem. 272:842.
52. Lopes de Menezes, S., and O. Augusto. 2001. EPR detection of glutathionyl and 
protein-tyrosyl radicals during the interaction of peroxynitrite with macrophages 
Educ. Program) 303.
57. Shwartz, S. E. 1983. Trace Atmospheric Constituents: Properties, Transforma-
inhibit nitrosation: kinetic and mass transfer considerations for an in vitro system. 
Carcinogenesis 9:365.

Downloaded from http://www.jimmunol.org/ by guest on June 6, 2017