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Effect of Nitric Oxide Donors on Oxygen-Dependent Cytotoxic Responses Mediated by Neutrophils

G. Andonegui,*,‡ A. S. Trevani,*† R. Gamberale,‡† M. C. Carreras,‡ J. J. Poderoso,‡ M. Giordano,*† and J. R. Geffner*†

We analyzed the effect of nitric oxide (NO) on oxygen-dependent cytotoxic responses mediated by neutrophils against unopsonized erythrocytes using three NO donors: S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), and sodium nitroprusside (SNP). Neutrophils were treated with these compounds for 1–2 min at 37°C and cytotoxicity was then triggered in the presence of NO donors by precipitating immune complexes, aggregated IgG, the chemotactic peptide FMLP, or opsonized zymosan. GSNO induced, in all cases, a marked increase in cytotoxic responses, while SNAP moderately increased cytotoxicity triggered by immune complexes, aggregated IgG, or Z, opsonized zymosan, without modifying those responses induced by FMLP. By contrast, SNP dramatically suppressed cytotoxicity triggered by all of the stimuli assayed. The enhancing effects mediated by GSNO and SNAP did not depend on the stimulation of guanylyl cyclase and were prevented by the NO scavengers hemoglobin and PTIO (2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide). The inhibitory activity of SNP, on the other hand, was not prevented by NO scavengers, suggesting that it cannot be ascribed to the release of NO. In another set of experiments, neutrophils were pretreated with GSNO or SNAP for different times. Then cells were washed to remove NO donors from the culture medium, and cytotoxicity was triggered by different stimuli. It was found that neutrophils must be pretreated with NO donors for at least 4 h to increase cytotoxic responses, and pretreatment for longer periods (i.e., 8 or 18 h) further increased cytotoxicity. Not only cytotoxic responses, but also the production of O2•− and H2O2, and the release of myeloperoxidase were increased under these conditions.


Growing evidence suggests that nitric oxide (NO)3 inhibits neutrophil function. Adherence, chemotaxis, aggregation, and LTB4 synthesis are inhibited in neutrophils treated with NO or NO-releasing compounds (1–4). Contradictory results, on the other hand, have been published regarding the effect of NO on the production of oxygen-reactive intermediates (IRO) as well as on neutrophil degranulation (3–9). Most of these modulatory actions of NO are believed to involve the activation of soluble guanylate cyclase. In addition, other mechanisms, such as the stimulation of the ADP-ribosylation of actin and the inactivation of the enzymes nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and protein kinase C, have been proposed (1–4, 6, 10, 11).

In vivo studies support the notion that NO inhibits the inflammatory activity of neutrophils. In fact, the suppression of basal NO tone increases the attachment of neutrophils to postcapillary venules and their emigration into peripheral tissues, suggesting that the impairment of NO production by constitutive NO synthases results in a pattern of leukocyte adhesion and emigration that is characteristic of acute inflammation (12–14). Administration of NO donors, on the other hand, has shown to inhibit the course of acute inflammatory processes. Andrews et al. (15) demonstrated that fewer neutrophils infiltrated the posts ischemic gastric mucosa after SNP administration, while Kurose et al. (16) showed that NO donors greatly inhibited leukocyte adhesion, emigration, and vascular dysfunction in posts ischemic mesentery.

Oxygen-dependent cytotoxic responses mediated by phagocytes play an important role in immune response against infectious agents (17, 18). They involve a number of different mechanisms, all of which require the production of O2•− by effector cells to induce target cell injury (17, 18). While O2•− can be directly cytotoxic, its chemical reactivity is, in general, limited compared with other free radicals. Cytotoxic potential of O2•− appears to be dependent on its conversion to much stronger oxidants by secondary reactions (17–20). Since, at inflammatory sites, cells can produce not only O2•−, H2O2, OH, and HOCl, but also NO (20, 21), in this study we focused our attention on the analysis of the interplay between IRO and NO in regard to neutrophil cytotoxic ability. For this purpose, we employed three NO donors: GSNO, SNAP, and SNP (1–6, 21).

Materials and Methods

Reagents

GSNO and SNP were obtained from Alexis (Leufelfingen Switzerland). SNAP, FMLP, zymosan, acridine orange, ethidium bromide, propidium
iodide, catalase (from bovine liver, 50,000 U/mg protein), superoxide dismutase (SOD) (from bovine erythrocytes, 5,000 U/mg), sodium azide, sodium cyanide, N2, 2′-0-dibutyryl guanosine 3′,5′-cyclic monophosphate (Bt,cGMP), 1H-(1, 2, 4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), cytochrome c, 4-aminooantipyrine, horseradish peroxidase, and 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide (PTIO) were from Sigma (St. Louis, MO). Precipitating immune complexes (IC) were prepared at the equivalence zone, as we previously described (22), using human IgG (Sigma) as Ag and specific rabbit IgG Abs to human IgG. Equivalence points were determined by quantitative precipitin titrations. In all cases, Ags and Abs were incubated for 1 h at 37°C and 18 h at 4°C. After this period, IC were centrifuged at 3000 × g for 10 min, the precipitate was recovered, and it was resuspended in culture medium. The IgG aggregates (algG) were prepared by heating human IgG at a concentration of 5 mg/ml for 12 min at 63°C. Then algG was centrifuged at 10,000 × g for 5 min and the precipitate was discarded. Oposinized zymosan (Z) was prepared by incubating 100 mg of zymosan with 10 ml of normal human serum for 30 min at 37°C. After washing, Z was resuspended in culture medium.

**Blood samples**

Blood samples were obtained from healthy donors who had taken no medication for at least 10 days before the day of sampling. Blood was obtained by venipuncture of the forearm vein, and it was drawn directly into heparinized plastic tubes.

**Effector cells**

Neutrophils were isolated by Ficoll-Hypaque gradient centrifugation (Ficoll Pharmacia, Uppsala, Sweden; Hypaque, Winthrop Products, Buenos Aires, Argentina) and dextran sedimentation, as described (23). Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells (more than 96% of neutrophils on May Grünwald/Giemsa-stained cyttops) were resuspended in RPMI 1640 (Life Technologies, Detroit, MI) supplemented with 1% of FCS (Life Technologies).

**Treatment with NO donors**

Neutrophils (3 × 106/ml in RPMI 1640 supplemented with 1% FCS) were incubated with NO donors for 1–2 min, 30 min, 1 h, 2 h, 4 h, 8 h, or 18 h at 37°C, in 5% CO2 -95% humidified air. Cytotoxic assay

In a first set of experiments, neutrophils were cultured in the absence or presence of NO donors for 1–2 min and, after this time, cytolysis responses were triggered by IC, algG, FMLP, or Z, without removal of NO donors from the culture medium. In a second set of experiments, neutrophils were pretreated with NO donors for different times (1–2 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 18 h). At each time point, neutrophils were washed to remove NO donors from the culture medium. In this second set of experiments, neutrophils were then washed four times with saline and resuspended in RPMI 1640 medium supplemented with 10% FCS, to a density of 4 × 106 cells/ml. One hundred microliters of this suspension were added to each well in 96-well flat-bottom polystyrene plates. Neutrophils were incubated in the presence or absence of NO donors for 1–2 min or 8 h. Then they were cultured with or without FMLP (10−7 M) for 15 min at 37°C in 5% CO2-95% humidified air. After this time, cells were washed three times with culture medium to remove nonadherent neutrophils. Adherent neutrophils were then lysed with 1 N NH4OH, and the radioactivity present in the lysates was measured. Cell adherence was expressed as the number of neutrophils that remained adherent to the plastic surface after washing.

**Quantitation of cellular apoptosis and viability by fluorescence microscopy**

Quantitation of cellular apoptosis and viability was performed as previously described, using the fluorescent DNA-binding dyes acridine orange (100 µg/ml), to determine the percentage of cells that had undergone apoptosis, and ethidium bromide (100 µg/ml), to differentiate between viable and nonviable cells (30). With this method, nonapoptotic cell nuclei show variations in fluorescent intensity that reflect the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. To assess the percentage of cells showing morphologic features of apoptosis, at least 200 cells were scored in each experiment.
Statistical analysis

Results were compared using the Student’s t test, and p values ≤0.05 were considered statistically significant.

Results

Effect of NO donors on neutrophil cytotoxic responses triggered by IC, aIgG, FMLP, and Z

Neutrophils were incubated for 1–2 min with NO donors, and, after this time, cytotoxic responses were triggered by the addition of different stimuli, without removing NO donors from the culture medium. As shown in Fig. 1, GSNO increased, in a concentration-dependent manner, cytotoxicity induced by all of the stimuli employed, the maximal effect being observed at 100 μM of GSNO. Higher concentrations did not further enhance cytotoxic responses (not shown). Data depicted in Fig. 2 show the effects of SNAP and SNP on cytotoxicity. It was found that SNAP moderately increased cytotoxicity triggered by IC, aIgG, or Z without modifying those responses induced by FMLP. By contrast, SNP dramatically inhibited cytotoxicity triggered by all of the stimuli used. It is important to note that: 1) in the absence of triggering stimuli, NO donors did not induce cytotoxic responses, and 2) the enhancement of cytotoxicity induced by NO donors was not impaired by high concentrations of serum. In fact, when cytotoxic reactions were performed in culture medium supplemented with 25% of FCS, cytotoxic responses triggered by 10−7 M FMLP were increased from 6 ± 3 (untreated cells) to 31 ± 6 (GSNO 100 μM-treated cells) (X ± SEM, n = 6, p < 0.001). Similar results were obtained using IC as stimulus (not shown).

To analyze whether NO accounts for the biological activity of NO donors, two NO scavengers were used, hemoglobin (20, 21) and PTIO (32). Cells were incubated with GSNO or SNP (100 μM) in the presence of hemoglobin (500 μM) or PTIO (250 μM). After 1–2 min of incubation at 37°C, cytotoxicity was triggered by the addition of IC without removing NO donors or NO scavengers from the culture medium. It was found that NO scavengers prevented the increase in cytotoxicity induced by both, GSNO (Fig. 3) and SNAP (not shown), but did not modify the inhibition induced by SNP (Fig. 3), suggesting that it cannot be ascribed to the release of NO.

The enhancing effect of NO donors on cytotoxicity does not involve the activation of soluble guanylate cyclase

The activation of soluble guanylate cyclase appears to be a cardinal mechanism of action of NO (21). In agreement with previous observations (2, 3), we found that neutrophil treatment with GSNO, SNAP, or SNP markedly enhanced cGMP production: fmol/107 cells = 215 ± 32, 1315 ± 215, 1468 ± 134, and 487 ± 79, for untreated and neutrophils treated with 100 μM of GSNO, SNAP, and SNP, respectively; mean ± SEM; n = 5; p < 0.05, untreated versus treated cells. To determine whether the enhancement exerted by GSNO and SNAP on neutrophil cytotoxic responses could be ascribed to the increase in intracellular concentrations of cGMP, we examined the effect of the cell-permeable analogue of cGMP, Bt2 cGMP. It was found that treatment with Bt2 cGMP, at concentrations of 10 μM (Fig. 4A) or 1 μM (not shown), did not modify neutrophil cytotoxic responses triggered by either IC, aIgG, FMLP, or Z. A possible role of cGMP in the enhancement of cytotoxicity induced by GSNO and SNAP was further examined using a selective inhibitor of the soluble form of the guanylyl cyclase enzyme, the compound ODQ (33). At concentrations known to inhibit NO-stimulated guanylyl cyclase activity (10 μM) (33), ODQ did not prevent the enhancing effect exerted by GSNO and SNAP on cytotoxic responses triggered by either IC, aIgG, FMLP, or Z (Fig. 4B).

Hydrogen peroxide plays a critical role in cytotoxicity mediated by either untreated or NO donor-treated neutrophils

To analyze the role of the respiratory burst in cytotoxic reactions, we employed neutrophils isolated from three patients with chronic granulomatous disease (CGD), which produced, in response to either FMLP or IC, O2− concentrations lesser than 1% compared to...
FIGURE 3. Effect of hemoglobin and PTIO on the enhancement of IC-triggered cytotoxicity mediated by NO donors. Neutrophils (3 × 10^6/ml in medium supplemented with 1% FCS) were incubated with GSNO or SNP (100 μM) in the absence or presence of hemoglobin (500 μM) or PTIO (250 μM). After 1–2 min of incubation at 37°C, cytotoxicity was triggered by the addition of IC (10 μg/ml), without removing NO donors or NO scavengers from the culture medium. Data are expressed as the arithmetic mean ± SEM of triplicates from five to seven donors. * , p < 0.001 vs cytotoxic responses assessed in the absence of hemoglobin or PTIO.

with normal cells, CGD neutrophils were incubated for 1–2 min with GSNO or SNAP (100 μM) and, after this time, cytotoxic responses were triggered by the addition of FMLP or IC, without removing NO donors from the culture medium. It was observed that neither untreated nor GSNO or SNAP-treated CGD neutrophils mediated significant levels of cytotoxicity (percentage of cytotoxicity <7, n = 3 for each NO donor and stimulus).

The role of O_2^- in cytotoxicity was analyzed by performing cytotoxic reactions in the presence of SOD. Taking into account that O_2^- rapidly combines with NO to form ONOO^- (34, 35), high concentrations of SOD (2, 500 U/ml) were employed. It was found that the addition of SOD did not decrease cytotoxicity mediated by normal neutrophils triggered by either IC, algG, FMLP, or Z, assessed in the absence or presence of GSNO or SNAP (data not shown). By contrast, in all cases, the addition of catalase (500 U/ml) almost completely suppressed cytotoxicity (percentage of inhibition >88%, n = 3–6 for each of the stimuli employed, either in the presence or absence of GSNO or SNAP), indicating that cytotoxic responses were dependent on the release of H_2O_2 by effector cells. Additional experiments were performed to analyze whether cytotoxic reactions conducted in the presence of SNAP were also dependent on H_2O_2 release. Since 100 μM of SNAP markedly inhibited cytotoxicity, these reactions were performed in the presence of 50 μM of SNP, which decreased cytotoxic responses triggered by IC and Z by 48 ± 7% and 59 ± 6%, respectively (n = 4). It was found that cytotoxic responses triggered by either IC or Z, in the presence of 50 μM of SNP, were not modified by the addition of SOD (2500 U/ml), but were almost completely suppressed by catalase (500 U/ml) (percentage of inhibition >92, n = 3 for both stimuli).

Long-term incubation with NO donors enhances neutrophil ability to mediate oxygen-dependent cytotoxicity in response to triggering stimuli

In all of the above-described experiments, cytotoxic responses were assessed in the presence of NO donors, after pretreatment for 1–2 min. Additional studies were performed to determine whether removal of NO donors before the addition of triggering stimuli impaired the enhancement of cytotoxicity. Neutrophils were treated with GSNO (100 μM) for different times. At each time point, cells were washed to remove NO donors from the culture medium, targets were added, and cytotoxicity was triggered by IC (Fig. 5A) or FMLP (Fig. 5B). Our results show that neutrophils must be pretreated with GSNO for at least 4 h to increase cytotoxic responses. Similar results were obtained using SNAP (not shown). Pretreatment for longer periods (i.e., 8 or 18 h) further increased cytotoxicity (Fig. 6, A and B). Taken together, these results suggest that long-term treatment of neutrophils with NO donors induces a...
priming effect on neutrophil cytotoxic capacity. It was also observed that: 1) in all cases, the addition of catalase (500 U/ml) almost completely suppressed cytotoxicity (percentage of inhibition 91%, n = 4–5 for each stimulus), while SOD had no inhibitory effect, and 2) the enhancement of cytotoxicity induced by NO donors was impaired by hemoglobin (500 μM) and was not modified by ODQ (10 μM), an inhibitor of the guanylyl cyclase enzyme (not shown).

Long-term treatment with GSNO enhances neutrophil ability to produce O2 and H2O2 in response to triggering stimuli

To analyze the mechanisms underlying increased cytotoxicity by NO donors, we examined whether treatment with GSNO (100 μM) enhanced the ability of neutrophils to generate IRO. To this aim, neutrophils were incubated with GSNO for 8 or 18 h and, after these times, cells were washed twice and cytotoxicity was triggered by the addition of different stimuli: IC (10 μg/ml), algG (50 μg/ml), FMLP (10−7 M), and Z (200 μg/ml). Data are expressed as the arithmetic mean ± SEM of triplicates from seven donors. *p < 0.001 vs cytotoxic responses mediated by neutrophils cultured in the absence of NO donors.

FIGURE 5. Short-term exposure of neutrophils to GSNO or SNAP does not increase cytotoxic responses. Neutrophils (3 × 10⁶/ml in medium supplemented with 1% FCS) were incubated in the presence or absence of GSNO (100 μM) for different times at 37°C, 1–2 min, 30 min, 60 min, 120 min, and 240 min. At each time point, neutrophils were washed to remove NO donors from the culture medium, and cytotoxicity was triggered by the addition of 10 μg/ml IC (A) or 10−7 M FMLP (B). Data are expressed as the arithmetic mean ± SEM of triplicates from five to six donors. *p < 0.05, untreated vs NO donor-treated neutrophils.

FIGURE 6. Long-term exposure of neutrophils to GSNO or SNAP increases cytotoxic responses. Neutrophils (3 × 10⁶/ml in medium supplemented with 1% FCS) were incubated in the presence or absence of GSNO or SNAP (100 μM) for 8 h (A) or 18 h (B) at 37°C. After these times, cells were washed twice and cytotoxicity was triggered by the addition of different stimuli: IC (10 μg/ml), algG (50 μg/ml), FMLP (10−7 M), and Z (200 μg/ml). Data are expressed as the arithmetic mean ± SEM of triplicates from seven donors. *p < 0.001 vs cytotoxic responses assessed in the absence of NO donors.

Table I. Effect of azide and cyanide on neutrophil cytotoxic responses

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GSNO</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>algG</td>
<td>FMLP</td>
</tr>
<tr>
<td>None</td>
<td>26 ± 4</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Azide (10 μM)</td>
<td>34 ± 6</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Cyanide (10 μM)</td>
<td>31 ± 5</td>
<td>19 ± 2</td>
</tr>
</tbody>
</table>

* Neutrophils (3 × 10⁶/ml) were incubated with GSNO (100 μM) in the absence or presence of azide or cyanide (10 μM) for 8 h at 37°C. Then cytotoxicity was triggered by the addition of different stimuli: IC (10 μg/ml), algG (50 μg/ml), FMLP (10−7 M), and Z (200 μg/ml). Data are expressed as the arithmetic mean ± SEM of triplicates from 6–8 donors.

*p < 0.005 vs cytotoxic responses assessed in the absence of azide or cyanide.

Treatment with GSNO switches the cytotoxic mechanisms triggered by algG and FMLP from a MPO-independent to a MPO-dependent pathway

As mentioned above, catalase almost completely suppressed cytotoxic responses conducted by untreated or NO donor-treated neutrophils, supporting a critical role for H2O2 in cytotoxicity. It is...
well known that H$_2$O$_2$ could directly mediate the lysis of the target cells or could serve as a component of the H$_2$O$_2$-halide-MPO system, which is capable of peroxiding Cl$^-$ to ClO$^-$, a species with a strong cytotoxic potential (36). To analyze the involvement of MPO in cytotoxicity, additional experiments were performed. Cells were cultured for 8 h with GSNO (100 μM) and, after this time, they were washed to remove the NO donor from the culture medium. Then azide or cyanide, two haem-enzyme inhibitors (36), was added and cytotoxic responses were triggered by IC, aIgG, FMLP, or Z. Results depicted in Table I show that cytotoxicity mediated by neutrophils cultured in the absence of GSNO involved a MPO-independent mechanism for all of the stimuli employed. Interestingly, neutrophil exposure to GSNO switched the cytotoxic mechanisms triggered by aIgG and FMLP from a MPO-independent to a MPO-dependent pathway. By contrast, cytotoxic mechanisms triggered by IC and Z remained MPO independent, as seen with control cells. Similar results were obtained when neutrophils were treated with GSNO for 18 h, before the addition of triggering stimuli (not shown).

The switch in the cytotoxic mechanisms triggered by aIgG and FMLP from a MPO-independent to a MPO-dependent pathway, induced as a consequence of GSNO treatment, could be related to the ability of NO to increase MPO release from stimulated neutrophils, as previously described (6). To analyze this point, neutrophils were treated with GSNO (100 μM) for 8 h and, after washing, the release of MPO was triggered. Treatment with GSNO significantly increased MPO release triggered not only by aIgG (percentage of MPO released: 1.6 ± 2.1 vs 7.5 ± 2.3) and FMLP (1.7 ± 0.9 vs 5.6 ± 1.6), but also by IC (17.6 ± 4.3 vs 28.5 ± 3.9) and Z (13.5 ± 2.9 vs 24.6 ± 3.4) (X ± SEM, n = 5–10, p < 0.05 untreated vs GSNO-treated neutrophils).

**FIGURE 7.** Long-term exposure of neutrophils to GSNO increases O$_2$ and H$_2$O$_2$ production triggered by FMLP. Neutrophils (3 × 10$^6$/ml in medium supplemented with 1% FCS) were incubated in the presence or absence of GSNO (100 μM) for 8 or 18 h at 37°C. After these times, cells were washed twice and adjusted to 10 × 10$^6$/ml. Then the production of O$_2$ (A) and H$_2$O$_2$ (B) triggered by FMLP (10$^{-7}$ M) was evaluated. Data are expressed as the arithmetic mean ± SEM from six donors. *p < 0.001 vs O$_2$ or H$_2$O$_2$ produced by cells cultured in the absence of GSNO.

**Discussion**

The results presented in this study show that oxygen-dependent cytotoxic responses mediated by neutrophils can be modulated by NO donors. Stimulatory or inhibitory effects were observed depending on the NO donor employed. Thus, cytotoxic responses were increased by GSNO and SNAP, while they were dramatically suppressed by SNP. The enhancement mediated by GSNO or SNAP was prevented by hemoglobin and PTIO, two NO scavengers, which did not modify the inhibition induced by SNP, suggesting that it cannot be ascribed to the release of NO. The molecular identity of the species responsible for this inhibitory effect

**FIGURE 8.** Effect of NO donors on neutrophil apoptosis. Neutrophils (3 × 10$^6$/ml in medium supplemented with 1% FCS) were cultured in the presence or absence of NO donors (100 μM) for 18 h at 37°C. The percentage of apoptotic cells was determined by fluorescence microscopy (A) and flow cytometry (B). A. Results are expressed as the mean ± SEM of 10 experiments. B. Histograms of a representative experiment (n = 10), showing the percentage (M1) of nuclei with hypodiploid DNA content.
remains to be established. It is well known that SNP attacks thiols and releases cyanide (34). This reaction could explain contrasting effects of SNP and other NO donors, such as GSNO and SNAP, on lymphocyte proliferation (37). However, cyanide does not seem to account for the suppression of cytotoxicity reported in this study since, as we have shown, haem-enzyme inhibitors, including cyanide, do not inhibit neutrophil cytotoxicity triggered by IC and Z (Table I and Ref. 38).

The mechanisms by which GSNO and SNAP enhance cytotoxic responses do not depend on the stimulation of guanylyl cyclase since: 1) treatment of neutrophils with the cell-permeable analogue of cGMP, 8-BrcGMP, has no effect on cytotoxicity, and 2) ODQ, a selective inhibitor of the soluble form of the guanylyl cyclase enzyme (33), does not impair the enhancing effect of GSNO and SNAP on cytotoxicity. These findings contrast with our previous observations regarding the modulatory action of NO donors on Ab-dependent cellular cytotoxicity mediated by neutrophils. We found that NO donors modulate Ab-dependent cellular cytotoxicity through mechanisms dependent on the increase in intracellular concentrations of cGMP (39). Taken together, these observations suggest that NO could modulate different cytotoxic responses mediated by neutrophils through guanylyl cyclase-dependent and independent pathways.

The role of NO in cytotoxic reactions is still unclear. It has been proposed that NO can directly cause cell death. However, primary cell cultures of various types exposed to high concentrations of NO (1 mM) for long periods show no adverse effects (35, 40). Taking into account that cells involved in inflammatory processes can produce not only O₂⁻, H₂O₂, OH, and HOCl, but also NO, several studies have focused their attention on the analysis of the interplay between IRO and NO in the mediation of cytotoxicity (20, 21, 34, 35). They showed that peroxinitrite (ONOO⁻), the product of the reaction of O₂⁻ with NO, has a strong oxidizing potential (34, 35). However, the combination of NO with O₂⁻ does not always increase cytotoxicity. Instead, in some models it may be H₂O₂ that synergizes more powerful with NO. In this regard, it has been shown recently that NO exacerbates H₂O₂-mediated killing of Escherichia coli as well as the increase in endothelial permeability induced by H₂O₂ (41, 42). While the above-mentioned observations suggest that NO potentiates oxidative injury, recent findings point out a more complex scenario. In fact, NO can also attenuate cell injury mediated by H₂O₂, as shown in different models (43–45). Considering that all of these studies have been performed in host cell-free systems (41–45), assumptions and extrapolations from them, regarding the interplay between IRO and NO and their effects on inflammation, should be reexamined in biological systems in which effector cells are included.

Our results showed that when neutrophils were treated with NO donors for 1–2 min, and cytotoxic responses were then triggered in their presence, there was a marked increase in cytotoxicity. Under these conditions, the enhancement of cytotoxicity induced by GSNO was higher compared with SNAP. This observation could be due to differences in the NO generation kinetics. In fact, it has been shown that SNAP discomposes more slowly than GSNO (46, 47). It was also observed that the removal of NO donors after 1–2 min of pretreatment, before the addition of triggering stimuli, completely impaired the enhancement of cytotoxicity, indicating that a brief exposure of neutrophils to NO donors is unable to prime their cytotoxic capacity. The enhancement of cytotoxicity conducted in the presence of NO donors does not appear to involve the participation of ONOO⁻ since high concentrations of SOD did not exert any inhibitory effect. A critical role for H₂O₂, on the other hand, was suggested by the fact that catalase almost completely abrogated cytotoxicity. However, the possibility that enhanced cytotoxicity may involve a direct effect of both NO and H₂O₂ acting together on target cells, should be ruled out since neither NO donors (100–1000 µM), H₂O₂ (10–100,000 µM), nor NO donors plus H₂O₂ induced cytotoxic effects on target cells when cultured in the absence of neutrophils (unpublished results).

Different mechanisms could account for the enhancement in cytotoxicity observed for reactions performed in the presence of NO donors. First, NO may enhance neutrophil ability to produce O₂⁻ and H₂O₂ by acting in the course of the cytotoxic reaction, which involves long-term coincubation of triggered neutrophils and target cells (18 h at 37°C). Second, NO may interact with H₂O₂ and/or other free radicals derived from H₂O₂ to form a new agent with stronger oxidative potential, as previously proposed (48). Third, considering the ability of NO and IRO to inhibit antioxidant enzymes such as glutathione peroxidase, catalase, and MPO (40, 48–54), H₂O₂ could act in synergy with NO by depleting target and/or effector cell antioxidant defenses. Inhibition of antioxidant defenses in target cells may result in enhanced susceptibility to the cytotoxic action of IRO. On the other hand, inhibition of MPO and catalase is associated with a marked increase in both O₂ consumption and H₂O₂ release during neutrophil activation (49, 50). Nevertheless, since NO donors were present during the course of the cytotoxic reaction, it was very difficult to ascertain which of the above-mentioned mechanisms account for the enhancement in cytotoxicity.

To further analyze the mechanisms by which NO donors enhance oxygen-dependent cytotoxic responses, we treated neutrophils with GSNO or SNAP (100 µM) for different times. At each time point, cells were washed to remove NO donors from the culture medium before triggering cytotoxicity with IC, algG, FMLP, or Z. It was observed that neutrophils must be pretreated with NO donors for at least 4 h to increase cytotoxic responses. Pretreatment for longer periods (i.e., 8 or 18 h) further increased cytotoxicity. Since these cytotoxic reactions were performed after removal of NO donors from the culture medium, the enhancement of cytotoxicity can be attributable to the induction of a priming effect on the

### Table II. Effect of NO donors on different neutrophil-mediated responses

<table>
<thead>
<tr>
<th>NO Donor</th>
<th>Shape Change (MFSC)</th>
<th>CD18 (MFI)</th>
<th>Adherence (no. of cells × 10⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FMLP</td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>247 ± 21</td>
<td>389 ± 36</td>
<td>285 ± 33</td>
</tr>
<tr>
<td>GSNO</td>
<td>251 ± 32</td>
<td>401 ± 32</td>
<td>237 ± 32</td>
</tr>
<tr>
<td>SNAP</td>
<td>240 ± 21</td>
<td>376 ± 29</td>
<td>280 ± 30</td>
</tr>
<tr>
<td>SNAP</td>
<td>242 ± 21</td>
<td>398 ± 32</td>
<td>289 ± 29</td>
</tr>
</tbody>
</table>

*Neutrophils were incubated in the presence or absence of GSNO, SNAP, or SNAP (100 µM) for 1–2 min at 37°C. Then, FMLP (10⁻⁶ M) was added and the cells were incubated for 15 min at 37°C. Shape change and CD18 expression were evaluated by flow cytometry. Adherence assays were performed employing neutrophils labeled with ⁵¹Cr. Data are expressed as the arithmetic mean ± SEM of duplicates or triplicates from six to eight donors.*
neutrophil itself. In this regard, we have also observed that pre-
treatment with GSNO for either 8 or 18 h markedly increased the ability of neutrophils to produce O₂⁻ and H₂O₂, as well as to release MPO to the extracellular medium in response to triggering stimuli. Both effects could explain, at least in part, the enhancement of cytotoxicity induced by NO donors. It should be pointed out that the increase in MPO release triggered by aIgG and FMLP was associated with a switch in the cytotoxic mechanism from a MPO-
dependent to a MPO-dependent pathway. Since aIgG and FMLP were unable to induce MPO release from control cells, we spec-
ulate that the switch in the cytotoxic mechanism, observed in GSNO-treated neutrophils, was due to the induction of MPO re-
lease. Regarding IC and Z, it should be noted that they were able to trigger MPO release not only from GSNO-treated cells, but also from untreated cells. In spite of this, cytotoxic responses were in all cases MPO-dependent. We speculate that this could be due, at least in part, to the scavenging of ClO⁻ by these agonists. In fact, this scavenging activity has been shown for Z (55), but remains to be evaluated for IC.

The physiologic relevance of our results can only be speculative at this time. It should be noted, however, that the concentrations of NO donors used in this work appear to be consistent with the levels of NO expected in vivo. GSNO and SNAP, at concentrations from 10–100 μM, produce 0.10–5.60 μM NO/min (59). Stimulated macrophages and endo-
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0.2 μM/min/10⁷ cells (60–62). These observations support the possibility that, at inflammatory areas, NO may modulate oxygen-
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ducted in the presence of NO synthase inhibitors or in l-larg-free culture medium (unpublished data).

In summary, we have shown that the physiologic NO donor GSNO, as well as SNAP exacerbate oxygen-dependent cytotoxic responses mediated by neutrophils. This effect appears to be re-
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