S-nitrosoglutathione enhances neutrophil DNA fragmentation and cell death

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Fortenberry, James D., Marilyn L. Owens, and Lou Ann S. Brown. S-nitrosoglutathione enhances neutrophil DNA fragmentation and cell death. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L435–L442, 1999.—Enhancing the cleavage of neutrophils by enhancing apoptotic cell death and macrophage recognition may be beneficial in acute lung injury. Exogenous nitric oxide gas depresses neutrophil oxidative functions and accelerates cell death (A. H. Daher, J. D. Fortenberry, M. L. Owens, and L. A. Brown. Am. J. Respir. Cell Mol. Biol. 16: 407–412, 1997). We hypothesized that S-nitrosoglutathione (GSNO), a physiologically relevant nitric oxide donor, could also enhance neutrophil DNA fragmentation. Neutrophils were incubated for 2–24 h in the absence and presence of GSNO (dose range 0.1–5 mM) and evaluated for cell death by a fluorescent viability/cytotoxicity assay. Neutrophil DNA fragmentation was assessed by cell death detection ELISA and by terminal deoxynucleotidyltransferase-mediated fluorescence-labeled dUTP nick end labeling assay. Neutrophil oxidative function was also determined. Incubation with GSNO increased cell death at 2, 4, and 24 h. GSNO incubation for 24 h significantly increased DNA fragmentation in a dose-dependent fashion at 0.5 (median 126% of control value; P = 0.002) and 5 mM (185% of control value; P = 0.002) by terminal deoxynucleotidyltransferase-mediated fluorescence-labeled dUTP nick end labeling and at 0.5 mM by ELISA (164% of control value; P = 0.03). The apoptosis-to-total cell death ratio increased with increasing GSNO concentration (P < 0.05). Effects were mitigated by coincubation with superoxide dismutase. Five millimolar GSNO decreased overall superoxide production and O2 consumption but not when adjusted for dead neutrophils. GSNO significantly enhances cell death and neutrophil DNA fragmentation in a dose-dependent fashion.

Nitric oxide (NO), primarily regarded as an endogenous vasodilator (38, 55), has been discovered to serve a multitude of immunomodulatory functions. NO inhibits platelet aggregation and basal neutrophil adherence to endothelium (24, 36). At concentrations used with inhaled NO therapy for respiratory failure and persistent pulmonary hypertension of the newborn, exogenous NO can also inhibit neutrophil oxidative function (9). NO may produce this effect, in part, by inducing apoptosis, as seen with neutrophils and monocytes. A recent study (11), however, suggested that NO gas, particularly with superimposed hyperoxia, induces both apoptotic and necrotic cell death. Necrotic cell death (41) brings the potential extrusion of large quantities of neutrophil proteases and oxidants into an already damaged lung (42). The role of apoptosis of either neutrophils or lung cells in acute inflammatory lung injury is less certain. The effects of apoptosis could range from mitigating to enhancing injury, dependent on the specific cell types affected, as well as the timing and nature of the insult (30).

Free NO is exquisitely reactive, with a half-life of seconds (44). NO reacts with ambient O2 and superoxide radicals to produce more toxic species, including nitrogen dioxide (NO2) and peroxynitrite (OONO−), which have been associated with toxicity in several forms of lung injury (3, 28). Alternatively, NO may react with endogenous or exogenous thiols to form more stable compounds. One such adduct is S-nitrosothiol (GSNO), an S-nitrosothiol synthesized by S-nitrosylation of glutathione (GSH), an abundant intracellular thiol (45). GSNO is more stable and much more abundant in plasma than reactive free NO (44). Gaston et al. (13) found endogenous bioactive GSNO in nanomolar to micromolar concentrations in the airways of normal human subjects and in patients with pneumonia or receiving inhaled NO therapy. The authors speculated that nitrosothiol formation in the lungs might serve a dual function both by stabilizing NO in bioactive form and by decreasing cytotoxicity. Exogenous GSNO is also bioactive. Pharmacological doses of GSNO potently inhibit platelet aggregation and adhesion in humans with forearm ischemia or undergoing coronary angioplasty (25), similar to the inhibitory effects of endogenous NO on platelet adhesion in vivo (36).

We therefore hypothesized that GSNO induces cell death and DNA fragmentation, consistent with apoptosis, in human neutrophils and concomitantly inhibits neutrophil oxidative function.
MATERIALS AND METHODS

Neutrophil Isolation

Blood was obtained after informed consent from normal adult human volunteers by standard venipuncture. Neutrophils were isolated from whole blood as previously described (5). Briefly, heparinized blood was centrifuged over a sodium diatrizoate (Sigma, St. Louis, MO) layer, followed by sedimentation with 6% dextran (Spectrum, Gardenia, CA) and hypotonic lysis of red blood cells. Isolated samples contained >95% neutrophils by Wright stain and differential count and demonstrated >95% trypan blue exclusion. Neutrophils were maintained in Dulbecco’s phosphate-buffered saline (DPBS) with 0.1% glucose (GIBCO BRL, Grand Island, NY).

Neutrophil Exposure

Neutrophils were incubated in the absence and presence of GSNO (0.1–5 mM; Sigma) or the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 1 mM; Sigma). Cells were incubated in Iscove’s modified Dulbecco’s medium (GIBCO BRL) for 2 or 24 h and then assessed for cell viability and DNA fragmentation. In another group of experiments, neutrophils were incubated with GSNO (0.5 mM) in the absence and presence of 80% O₂ to determine whether hyperoxia produced synergistic effects with GSNO. In separate experiments, neutrophils were incubated for 24 h with GSH (Sigma) to determine whether GSNO effects were related to the presence of GSH as a thiol antioxidant.

To determine the role of endogenous NO in intrinsic regulation of neutrophil cell death, neutrophil exposures were also performed in the absence and presence of the L-arginine analog NO synthase inhibitor N’-monomethyl-L-arginine (L-NMMA; 0.5 mM; Calbiochem, San Diego, CA) for 24 h.

To determine whether peroxynitrite produced by interactions between superoxide radicals and NO released from GSNO played a role in cell death, neutrophils were also coincubated in separate experiments with manganese superoxide dismutase (SOD; Sigma; nonpyrogen free, 100 μg/ml) and cytochalasin B (0.5 μg/ml) for 24 h.

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Cell Death Assessment

Cell necrosis was evaluated by a fluorescent viability/cytotoxicity assay (Eukolight, Molecular Probes, Eugene, OR) (29). Briefly, exposed cells were placed on glass slide covers within polyethylene six-well dishes that allowed neutrophil adherence, then stained with a mixture of the fluorescent probes calcine AM and ethidium homodimer. After uptake, only viable cells containing functioning esterases can cleave the ester group on calcine AM to generate a characteristic green fluorescence under fluorescent microscopy. Ethidium homodimer penetrates the permeable membranes of nonviable cells and binds with nucleic acids, identifiable by red-orange fluorescence. For each experiment, ~100 neutrophils were counted from three high-power fields on each slide cover for green and red fluorescence and averaged. Values are expressed as the percentage of calcine (green)-positive cells divided by total calcine- and ethidium (red)-positive cells counted.

DNA Fragmentation Assessment

Samples were evaluated for evidence of DNA fragmentation associated with apoptosis by the following techniques. ELISA. We used an apoptotic cell death detection ELISA (Boehringer Mannheim, Indianapolis, IN) to quantitatively determine cytoplasmic histone-associated DNA oligonucleosome fragments associated with apoptotic cell death. This ELISA has demonstrated correlation with gel electrophoresis and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays in measurement of apoptotic cell death in HL-60 cell lines treated with camptothecin (Boehringer Mannheim; data on file) and with histological evidence of apoptosis (4, 27). We did not repeat these studies for neutrophils. Briefly, neutrophil samples were sonicated to obtain cytoplasmic lysates. Samples were incubated with microtiter plates adsorbed with mouse antihistone antibody (clone H11-4) to bind histone-associated DNA oligonucleosomes uncovered by endonuclease-mediated DNA nicking. Plates were washed, and nonspecific binding sites were saturated with blocking buffer. Bound samples were then reacted with anti-mouse DNA monoclonal antibody (MCA-33) and then conjugated with peroxidase. To determine the amount of retained peroxidase, 2,2’-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) was added as a substrate, and the complex was measured by spectrophotometer at 405 nm (Anthos HTII, Anthos Labtec Instruments, Frederick, MD). Results are expressed as the ratio of sample absorbance to absorbance of room air control sample measured daily.

TUNEL assay. Specific 3’-hydroxyl ends of DNA fragments generated by endonuclease-mediated apoptosis are preferentially repaired by TdT (14). The TUNEL assay (Boehringer Mannheim) labels these strand breaks with fluorescent nucleotides, and provides a sensitive measure of DNA fragmentation consistent with apoptosis in individual cells (14, 17). After exposure, cells were fixed in 4% paraformaldehyde and permeabilized with 1% Triton X-100 and 0.1% sodium citrate. Samples were then incubated for 60 min at 37°C in the absence and presence of exogenous TdT and incubated with fluorescein-conjugated dUTP for repair of nicked 3’-hydroxyl DNA ends. Mean cell fluorescence of 10,000 neutrophils and percentage of TUNEL-positive cells were assessed by flow cytometry (FACSscan, Becton Dickinson, Bedford, MA) for each condition. To estimate the relative contribution of apoptotic cell death to overall cell death, an apoptosis-to-total cell death ratio was calculated by dividing the percentage of TUNEL-positive neutrophils in a sample by the percentage of dead neutrophils as determined by viability/cytotoxicity assay.

Microscopy. Exposed neutrophils were treated with Wright stain and examined by phase microscopy (×150) to qualitatively assess for histological findings consistent with apoptosis.

Assessment of Neutrophil Oxidative Function

Superoxide anion generation. Superoxide anion generation was determined by a standard assay (16) after 24 h of GSNO exposure. Briefly, phorbol 12-myristate 13-acetate (PMA; Sigma), a potent neutrophil stimulant, and horse cytochrome c (Sigma) were added to isolated neutrophil suspensions after GSNO exposure and washing with DPBS to remove residual NO donor compounds. Cytochrome c reduction by generated superoxide was then determined by spectrophotometric absorbance (Beckman DU 64 spectrophotometer, Fullerton, CA) at 550-nm wavelength. Results are expressed as nanomoles of superoxide anion per 1 × 10⁶ neutrophils. This reaction could be quenched by the addition of superoxide dismutase before PMA, confirming that a sensitive measure of cytochrome c reduction was mediated by superoxide anion (data not shown). The superoxide generation assay was then performed in room air conditions in the absence of NO or hyperoxia.

O₂ consumption. Neutrophil O₂ consumption was measured as an indicator of overall respiratory burst. O₂ consumption was determined with a Clark-type O₂ membrane electrode (Yellow Springs Instrument, Yellow Springs, OH) as described originally by Chance and Williams (6) and used for...
neutrophil measurements by Rossi et al. (39). Briefly, neutrophils were exposed to the conditions in Superoxide anion generation and then washed in DPBS. Cells \(1 \times 10^7\) were added to DPBS with constant stirring and maintained at 37°C in a glass chamber, with DPBS bathing the electrode membrane. After the addition of PMA to the chamber (final concentration 500 nM), the slope of the electrode response \(\text{O}_2\) consumption/time was recorded for 5–6 min on a strip-chart recorder and used to calculate \(\text{O}_2\) consumption, assuming an \(\text{O}_2\) solubility coefficient of 0.024 µmol/ml medium. Results are expressed as nanomoles of \(\text{O}_2\) per minute per \(10^7\) neutrophils.

**Statistics**

Assays were performed in triplicate for each sample exposure, and an average value was determined. Results are expressed as means \(\pm\) SE. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Student-Newman-Keuls comparison for parametric data sets. Data not meeting parametric characteristics were analyzed with Kruskal-Wallis one-way ANOVA on ranks and Student-Newman-Keuls or Dunn's test for comparisons. \(P < 0.05\) was considered significant.

**RESULTS**

Effects of Exogenous NO Donor and Inhibitor Exposure on Cell Death and DNA Fragmentation

GSNO decreased neutrophil viability in a time-dependent and concentration-dependent manner at 2, 4, 12, and 24 h as assessed by viability/cytotoxicity assay (Fig. 1). GSNO exposure increased neutrophil DNA fragmentation in a concentration-dependent fashion. GSNO effects on DNA fragmentation were seen by ELISA at 12 \((P < 0.05)\) and 24 h \((P < 0.03;\) Fig. 2) but not at 2 or 4 h compared with control cells (data not shown). GSNO exposure alone did not enhance cell death or DNA fragmentation at any concentration (data not shown).

TUNEL staining also demonstrated GSNO effects on DNA fragmentation. Mean fluorescence intensity from dUTP binding was increased at 24 h, but not at 12 h, in neutrophils incubated in GSNO (Figs. 3 and 4). GSNO produced a dose-dependent DNA fragmentation response; 0.5 and 5 mM GSNO increased fragmentation above that in 24-h control cells \((P < 0.05\) by Kruskal-Wallis ANOVA on ranks, \(n = 4–10\) experiments/group) and SNAP-treated cells \((P = 0.002; n = 6\) experiments). GSNO exposure increased the total percentage of TUNEL-positive neutrophils at 24 h compared with control cells (Fig. 5A). The apoptosis-to-total cell death ratio also increased with increasing GSNO concentration, from 1.7 ± 0.3 (control) to 30.7 ± 4.0% \((5\) mM GSNO) after 24 h of exposure (Fig. 5B). By light microscopy, neutrophils exposed to GSNO showed histological evidence of apoptosis, demonstrated by nuclear chromatin condensation and cell involution at 12 h (Fig. 6).

Incubation of neutrophils with SNAP did not increase cell death compared with those in room air at 24 h as assessed by cytotoxicity assay (Fig. 1). SNAP-exposed cells were also evaluated by TUNEL to rule out an early effect on DNA fragmentation without secondary necrosis. SNAP did not enhance apoptosis compared with control value as measured by TUNEL (105% of control value, range 99–126%; \(P = 0.28; n = 5\) experiments) or by ELISA \((P > 0.05\); \(n = 5\) experiments). SNAP exposure with hyperoxic conditions also
L-NMMA exposure for 24 h did not decrease cell viability (Fig. 1). Likewise, L-NMMA pretreatment did not halt the development of apoptosis at 2 or 24 h. At 24 h, neutrophil GSNO dose response at 12 and 24 h expressed as percentage of control mean fluorescence intensity (%MFI) by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. Boxes, 25th to 75th percentile, with centerline as median; SE bars, 10th to 90th percentile; ○, outliers. %MFI, as a measure of DNA fragmentation, was significantly increased with GSNO exposure at 0.5 and 5 mM at 24 h but not at 12 h, *P < 0.05 compared with control value by ANOVA on ranks and Student-Newman-Keuls test.

Fig. 5. A: percent TUNEL-positive (high fluorescence-stained) neutrophils at 24 h after GSNO exposure. *Significant increases compared with control value, P < 0.05 by ANOVA on ranks and Student-Newman-Keuls test. B: ratio of apoptosis-to-total cell death as determined by percent TUNEL-positive neutrophils and fluorescent viability/cytotoxicity assay in cells exposed to GSNO for 24 h (n = 10 experiments/group). With increasing concentrations of GSNO, contribution of apoptotic neutrophil number to total cell death was significantly increased: *P < 0.05 compared with control value; **P < 0.05 compared with 0.1 and 0.5 mM GSNO by ANOVA and Student-Newman-Keuls test.

Fig. 3. Neutrophil GSNO dose response at 12 and 24 h expressed as percentage of control mean fluorescence intensity (%MFI) by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. Boxes, 25th to 75th percentile, with centerline as median; SE bars, 10th to 90th percentile; ○, outliers. %MFI, as a measure of DNA fragmentation, was significantly increased with GSNO exposure at 0.5 and 5 mM at 24 h but not at 12 h, *P < 0.05 compared with control value by ANOVA on ranks and Student-Newman-Keuls test.

Fig. 4. Representative flow cytometric histograms of TUNEL assay results from neutrophil specimens incubated for 24 h in control conditions and with indicated concentrations of GSNO. Graphs depict relative cell number as function of MFI. Note increase in relative number of cells with higher MFI as well as shift of this cell population to the right, with increasing MFI, in GSNO-exposed neutrophils.
h, neutrophil DNA fragmentation as measured by ELISA was markedly increased in neutrophils exposed to L-NMMA at both 0.5 [4,904% of time 0 (t0) baseline] and 5 mM (6,908% of t0 baseline; P < 0.05) and was not different from that seen with control neutrophils at 24 h (2,538% of t0 baseline; P = 0.136; n = 6 experiments/group). At 24 h, L-NMMA-treated neutrophil DNA fragmentation (96% of control) measured by TUNEL assay was not different from that in control cells (P > 0.05).

GSNO exposure at 0.5 mM GSNO with hyperoxic conditions (GSNO-O2) did not increase cell death compared with GSNO in room air (80 ± 6 vs. 81 ± 4%). GSNO-O2 (1,364% of control value at 24 h, range 524–2,430%) also did not enhance apoptosis to a greater extent than GSNO in room air conditions (1,010% of control value, range 390–2,840%; not significant).

Coincubation of GSNO-exposed neutrophils with MnSOD decreased DNA fragmentation. By ELISA, addition of MnSOD decreased 24-h DNA fragmentation (median 115% of control value, range 55–172%) compared with GSNO alone (250% of control value, range 189–293%; P < 0.05 by ANOVA on ranks and Student-Newman-Keuls test).

Effects of GSNO on Neutrophil Oxidative Function
Neutrophil oxidative function was affected only at high GSNO concentrations. Five millimolar GSNO decreased superoxide generation (75 ± 6 nmol/106 neutrophils vs. 233 ± 6 nmol/106 control neutrophils; P < 0.05 by ANOVA and Student-Newman-Keuls test; n = 18 experiments). O2 consumption in response to PMA was also diminished by 5 mM GSNO (5 ± 1.3
nmol · ml⁻¹ · min⁻¹ · 10⁷ neutrophils⁻¹ vs. 15 ± 2 nmol · ml⁻¹ · min⁻¹ · 10⁷ control neutrophils⁻¹; P < 0.05; n = 11 experiments). However, no significant differences were seen when these results were adjusted for total live neutrophils as determined by cytotoxicity assay. Exposure to other GSNO concentrations did not significantly affect neutrophil superoxide generation or O₂ consumption. Oxidative function with SNAP was not tested.

**DISCUSSION**

GSNO is formed from the reaction of NO and GSH in the presence of O₂ to form a relatively stable compound (46). Several authors (15, 43) have postulated that the S-nitrosylation reaction represents a very convenient endogenous method to store, transport, and release NO. S-nitrosothiol adducts may not only serve as physiologically active forms of NO in human plasma (44), but their formation may provide a physiological scavenging mechanism to minimize toxicity (52). Packaging of NO in this form could potentially serve both to preserve the bioactivity of NO and to limit its potential for O₂-dependent toxicity.

We found a time- and dose-dependent effect of GSNO exposure on DNA fragmentation and cell death. These findings are consistent both with our previous study (11) with NO gas exposure and with recent preliminary findings (53) that the NO donor GEA-3162 and the combined NO-superoxide anion donor 3-morpholinosydnonimine induced a cGMP-independent increase in neutrophil apoptosis. Our results are also similar to findings with NO gas (11). Evaluation of GSNO as an NO donor is important because of its endogenous existence in the lung (13) and its potential for systemic delivery. GSNO also induced apoptosis in the absence of hyperoxia compared with the limited effects of NO gas alone in our previous study (11).

The TUNEL method for determining DNA fragmentation has previously been considered to correlate well with histological findings of apoptosis. However, some recent studies (18, 48) have suggested that TUNEL may not always be specific for apoptosis, particularly in autopsy material or with prolonged fixation time. Our samples were rapidly fixed after exposure and would be less likely to show such artifacts. TUNEL results were supported by elevated DNA fragmentation ELISA levels and by photomicrographs supporting the development of apoptosis. It is likely that DNA fragmentation seen in the current study does not solely represent apoptotic cell death but also primary or secondary necrosis.

We did not evaluate the effects of GSNO and hyperoxia on other cell types. Recent studies have found that NO donors in the presence of hyperoxia produced surfactant dysfunction and increased neutrophil chemotactic activity in vivo (37) and cytotoxicity to alveolar epithelial cells and lung vascular endothelial cells in vitro (35). However, other studies (23, 31) of ex vivo isolated lung models and in vivo models found that inhaled NO exposure actually decreased lung injury, suggesting that NO may not injure the lung in a more complex environment and at more clinically relevant gas concentrations. Further studies are ongoing in our laboratory to determine effects of both GSNO and inhaled NO at clinically relevant concentrations on alveolar epithelium to help address this disparity of results.

In our study, GSNO demonstrated fragmentation effects only at concentrations significantly above previous serum and airway GSNO concentrations in vivo (13), although intracellular GSNO levels have not been reported. Endogenous S-nitrosothiols would therefore be unlikely to produce the effects on cell death that we observed with higher concentrations. Exogenous GSNO at these higher concentrations could potentially diminish pulmonary inflammation secondarily induced by neutrophils, dependent on the timing of administration. Moilaen et al. (34) found that NO donors, including GSNO, inhibited polymorphonuclear leukocyte function, whereas Lefer et al. (26) showed a beneficial antineutrophil effect from GSNO in a canine model of acute myocardial ischemia and reperfusion.

The effects of GSNO on DNA fragmentation compared with those of SNAP could result from an enhanced ability to transport NO intracellularly. The mechanism of nitrosothiol transport and metabolism, however, remains uncertain. Recent evidence suggests that γ-glutamyl transpeptidase (GGT), a dipeptide transporter enzyme involved in GSH metabolism and transport (33), may play a role in the cytostatic effect of GSNO on Salmonella typhimurium (10) and infers that intracellular transport of GSNO occurs with subsequent S-nitrosylation of cytoplasmic proteins. GGT hydrolyzes the γ-glutamyl moiety of GSNO to form glutamate and S-nitroscysteinylglycine (20), which is much more susceptible to release of free NO. Although GGT is predominantly membrane bound and localized on the external surface of epithelial cells (33), GGT activity is also found in leukocytes, primarily in immature myeloid cells but also in mature granulocytes (47). GGT is released into a surfactant-associated pool within the lung alveolus (22), which could potentially enable NO to be generated in the alveolar space. SNAP effects may require higher concentrations because a previous study (35) found that SNAP at a concentration of 2 mM (compared with 1 mM in our study) in hyperoxic conditions induced apoptosis in an alveolar epithelial cell line. We also did not measure NO production in our donor experiments, and it is possible that released NO from our lower SNAP dose was inadequate.

The mechanism of this NO effect on apoptosis remains uncertain. NO inactivation of the gene transcription factor nuclear factor-κB may induce apoptosis in some cell types (49, 50). A preliminary study (12) in our laboratory showed that GSNO inhibits nuclear factor-κB activation in human neutrophils. In the current study, MnSOD inhibited DNA fragmentation with NO, suggesting that oxidants could be participating in cell death. A speculative mechanism could involve production of the toxic metabolite peroxynitrite from the reaction of NO and superoxide anion (2) because peroxynitrite can induce apoptosis in other cell types (27).
S-NITROSGLUTATHIONE AND NEUTROPHIL CELL DEATH

L441

However, we found that superoxide generation was actually decreased in GSNO-exposed neutrophils, making this possibility much less likely.

NO synthase inhibition did not slow the intrinsic progression of neutrophil DNA fragmentation in our study, in contrast to the effects of NO synthase inhibition in murine macrophages. Albina et al. (1) found that NO synthase inhibition prevented interferon- and/or endotoxin-mediated apoptosis in murine peritoneal macrophages, implying that endogenous NO induced apoptosis. Differences among cell types in intrinsic apoptosis are likely related to quantitative and qualitative differences in molecular signaling mechanisms. Neutrophils express the Fas/Apo-1 antigen but lack the anti-apoptotic gene bcl-2 that is found in lymphocytes and monocytes (21). The absence of bcl-2 could allow the inexorable progression toward apoptosis seen in neutrophils but not in other cellular blood elements.

In summary, GSNO enhances DNA fragmentation in human neutrophils by a mechanism that may be, in part, mediated by interactions of NO with superoxide. Further studies are necessary to determine whether this in vitro pharmacological effect significantly affects neutrophil clearance in models of lung injury.

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