Biochemical Pharmacology, Vol. 59, pp. 305–314, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00329-9

Induction of Human Neutrophil Apoptosis by Nitric Oxide Donors: Evidence for a Caspase-dependent, Cyclic-GMP-independent, Mechanism

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ABSTRACT. This study investigated the regulatory effects of the major inflammatory mediator, nitric oxide (NO), on human neutrophil apoptosis *in vitro*. Co-culture of human neutrophils with the NO donors GEA 3162 (1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride) (10–100 μM) and 3-morpholino-sydnonimine (SIN-1) (0.3–3 mM) caused a dramatic and concentration-dependent induction of apoptosis. However, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced neutrophil activation (actin reorganization and chemotaxis) was inhibited by GEA 3162 treatment. The pro-apoptotic effects of the NO donors were (i) unaffected by the soluble guanylate cyclase inhibitor LY-83583 (6-anilino-5,8-quinolinedione; 100 μM), (ii) antagonized by superoxide dismutase (6 μg/mL), (iii) mimicked by exogenous peroxynitrite (at concentrations >100 μM), and (iv) inhibited by the caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (100 μM). The pro-apoptotic effect of the NO donors was not mimicked by the cell-permeable cyclic nucleotide analogue, N^6,2-*O*-dibutyrylguanosine-3′,5′-cyclic monophosphate (dibutyryl-cGMP) at concentrations ≤0.2 mM. Indeed, at high concentrations (≥2 mM), dibutyryl-cGMP caused an inhibition of apoptosis. These results suggest that NO-mediated apoptosis, although caspase-dependent, is mediated by a cGMP-independent mechanism and involves the concurrent generation of oxygen free radicals and, potentially, peroxynitrite. Our data reveal a unique role for NO in inflammatory responses with differential effects upon neutrophil activation and survival, with important implications for the successful resolution of inflammation. BIOCHEM PHARMACOL **59**;3:305–314, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. neutrophil; apoptosis; nitric oxide; inflammation; caspase

Apoptosis represents a physiological form of programmed cell death that permits efficient phagocytic removal of intact senescent cells. In contrast, cell death by necrosis is typified by the loss of membrane integrity and hence represents a potentially pro-inflammatory and injurious mode of cell deletion [1, 2]. In the neutrophil, apoptosis is characterized by a set of distinct morphological and biochemical changes [3–6] which results in functional down-regulation [5] and the rapid ingestion and removal of these cells by a process that uniquely fails to incite an inflammatory phagocytic response [7]. In view of the enormous capacity of the neutrophil to release an array of histotoxic products during necrotic cell death, apoptosis therefore offers a physiological, efficient, and non-inflammatory mechanism for neutrophil removal. Granulocyte apoptosis is thus regarded as a crucial process underlying the control and successful resolution of inflammation.

Despite clear evidence that NO‡ plays an important role in a number of physiological and pathological processes, displaying pro-inflammatory or anti-inflammatory effects depending on its effective concentration at the inflammatory site [8–10], the regulatory effects of NO on human neutrophil apoptosis are unknown. Furthermore, the signaling events regulating neutrophil apoptosis remain ill defined. Many of the known modulators of apoptosis in other cell types have very different effects on neutrophil apoptosis; for example, the glucocorticoid dexamethasone and agents that elevate cytosolic free Ca^{2+} or intracellular cAMP inhibit neutrophil apoptosis [11–15], yet induce apoptosis in other myeloid cells [13, 14, 16, 17]. Inflamma-

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Received 20 April 1999; accepted 3 August 1999.

‡ *Abbreviations:* db-cAMP, N^6,2-*O*-dibutyryladenosine-3′,5′-cyclic monophosphate; db-cGMP, N^2,2-*O*-dibutyrylguanosine-3′,5′-cyclic monophosphate; MDM, modified Dulbecco's medium; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GEA 3162, 1,2,3,4-oxatriazolium,5-amino-3-(3,4-dichlorophenyl)-chloride; HBSS, Hank's balanced salt solution; LY-83583, 6-anilino-5,8-quinolinedione; NO, nitric oxide; $ONOO^-$, peroxynitrite; O_2^-, superoxide anion; sGC, soluble guanylate cyclase; SIN-1, 3-morpholino-sydnonimine; SOD, superoxide dismutase; and ZVAD-fmk, Z-Val-Ala-DL-Asp-fluoromethylketone.

tory mediators also differentially regulate neutrophil apoptosis; for example, tumor necrosis factor induces apoptosis at early time points after culture [18], whereas granulocyte-macrophage colony-stimulating factor, C5a, and lipopolysaccharide all inhibit the rate of apoptosis [4, 19]. Therefore, these data relating to the effects of NO on apoptosis in other cell types cannot be extrapolated to the neutrophil.

We therefore sought to define the role of NO in regulating human neutrophil apoptosis *in vitro*. We examined the direct effects of the well-characterized NO donors GEA 3162 [20] and SIN-1 [21], together with the cell-permeable cyclic nucleotide analogue db-cGMP, LY-83583, an inhibitor of the cGMP-protein kinase G pathway [22], exogenous SOD, and $ONOO^-$ (a product of $O_2^{\cdot}$ and NO interaction). We present evidence demonstrating that in contrast to its effect on neutrophil activation NO mediates a profound pro-apoptotic effect on neutrophils. These data represent a further investigation of our initial preliminary observations of NO-mediated apoptosis in neutrophils [23], which have been confirmed in recent reports by Blaylock *et al.* [24] and Fotenberry *et al.* [25], who also demonstrated that NO induces apoptosis in human neutrophils. Our results describe additional mechanistic data on the NO-mediated pro-apoptotic effect. We report for the first time that NO induces apoptosis in human neutrophils by a mechanism involving a caspase-dependent pathway that is not mediated by the guanylate cyclase/cGMP pathway. Our studies suggest that NO-mediated effects may have significance in inflammatory conditions where the mechanisms controlling neutrophil activation and survival are normally tightly regulated.

MATERIALS AND METHODS

Reagents

Sodium citrate was purchased from Phoenix Pharmaceuticals Ltd. Percoll and dextran were obtained from Pharmacia Biotechnologies. Iscove's MDM, PBS, HBSS, and supplements (penicillin and streptomycin) were from Life Technologies. Sterile tissue culture plasticware and flexiwell plates were purchased from Corning–Costar and Falcon. Methanol was purchased from Fisons Scientific. Diff-Quik stain was obtained from Baxter. db-cAMP, db-cGMP, SOD, SIN-1, N^G-nitro-L-arginine methyl ester (L-NAME), FMLP, and trypan blue were purchased from Sigma Chemical Co. Ltd. GEA 3162, N^G-monomethyl-L-arginine monoacetate (L-NMMA), LY-83583 were from Alexis Corporation. ZVAD-fmk was from Bachem.

Neutrophil Preparation

Human neutrophils were isolated and purified from peripheral venous blood taken from healthy adult volunteers as previously described [26]. In brief, aliquots (40 mL) of blood were anticoagulated with 4 mL 3.8% sodium citrate and centrifuged (300 *g*; 25°; 20 min). The leukocyte-rich fraction was separated from erythrocytes using dextran sedimentation and the neutrophils separated from mononuclear cells using discontinuous PBS–Percoll gradients. The purified neutrophils were washed in platelet-poor plasma, followed by HBSS without Ca^{2+} and Mg^{2+}, and finally resuspended in HBSS with Ca^{2+} and Mg^{2+}. Cell purity was determined by examining cytocentrifuged preparations (fixed in methanol and stained with Diff-Quik) and viability assessed by trypan blue exclusion. The neutrophils were routinely >95% pure (<0.5% monocyte contamination) and >99.5% viable.

Assessment of Neutrophil Apoptosis by Cytology

Neutrophils (5×10^6/mL) were suspended in Iscove's MDM supplemented with 10% autologous serum, 10 U/mL penicillin, and 50 μg/ml streptomycin (unless otherwise stated). Iscove's MDM was replaced with PBS containing Ca^{2+} and Mg^{2+} for cultures with $ONOO^-$ (see below). Neutrophils (3.75×10^5) were cultured in the presence or absence of the reagents indicated in a final volume of 150 μL at 37° in a humidified 5% CO_2 atmosphere in flat-bottomed 96-well flexiwell plates. At 4, 6, and 20 hr, neutrophils were harvested from culture and assessed for recovery, viability (trypan blue exclusion), and apoptosis. Apoptosis was assessed morphologically by oil immersion light microscopy (×100 objective) of fixed and stained cytocentrifuge preparations of the recovered neutrophils. Apoptotic neutrophils were defined as cells containing one or more hyperchromatic pyknotic nuclei [3]. All treatments were performed in triplicate with at least 500 cells counted per slide and the observer blinded to the experimental condition. Results are expressed as the percentage of cells displaying characteristic apoptotic morphology.

Assessment of Neutrophil Apoptosis by Annexin V Binding

A separate and independent assessment of apoptosis was performed by flow cytometry using fluorescein isothiocyanate-labeled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells [27]. Neutrophils (5×10^6/mL) were cultured as described above and at the time indicated an aliquot (25 μL) of recovered cells was added to 75 μL of a 1:200 dilution of annexin V (Bender Med Systems). Following a 10-min incubation at 4°, samples were analyzed using an EPICS Profile II (Coulter).

Neutrophil Shape Change

The effects of NO donors on FMLP-induced shape change were examined by preincubating neutrophils (450 μL at 2×10^6/mL in PBS containing Ca^{2+} and Mg^{2+} in the absence of serum) with the corresponding NO donor for 45 min at 37° in a gently shaking water bath before the addition of 10 nM FMLP for a further 10 min. Incubations were terminated by the addition of 500 μL 2.5% gluteral-

dehyde. Samples were analyzed for shape change by flow cytometry (Coulter EPICS Profile II; Coulter) as previously described [28].

Neutrophil Chemotaxis

FMLP (100 nM in 35 μL Iscove's MDM containing 10% autologous serum) was placed in the bottom wells of a Neuroprobe 96-well chemotaxis chamber (Porvair Filtronics), with neutrophils (225 μL at 3×10^6/mL in Iscove's MDM containing 10% autologous serum) that had been incubated in the presence or absence of the indicated concentration of the NO donor for 45 min at 37° added to the top wells. The chamber was then incubated for 90 min at 37° in a humidified 5% CO_2 atmosphere. The polycarbonate filter (with 3-μm pores) was then removed, the top surface scraped to remove adherent cells, and washed (0.9% saline), dried, fixed and stained (Diff-Quik) prior to reading in a Dynatech MR5000 ELISA plate reader for O.D. at 550 nm. Results are expressed as a percentage of the control well O.D. values. Neutrophils that had migrated through the filter into the bottom wells were also counted under light microscopy using a hemocytometer for verification.

Peroxynitrite ($ONOO^-$) Synthesis

$ONOO^-$ was synthesized by the method as detailed by [29]. It is important to note that, due to the chemical instability of $ONOO^-$ when incubated under neutral or acidic conditions, all experiments examining the potential role of $ONOO^-$ in mediating NO-induced apoptosis, including control incubations, were performed in PBS containing Ca^{2+} and Mg^{2+} in the absence of serum at a pH of 8.0.

Analysis of Results

The results are expressed as means ± SEM of the number (N) of independent experiments each using cells from different donors. Statistical analysis was performed by ANOVA with comparisons between groups made using the Newman–Keuls procedure. Differences were considered significant when $P < 0.05$.

RESULTS

Effects of NO Donors on Neutrophil Apoptosis

Neutrophil apoptosis is accompanied by dramatic morphological changes; non-apoptotic neutrophils contain multilobular nuclei, whereas apoptotic neutrophils exhibit hyperchromatic pyknotic nuclei ([3] and Fig. 1). It is important to note that neutrophils cultured *in vitro* undergo constitutive apoptosis; for example, in the current experiments, 2.8 ± 0.6% and 61.5 ± 4.5% of neutrophils cultured for 6 and 20 hr, respectively exhibited apoptosis even in the absence of NO donors (N = 11). Cellular necrosis at 20 hr was negligible with >98% cell viability, assessed by trypan blue exclusion. Non-apoptotic neutrophils cultured for 6 hr are shown in Fig. 1A (control). In comparison, the apoptotic morphology of neutrophils is demonstrated in Fig. 1B, where the cells had been cultured for 6 hr with the NO donor GEA 3162 (100 μM). The induction of apoptosis by GEA 3162 was confirmed by analyses of annexin V binding to the cell surface of apoptotic neutrophils, where the NO donor caused a concentration-dependent increase in the number of cells binding annexin V after 6 hr culture (Fig. 1, C, D, and E). These findings indicate that GEA 3162 causes cell surface changes (exposure of phosphatidylserine molecules) as well as the nuclear changes (Fig. 1B) associated with apoptosis. The effects of two structurally distinct NO donors, GEA 3162, and SIN-1, on neutrophil apoptosis at 6 and 20 hr is shown in Fig. 2. GEA 3162 (10–100 μM) (Fig. 2A) and SIN-1 (0.3–3 mM) (Fig. 2B) caused a concentration-dependent induction of apoptosis at both time points. There was little evidence of necrosis under any condition except after treatment with GEA 3162 (100 μM) for 20 hr (control, 99 ± 1% viable, 100 μM GEA 3162, 54 ± 15% viable, as assessed by trypan blue exclusion). The markedly different potency and effectiveness of the two NO donors probably reflects the lipophilic nature of GEA 3162 and the fact that it is a highly effective NO donor when used in intact cells in comparison to SIN-1 [30, 31].

Cyclic GMP-independent Effect of NO Donors on Neutrophil Apoptosis

To investigate whether the pro-apoptotic effects of NO donors are mediated via a cGMP-dependent pathway, we (i) examined the effects of the cell-permeable cGMP analogue db-cGMP on the constitutive rate of neutrophil apoptosis and (ii) co-cultured cells with NO donors in the presence of the sGS inhibitor LY-83583. As shown in Fig. 3A, addition of db-cGMP at 0.2 or 2 mM failed to induce apoptosis, but a concentration of 2 mM inhibited apoptosis at 6 and 20 hr to a similar extent as that observed with db-cAMP [12]. Concentrations of db-cGMP <0.2 mM also had no effect on the rate of apoptosis (data not shown). Figure 3B shows that a concentration of 100 μM LY-83583, which inhibits agonist-induced cGMP-dependent protein kinase activation and degranulation in human neutrophils [22], did not significantly affect SIN-1 (3 mM)- or GEA 3162 (30 μM)-induced apoptosis assessed at 6 hr. Taken together, these results suggest that NO donors trigger apoptosis independently of cGMP signaling pathways. Interestingly, when neutrophils were co-cultured with GEA 3162 in the presence of the caspase inhibitor ZVAD-fmk (100 μM), NO-induced apoptosis was inhibited by >70% (Fig. 3C). This experiment was performed at the earlier time point of 4 hr, when the constitutive rate of apoptosis was extremely low. This ensured that the NO-induced apoptosis was blocked rather than constitutive apoptosis.

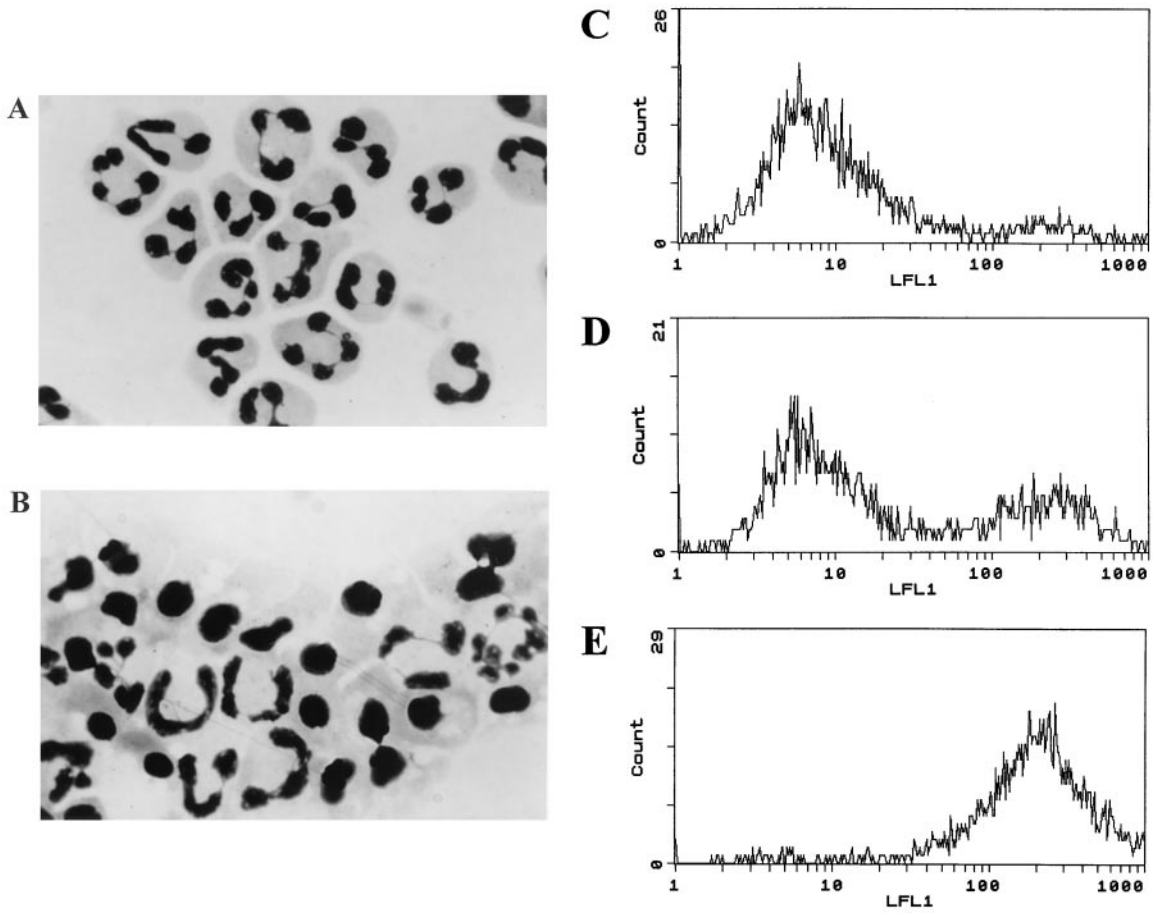

FIG. 1. Effect of the NO donor GEA 3162 on human neutrophil apoptosis. Neutrophils (5×10^6/mL) were cultured with (A) vehicle control or (B) GEA 3162 (100 μM) for 6 hr and cytocentrifuge preparations made (magnification ×1000). Neutrophils were cultured for 6 hr as above and treated with (C) vehicle control, (D) GEA 3162 (30 μM), or (E) GEA 3162 (100 μM), and samples were processed for annexin V binding to determine phosphatidylserine expression (LFL1, log fluorescence).

Effects of NO Donors Potentially Mediated by the Generation of Oxygen free Radicals

The capacity for certain NO donors to co-release $O_2^{\cdot}$ is well recognized. However, neither receptor-mediated generation of $O_2^{\cdot}$ nor the application of exogenous reactive oxygen species (e.g. hydrogen peroxide) is able to induce comparable apoptotic responses in neutrophils [32]. To examine whether the generation of $O_2^{\cdot}$ may be contributing to the pro-apoptotic effect of the NO donors, the effects of SOD (6 μg/mL) on SIN-1- and GEA 3162-induced apoptosis were assessed. Figure 4 demonstrates that SOD abolished the pro-apoptotic effect of SIN-1 (which releases more $O_2^{\cdot}$ than GEA 3162) and partially inhibited the effect of the latter, this being particularly evident at submaximal GEA 3162 concentrations. We investigated whether $ONOO^-$, a major product of $O_2^{\cdot}$ and NO interaction, could trigger neutrophil apoptosis directly. Figure 5 shows that $ONOO^-$ (≥100 μM) caused a dramatic and concentration-dependent induction of neutrophil apoptosis at 4 hr (e.g. 500 μM gave approximately 40% apoptosis), although at higher concentrations and longer incubation periods evidence of necrosis is evident, as assessed by trypan blue exclusion (e.g. 855 μM induced >30% necrosis at 4 hr). Given the high rates of apoptosis and the onset of necrosis by 4 hr, the 6-hr timepoint was not examined.

Effects of the NO Donor GEA 3162 on Neutrophil Activation

The rapid and dramatic pro-apoptotic effects of NO donors raised the possibility of initiation of signals within neutrophils that lead to engagement of the apoptotic machinery. We therefore examined whether these agents affected neutrophil polarization and directed migration as indices of neutrophil activation. After 45-min pretreatment in media alone, the mean percentage shape change for control neutrophils was 14.2 ± 1.8% (N = 3), as determined by a flow cytometric method to assess shape change using forward and side scatter parameters (Fig. 6, A–D). Treatment with FMLP (10 nM, 10 min) increased shape change to 89.5 ± 1.9% (N = 3). Preincubation of neutrophils with GEA 3162 (1–100 μM) caused a concentration-dependent inhibition (IC_{50} = 9 μM) of FMLP (10 nM, 10 min)-induced shape change (Fig. 6E). Note that these experiments were performed in PBS, since the presence of serum itself can induce cell polarisation. Optimal conditions for

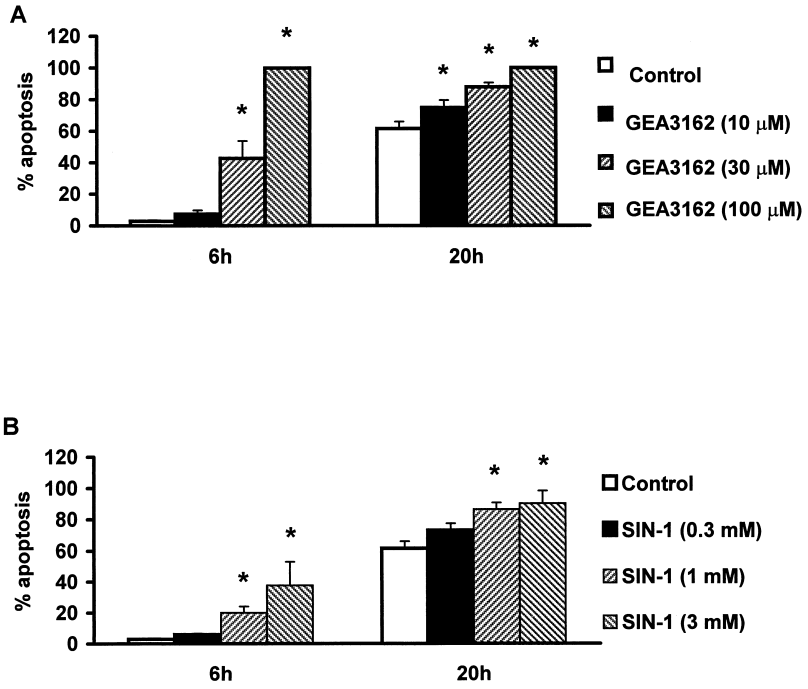

FIG. 2. Effect of the NO donors GEA 3162 and SIN-1 on human neutrophil apoptosis. Neutrophils (5×10^6/mL) were cultured with (A) GEA 3162 (10–100 μM) or (B) SIN-1 (0.3–3 mM) for 6 or 20 hr and apoptosis assessed morphologically. Results are expressed as the mean ± SEM percent apoptosis of 3–4 separate experiments. Significant difference ($P < 0.05$) from the appropriate control (open bars) is indicated by *.

chemotaxis were achieved using neutrophils suspended in Iscove's MDM containing 10% autologous serum. Mean O.D. values under control conditions (reflecting spontaneous chemokinesis) and maximal FMLP-stimulated conditions (directed chemotaxis) were 0.55 ± 0.04 and 1.17 ± 0.14, respectively (N = 9). Preincubation of neutrophils with GEA 3162 (1–100 μM) inhibited FMLP (100 nM)-induced chemotaxis in a concentration-dependent fashion ($IC_{50} \approx 7$ μM) (Fig. 7). SIN-1 (IC_{50} 1 mM) caused a similar inhibition of FMLP (100 nM)-induced chemotaxis (data not shown).

DISCUSSION

Our results clearly show the capacity of structurally distinct NO donors (GEA 3162 and SIN-1) to induce apoptosis in human neutrophils. These data corroborate and extend our initial findings [23] and also those recently reported [23–25]. These results are of particular interest since neutrophils are relatively resistant to receptor-mediated or pharmacological induction of apoptosis and fail to undergo augmented apoptosis in response to stimuli such as glucocorticoids, agents that increase cAMP or cytosolic free Ca^{2+}, and hypoxia [11–15, 32]. Although some variability occurred in the levels of induction of apoptosis between experiments, this was mainly due to donor variation and differences in activity between batches of GEA 3162 and SIN-1 (data not shown). To examine the mechanism of NO-induced neutrophil apoptosis, we initially investigated whether such effects were mediated via activation of sGC with the subsequent generation of cGMP. Although neutrophils possess a sGC [33], the mechanism of induction of apoptosis by NO does not appear to utilize the sGC–cGMP pathway, since the cyclic nucleotide analogue db-cGMP (0.002–200 μM) did not induce apoptosis and the GEA 3162 and SIN-1 pro-apoptotic effect could not be blocked by the sGC inhibitor LY-83583. On the contrary, db-cGMP at a higher concentration of 2 mM inhibited apoptosis. Preliminary data indicate that the ability of db-cGMP to inhibit neutrophil apoptosis at higher concentrations may be mediated via cross-activation of protein kinase A, since the protein kinase A inhibitor H89 blocks the inhibition of apoptosis by db-cGMP as well as the effect of agents that elevate cAMP [12 and *].

We next examined whether the pro-apoptotic effect of the NO donors involved, or was mediated by, the formation of oxygen-derived free radicals. Human neutrophils have the capacity to generate NO and $O_2^{\cdot}$, which may react to form $ONOO^-$ [34, 35] with the potential for the induction of apoptosis [36]. In the presence of NO donors, neutrophil-derived extracellular $O_2^{\cdot}$ -mediated generation of $ONOO^-$ can be prevented by the presence of exogenous SOD. Furthermore, direct addition of $ONOO^-$ to neutrophils resulted in a rapid and marked induction of apoptosis, again suggesting that GEA 3162- and SIN-1-induced apoptosis may be mediated, at least in part, by $ONOO^-$ formation, particularly in the case of SIN-1, which releases equimolar concentrations of NO and $O_2^{\cdot}$. The resulting protection against SIN-1- and GEA 3162-induced apoptosis provides strong evidence in support of this hypothesis, not least because SOD is not cell-permeable, its effects thus being restricted to protecting against extracellular $O_2^{\cdot}$. The finding that the pro-apoptotic effect of GEA 3162 at 100 μM was not blocked by SOD suggests that either GEA

* Wong TH and Rossi AG, unpublished observations.

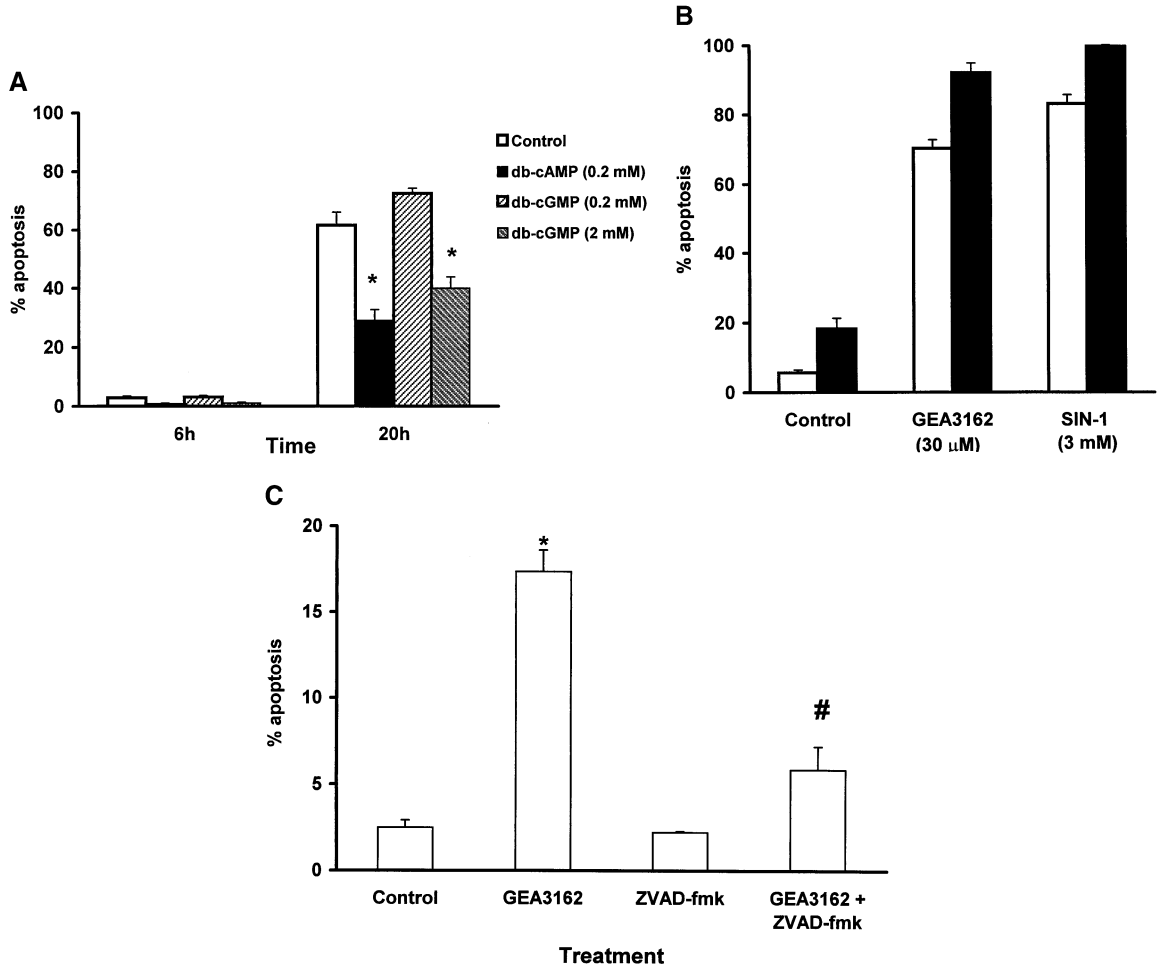

FIG. 3. (A) Effect of the cyclic nucleotide analogues db-cAMP and db-cGMP on human neutrophil apoptosis. Neutrophils (5 × 10^6/mL) were cultured with db-cAMP (0.2 mM) and db-cGMP (0.2 and 2 mM) for 6 or 20 hr and apoptosis assessed morphologically. Results are expressed as the mean ± SEM percent apoptosis of 6 separate experiments. Significant difference ($P < 0.05$) from the appropriate control (open bars) is indicated by *. (B) Effect of the sGC inhibitor LY-83583 on NO donor-induced human neutrophil apoptosis. Neutrophils (5 × 10^6/mL) were cultured with GEA 3162 (30 μM) or SIN-1 (3 mM) in the absence (open bars) or presence of 100 μM LY-83583 (filled bars) for 6 hr and apoptosis assessed morphologically. Data represent means ± SD of triplicate determinations from a single representative experiment. (C) Effect of the caspase inhibitor ZVAD-fmk on GEA 3162-induced human neutrophil apoptosis. Neutrophils (5 × 10^6/mL) were cultured with GEA 3162 (30 μM) in the presence or absence of 100 μM ZVAD-fmk for 4 hr and apoptosis thereafter assessed morphologically. Results are expressed as the mean ± SEM percent apoptosis of 3 separate experiments. Significant difference ($P < 0.05$) from the control and from GEA 3162 is indicated by * and #, respectively.

3162 triggers apoptosis by both $ONOO^-$-dependent and $ONOO^-$-independent routes or that SOD is unable to provide complete protection against $ONOO^-$ formation at this concentration of NO donor. Whether $O_2^{\cdot}$ is produced as a byproduct of NO during its oxidation to NO_2 by dioxygen or released after endogenous production by neutrophils themselves, the presence of SOD in these experiments should prevent the formation of oxygen-derived free radicals. In addition, further preliminary data suggest that the generation of endogenous NO by the neutrophil does not appear to play a critical role in regulating the constitutive rate of apoptosis in these cells since (i) depletion of the NO synthase substrate L-arginine had no significant effect on the rate of apoptosis at 20 hr and (ii) incubation of neutrophils with the NO synthase inhibitors L-NMMA (N^G-monomethyl-L-arginine) (0.3 mM) and L-NAME (N^G-nitro-L-arginine methyl ester) (0.3 mM) likewise did not affect the rate of apoptosis compared to control untreated cells (data not shown). Interestingly, the sGC inhibitor LY-83583 (100 μM) reproducibly increased the rate of constitutive neutrophil apoptosis. The data shown (Fig. 3, A and B) suggest that cGMP, while not involved in NO-induced apoptosis, can clearly enhance the survival of neutrophils. This supports the view that activation of sGC may play a role in regulating inhibition of apoptosis, particularly since the inhibitor enhances the increase in the rate of apoptosis achieved by NO alone.

A number of possible molecular mechanisms may account for the pro-apoptotic effect of $ONOO^-$ and NO donors. Firstly, reactive oxygen and nitrogen radicals can lead to direct 'free radical-mediated' DNA damage, including deamination and DNA strand breaks [37, 38]. Sec-

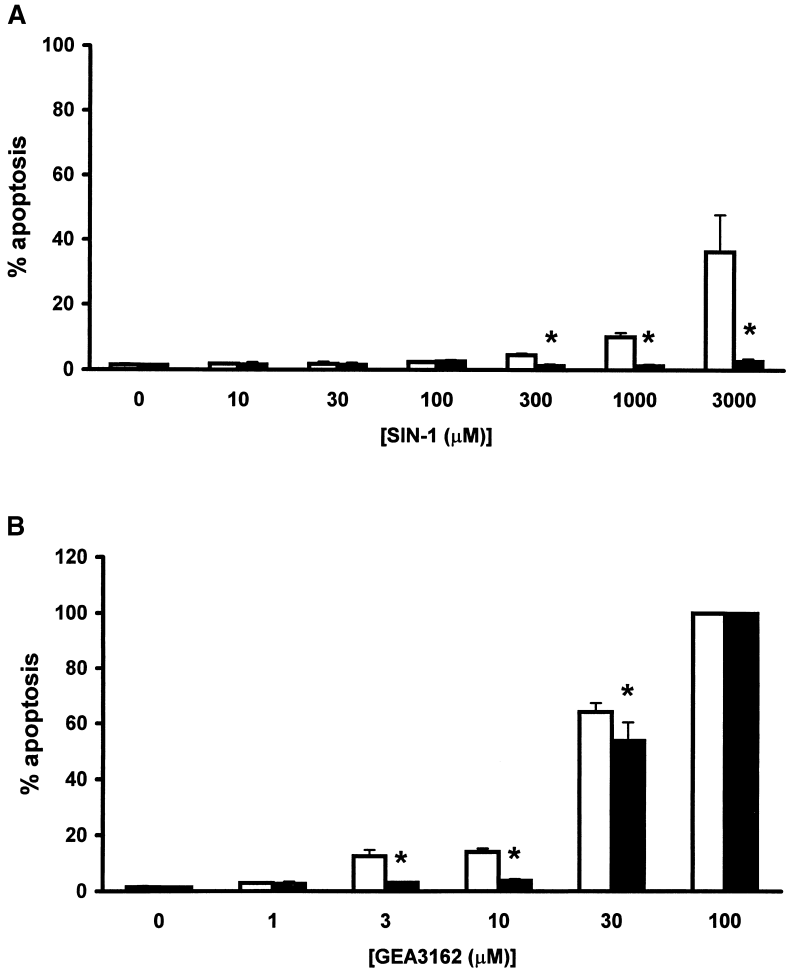

FIG. 4. Effect of SOD on GEA 3162- and SIN-1-induced human neutrophil apoptosis. Neutrophils (5×10^6/mL) were cultured with (A) SIN-1 (10–3000 μM) and (B) GEA 3162 (1–100 μM) in the absence (open bars) or in the presence (filled bars) of 6 μg/mL SOD for 6 hr and apoptosis assessed morphologically. Similar results were obtained after 20-hr culture (data not shown). Results are expressed as the mean ± SEM percent apoptosis of 3 separate experiments. Significant difference ($P < 0.05$) from the appropriate control (open bars) is indicated by *.

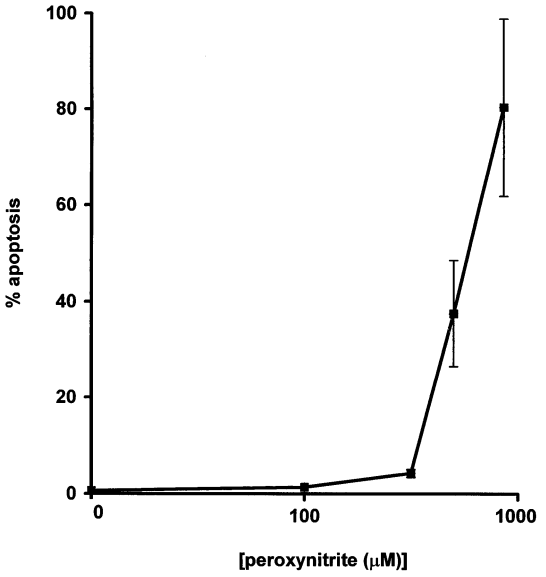

FIG. 5. Effect of $ONOO^-$ on human neutrophil apoptosis. Neutrophils (5×10^6/mL in PBS in the absence of serum) were cultured with $ONOO^-$ for 4 hr and apoptosis assessed morphologically. Results are expressed as the mean ± SEM percent apoptosis of 3 separate experiments.

ondly, recent evidence indicates that NO effects could be mediated by inhibition of cytosolic ADP-ribosyltransferase, an enzyme that catalyzes the transfer of the ADP-ribose moiety of NAD^+ to other proteins [39]. Finally, conversion of NO to $ONOO^-$ may deplete intracellular Ca^{2+} stores [40]. This effect could play a crucial role in neutrophil apoptosis, since elevation of cytosolic free Ca^{2+} delays neutrophil apoptosis [11, 14] whereas depletion may induce apoptosis [11]. Our results suggest that the effects of NO donors are not due to direct DNA damage, since the caspase inhibitor ZVAD-fmk dramatically inhibited the pro-apoptotic effect of NO donors. NO has been reported to inhibit the activity of the transcription factor nuclear factor-kappaB (NF-κB) [41], and interestingly we have recently demonstrated that inhibition of this transcription factor results in the onset of neutrophil apoptosis [42]. We are currently investigating whether NO can inhibit NF-κB in neutrophils, thus providing another possible mechanism for NO-induced apoptosis in these cells. Results in Figs. 6 and 7 clearly demonstrate that GEA 3162 inhibits FMLP-induced shape change and chemotaxis consistent with inhibitory actions of NO upon neutrophil activation [20, 43–45]. Clearly, further work is required to ascertain the precise signaling pathways involved and whether the reported effects of NO on cytoskeletal elements via ADP-

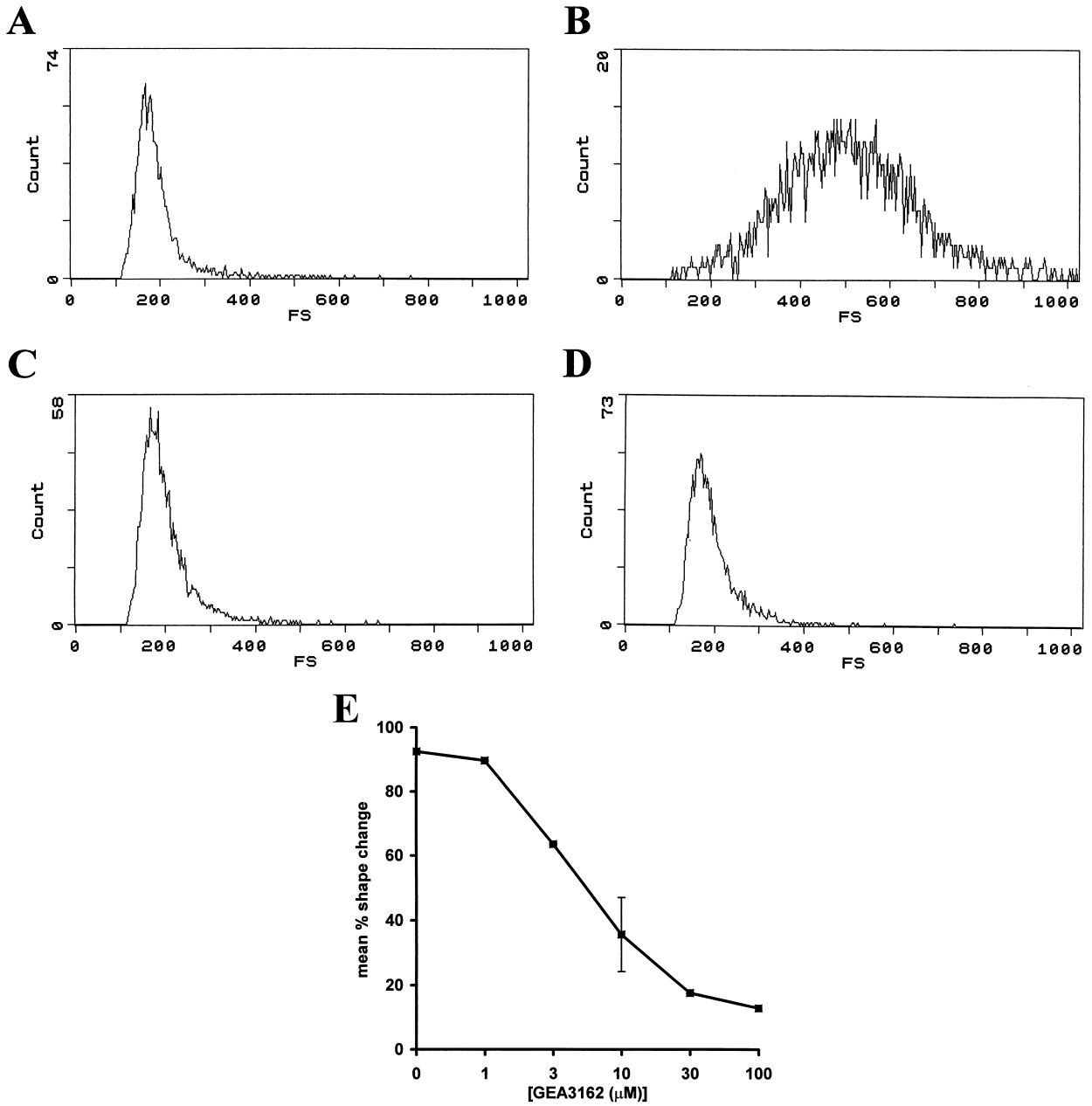

FIG. 6. Effect of GEA 3162 on FMLP-induced shape change. Neutrophils (2 × 10^6/mL in PBS in the absence of serum) were preincubated with GEA 3162 for 45 min at 37° before the addition of 10 nM FMLP for 10 min. The incubations were terminated by the addition of 500 μL 2.5% gluteraldehyde. Samples were assessed by an EPICS (Profile II) flow cytometer. Representative flow cytometry histograms of control cells (A), FMLP-stimulated cells (B), cells preincubated with GEA 3162 alone (C), and cells preincubated with GEA 3162 followed by FMLP stimulation (D) are shown, where the x-axis shows mean forward light scatter (FS) and the y-axis is the relative cell number. The mean percentage shape change (E) was calculated from the FS of each sample by gating out the population of non-shape-changed neutrophils. Results are expressed as the mean ± SEM percentage shape change of 3 separate experiments.

ribosylation of actin [46] are responsible for the effects on neutrophil activation and apoptosis. Our results, however, show that NO donors can functionally uncouple neutrophil responsiveness to external stimuli by two potential mechanisms: by directly inhibiting neutrophil function (inhibition of shape change and chemotaxis) and by stimulating programmed cell death (inducing apoptosis).

In conclusion, we have demonstrated that structurally distinct NO donors have the unique ability to induce neutrophil apoptosis and inhibit neutrophil activation. The induction of apoptosis by NO donors appears to be mediated via a caspase-dependent mechanism as well as a cGMP-independent pathway, possibly involving the formation of $ONOO^-$. Since the induction of neutrophil apoptosis and the functional uncoupling of these cells appear to be prerequisites for the successful resolution of inflamma-

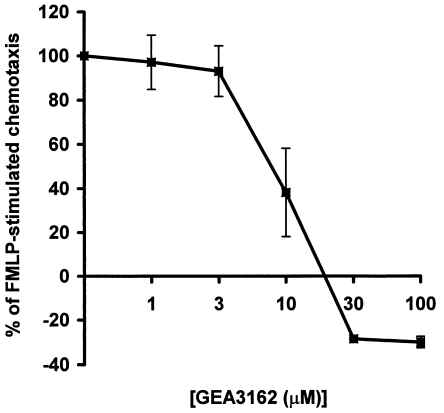

FIG. 7. Effect of GEA 3162 on FMLP-induced chemotaxis. Neutrophils (3 × 10^6/mL) were preincubated with vehicle control or GEA 3162 (1–100 μM) for 45 min at 37° before chemotaxis to FMLP (100 nM; 90 min; 37°) was assessed. Results are expressed as a mean ± SEM percentage of control O.D. values obtained at 550 nm of 3 separate experiments. Similar results were obtained with SIN-1 (data not shown).

tion, the ability of NO donors to trigger both these events may provide a novel pharmacological approach to promote resolution of inflammation.

The Medical Research Council, U.K., Programme Grant Number G9016491 and The Wellcome Trust supported this work. E.R.C. is a Wellcome Senior Researcher in Clinical Science. A.G.R. and E.R.C. contributed equally to this manuscript. C.W. and T.H.W. are joint first authors. We thank Dr. Ian Dransfield for constructive criticism of this manuscript.

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