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3-Morpholino-Sydnonimine-Induced Suppression of Human Neutrophil Degranulation is Not Mediated by Cyclic GMP, Nitric Oxide or Peroxynitrite: Inhibition of the Increase in Intracellular Free Calcium Concentration by *N*-Morpholino-iminoacetonitrile, a Metabolite of 3-Morpholino-Sydnonimine

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SUMMARY

This study was designed to clarify the mechanism of the inhibitory action of a nitric oxide (NO) donor 3-morpholino-sydnonimine (SIN-1) on human neutrophil degranulation. SIN-1 (100–1000 μM) inhibited degranulation (β-glucuronidase release) in a concentration-dependent manner and concomitantly increased the levels of cGMP in human neutrophils in suspension. However, further studies suggested that neither NO nor increase in cGMP levels were mediating the inhibitory effect of SIN-1 on human neutrophil degranulation because 1) red blood cells or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl added as NO scavengers did not inhibit the effect; 2) inhibitors of cGMP synthesis (methylene blue) or phosphodiesterases (3-isobutyl-1-methylxanthine) did not produce changes in cell function correlating with the changes in cGMP. SIN-1 releases both nitric oxide and superoxide, which together form peroxynitrite. Chemically synthesized peroxynitrite (1–100 μM) did not inhibit, but at high concentrations (1000–2350 μM), it potentiated FMLP-induced β-glucuronidase release from neutrophils. Thus formation of peroxynitrite from SIN-1 does not explain its inhibitory effects on neutrophil degranulation. The NO-deficient metabolite of SIN-1, SIN-1C (330–1000 μM) inhibited human neutrophil degranulation in a concentration-dependent manner similar to that of SIN-1 and reduced the increase in intracellular free calcium induced by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine. C88–3934 (330–1000 μM), another NO-deficient sydnonimine metabolite, also inhibited human neutrophil degranulation. In conclusion, the data shows that the NO-donor SIN-1 inhibits human neutrophil degranulation in a cGMP-, NO-, and peroxynitrite-independent manner, probably because of the formation of more stable active metabolites such as SIN-1C. The results demonstrate that studies on the role of NO and/or peroxynitrite carried out with SIN-1 and other NO-donors should be carefully re-evaluated as to whether the effects found are really attributable to NO or peroxynitrite and that in future studies, it will be crucial to carry out control experiments with the NO-deficient metabolites in any studies with sydnonimine NO-donors.

EDRF is a labile substance produced by vascular endothelium (1). Pharmacological and chemical evidence indicates that EDRF is identical or closely related to nitric oxide (NO), free radical gas, synthesized from L-arginine by a family of isoenzymes called NO synthases (2, 3) The known biological functions of EDRF/NO include vasodilatation, inhibition of platelet aggregation, neurotransmission, and regulation of the immune response. These effects are believed to be mediated mainly by the activation of soluble guanylate cyclase and an increase in the intracellular concentrations of cGMP (3, 4).

Because of the instability and inconvenient handling of aqueous solutions of authentic NO, there has been increased interest in using compounds that generate NO *in situ* (5). Ever since the NO-releasing properties of SIN-1 (the ac-

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ABBREVIATIONS: NO, nitric oxide; EDRF, endothelium-derived relaxing factor; Bt_2, N^2,2′-*O*-dibutyryl; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IBMX, 3-isobutyl-1-methylxanthine; PGE_2, prostaglandin E_2; PMA, phorbol myristate acetate; SIN-1, 3-morpholino-sydnonimine; SIN-1C, *N*-morpholinoiminoacetonitrile; SOD, superoxide dismutase; $[Ca^{2+}]_i$, intracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; RBC, red blood cell.

tive metabolite of the antianginal drug *N*-ethoxycarboxy-3-morpholino-sydnonimine-ethyl ester (molsidomine) were described (6, 7), it has been used in numerous studies assessing the physiological and pharmacological role of NO. The vast majority of these studies have used concentrations of SIN-1 higher than 100 μM. SIN-1 decomposes to produce NO in an oxygen-dependent process involving hydroxyl-driven hydrolytic ring opening to *N*-morpholino-*N*-nitrosoaminoacetonitrile and further to a more stable SIN-1C (Fig. 1) (8, 9). The simultaneous release of NO from sydnonimines and conversion of oxygen to superoxide ($O_2^{\cdot-}$) occurs in a stoichiometric manner (5, 9, 10). The release of $O_2^{\cdot-}$ together with NO may lead to the formation of a strong biological oxidant, peroxynitrite ($OONO^-$) and its subsequent degradation products, such as hydroxyl radical ($OH^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$) (5, 10–12).

The modulatory effects of NO and its second messenger, cGMP, on neutrophils have been extensively studied, although the results remain controversial (4). Inhibitors of NO synthesis have been reported to inhibit neutrophil migration and degranulation (13, 14). In addition, SIN-1 and some other NO-releasing compounds have been found to be inhibitors of neutrophil functions such as degranulation, chemotaxis, leukotriene B_4 synthesis, and superoxide production (15–24). The mechanism by which SIN-1 inhibits neutrophil function remains unclear. NO has been reported to inhibit directly the activity of the superoxide-producing NADPH oxidase (25), 5-lipoxygenase (26) and to ADP-ribosylate actin in human neutrophils (27). Some authors have proposed that cGMP could mediate the SIN-1–induced modulation of neutrophil activation (15, 16, 20), whereas most studies have left open the question on the role of cGMP.

The present study was designed to determine the mechanism of the inhibitory action of NO- and peroxynitrite-donor SIN-1 on human neutrophil degranulation. Unexpectedly, the present data indicates that SIN-1 inhibits degranulation *in vitro* in a cGMP-, NO-, and peroxynitrite-independent manner in human neutrophils in suspension. The active compound(s) is a more stable break-down product of SIN-1, such as SIN-1C, which is shown to inhibit human neutrophil degranulation in a manner involving inhibition of the increase in intracellular free calcium concentration.

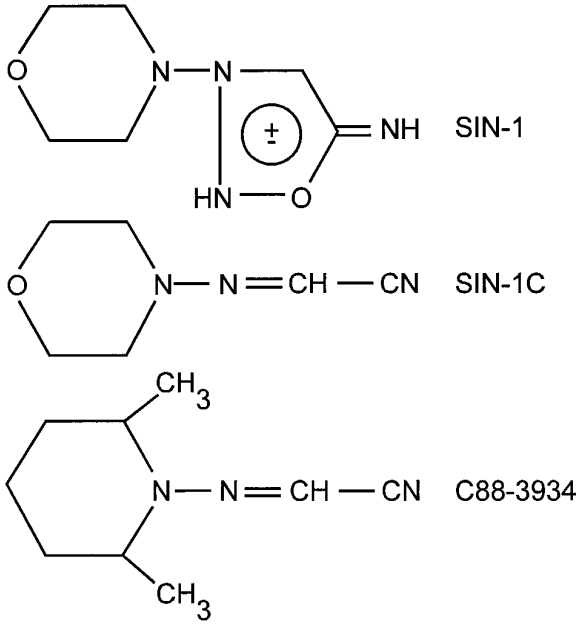

Fig. 1. The chemical structures of SIN-1, SIN-1C, and C88–3934.

Experimental Procedures

Materials. A23187, bovine hemoglobin, 8-bromo-cAMP, 8-bromo-cGMP, Bt_2-cAMP, Bt_2-cGMP, FMLP, Fura-2/AM, HEPES, $KO_2^-MnO_2$, phenolphthalein-β-D-glucuronide, PMA, pyrogallol, SOD, and Triton X-100 were purchased from Sigma Chemical (St. Louis, MO). Carboxy-PTIO, LY-83,583 [6-(phenylamino)-5,8-quinolinedione-6-anilino-5,8-quinolinedione] and (*S*)-nitrosoglutathione were purchased from Alexis (Läufelfingen, Switzerland). 8-(*p*-chlorophenylthio)-cGMP and SIN-1 were kindly provided by GEA (Copenhagen, Denmark). SIN-1C and C88–3934 were kind gifts from Dr. K. Schönafinger (Hoechst Marion Roussel, Frankfurt am Main, Germany). Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden); IBMX (Ega-Chemie, Steinheim, Germany), ^{125}I-labeled cGMP (Du Pont-NEN Research Products, Boston, MA), methylene blue (Merck, Darmstadt, Germany) and PGE_2 (Cayman Chemical, Ann Arbor, MI) were obtained as indicated. Oxyhemoglobin was prepared according to Feelisch and Noack (6), stored in liquid nitrogen, and used within 1 hr after thawing. Peroxynitrite was prepared basically according to Beckman *et al.* (11), stored at $-20°$ for < 2 weeks and the concentration was determined by absorbance at 302 nm.

Isolation of human neutrophils. Blood was collected by venipuncture from healthy volunteers who had abstained from any drugs for at least 1 week before sampling. Neutrophils were isolated by density-gradient centrifugation on Ficoll-Paque as previously described (23). After the isolation procedure, the viability of the cells was >97% as determined by trypan blue exclusion. The neutrophil suspension contained less than 2% contaminating mononuclear leukocytes. SIN-1 (1000 μM) alone or combined with other compounds studied did not affect the viability of the cells as assessed by trypan blue exclusion.

β-Glucuronidase release and assay. The cell suspensions [10×10^6 neutrophils/ml of DPBS (0.9 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 137 mM NaCl, 8 mM Na_2HPO_4)] were first incubated with different concentrations of SIN-1 (30 min at 37°) in the presence of 10 μM cytochalasin B, after which the cells were activated by FMLP (10–1000 nM, 10 min at 37°), PMA (160 nM, 20 min) or calcium ionophore A23187 (1 μM, 10 min). The β-glucuronidase (EC 3.2.1.31) activity in cell-free supernatants was assayed spectrophotometrically as described previously (23). The enzyme activity released from 10^6 neutrophils is expressed as nmol phenolphthalein formed from the substrate as calculated on the basis of a phenolphthalein standard curve. The direct effects of all compounds studied on enzyme activity were tested in β-glucuronidase-containing samples of Triton X-100 lysed cells and were found to be negligible.

Cyclic nucleotide assays. Isolated human neutrophils (5×10^6/500 μl DPBS) were incubated with SIN-1 (30 min) at 37° in the presence or absence of red blood cells, 100 μM IBMX or 10 μM LY-83,853. The incubation conditions were those used in the degranulation experiments. Incubation was stopped by addition of 50 μl of ice-cold trichloroacetic acid (final concentration 6%) and samples were centrifuged ($10{,}000 \times g$ for 10 min). The supernatants were washed four times with water-saturated ethyl ether and stored at $-20°$ until assayed for cGMP. For the cGMP determinations, the samples were acetylated and measured by radioimmunoassay as described previously (23, 28).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ concentrations were measured according to Grynkiewicz *et al.* (29) as previously described (30). Briefly: Isolated neutrophils (50×10^6/ml in 10 mM HEPES buffer with 1.0 mM Ca^{2+}) were loaded with the acetoxymethyl ester of the fluorescent probe Fura-2 (10 μM) for 30 min at 37° in a shaking

waterbath. Neutrophils were diluted with HEPES buffer (1:3) and kept at room temperature for 10 min to allow them to re-equilibrate. Thereafter, neutrophils were washed twice and finally suspended in HEPES buffer to obtain a cell suspension containing 5×10^6 neutrophils/ml of buffer. The changes in fluorescence were recorded with a Shimadzu RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan) in thermostatted (37°) quartz cuvettes with continuous stirring. The excitation wavelengths were set at 340 nm and 380 nm, and the emission wavelength was 500 nm. The increases in $[Ca^{2+}]_i$ were stimulated by 100 nM FMLP. Calibration of the signal was performed basically according to the method described by Grynkiewicz *et al.* (29). The maximal fluorescence (F_{max}) was measured after adding 2 μM ionomycin; the minimum fluorescence (F_{min}) was measured in the presence of 25 mM EGTA, pH 8.6, and 0.1% Triton X-100. The $[Ca^{2+}]_i$ was calculated from the equation: $[Ca^{2+}]_i$ (nM) = $R \times 224 \times (F - F_{min})/(F_{max} - F)$, where 224 represents the dissociation constant for Fura-2, F is the fluorescence of the intact cell suspension and R is the ratio of F_{max}/F_{min} at 380 nm.

Statistics. The results are expressed as mean ± standard errors. Analysis of variance for repeated measures supported by Tukey-Kramer multiple comparison test was used when the effects of the studied compounds were analyzed. Differences were considered significant when $p < 0.05$.

Results

Effects of SIN-1 on human neutrophil degranulation and cGMP levels. SIN-1 inhibited FMLP-induced degranulation of human neutrophils in suspension in a dose-dependent manner (Fig. 2A). The inhibition of β-glucuronidase release by SIN-1 did not depend on the concentration of FMLP (10–1000 nM; $n = 5$). At these concentrations SIN-1 also induced an increase in cGMP levels in neutrophils in a dose-dependent manner (Fig. 2B).

To find out whether cGMP could mediate inhibition of neutrophil degranulation we studied the effects of the 8-bromo-, Bt_2- and 8-*p*-chlorophenylthio- analogues of cGMP on FMLP (100 nM) induced degranulation. All of these cGMP analogues inhibited the neutrophil degranulation induced by FMLP (100 nM). The values for β-glucuronidase release without and with cGMP analogue were 10.0 ± 0.3 and 7.5 ± 0.6 ($p < 0.05$), 10.6 ± 1.2 and 8.2 ± 0.9 ($p < 0.05$), and 9.4 ± 0.9 and 4.7 ± 0.3 ($p < 0.0001$) nmol phenolphthalein released/10^6 cells for 8-bromo- (3 mM), Bt_2- (1 mM) and 8-p-chlorophenylthio- (300 μM) cGMP, respectively ($n = 4$–10). cGMP analogues at such high concentrations may produce nonspecific effects through activation of cAMP-dependent protein kinase (31, 32). Therefore, the effects of 8-bromo-cAMP (3 mM), Bt_2-cAMP (1 mM), and PGE_2 (1 μM, a cAMP elevating agent) were studied. The values for β-glucuronidase release without and with cAMP analogues or PGE_2 were 10.0 ± 0.3 and 8.7 ± 0.6 (8-bromo-cAMP, $p < 0.05$), 10.5 ± 1.3 and 7.2 ± 1.0 (Bt_2-cAMP, $p < 0.01$) and 5.6 ± 1.1 and 4.3 ± 0.8 (PGE_2, $p < 0.01$) nmol phenolphthalein released/10^6 cells ($n = 4$–6). Thus it seems that an increase in cGMP levels could, either directly or through cross-activation of protein kinase A (31, 32), negatively modulate neutrophil degranulation.

To fulfill the criteria for a second mediator (33), changes in the levels of cGMP should be reflected as expected alterations in the cell function studied. To test this, we used an inhibitor of guanylate cyclase, methylene blue (10 μM), which partly reversed the inhibitory action of SIN-1 on neutrophil degranulation (Fig. 2A). However, it unexpectedly potentiated the SIN-1–induced increases in cGMP levels (Fig. 2B). Another

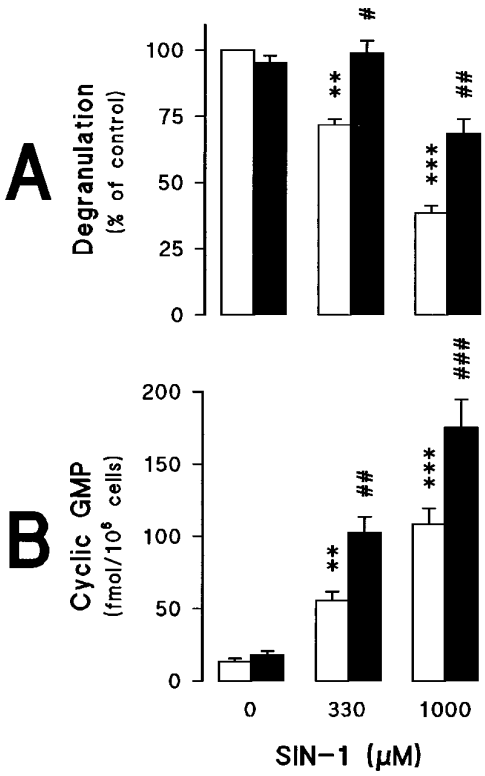

Fig. 2. The effects of SIN-1 (□; 30 min at 37°) and SIN-1 with methylene blue (■; 10 μM) on human neutrophil degranulation (β-glucuronidase release induced by 100 nM FMLP) (A) and cGMP levels (B). The data represent the means ± standard error of duplicate experiments with cells from 5–10 different donors. ** ($p < 0.01$) and *** ($p < 0.001$), differences from the corresponding control value without SIN-1 and methylene blue. # ($p < 0.05$), ## ($p < 0.01$), and ### ($p < 0.001$), differences from the corresponding value with same SIN-1 concentration but without methylene blue.

guanylate cyclase inhibitor, LY-83,853 (10 μM), did not significantly alter the effect of SIN-1 (330–1000 μM) on either degranulation or cGMP ($n = 6$, data not shown). Higher concentrations of LY-83,853 were excluded because of increasing toxicity. IBMX (100 μM), an inhibitor of phosphodiesterases, did not potentiate SIN-1–induced inhibition of neutrophil degranulation ($n = 5$; data not shown). However, it potentiated SIN-1–induced (330–1000 μM) increases in cGMP levels up to 1.4–1.6-fold ($n = 5, p < 0.001$). The results above suggest that manipulation of the intracellular cGMP levels by methylene blue or IBMX does not result in expected changes in the degranulation response to support the concept of cGMP mediating SIN-1 activity on neutrophil degranulation.

Is the inhibitory action of SIN-1 on neutrophil degranulation caused by the NO released from it? Neither oxyhemoglobin (10 μM, $n = 6$; data not shown) nor red blood cells (1 RBC: 1 neutrophil or 10 RBC: 1 neutrophil) were able to reverse the inhibition of neutrophil degranulation induced by SIN-1 (Fig. 3A). However, autologous red blood cells (1:1 and 10:1 RBC: neutrophil) significantly reduced SIN-1–induced increase in cGMP levels (Fig. 3B). Because inactivation of NO released from SIN-1 by erythrocyte hemoglobin did not affect the degranulation response, we utilized a novel NO-scavenging compound, carboxy-PTIO, which has been reported to oxidize NO· to $\cdot NO_2$ (34, 35). Unexpectedly, carboxy-PTIO (330–1000 μM) further potentiated the inhibition

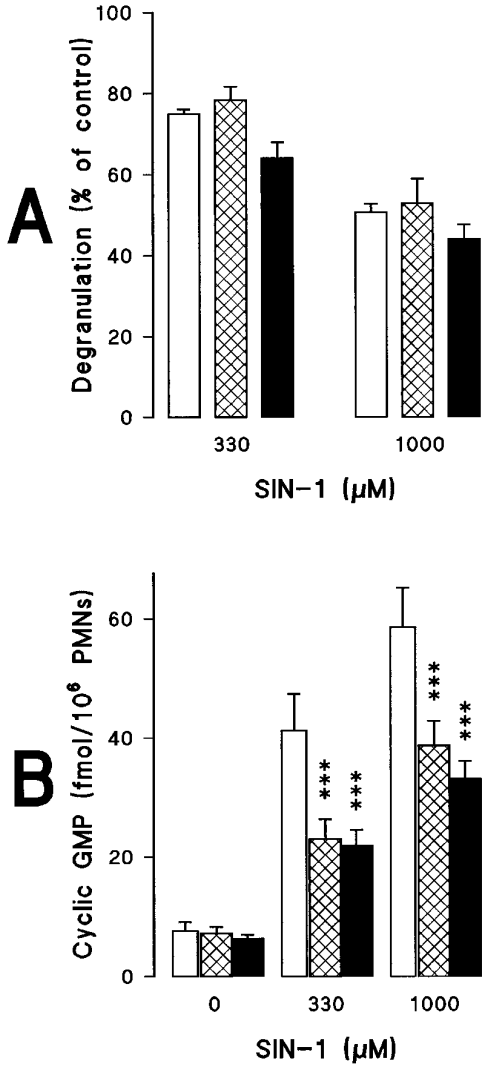

Fig. 3. The effects of autologous RBC (▨, 1 RBC:1 neutrophil; ■, 10 RBC:1 neutrophil) on SIN-1–induced (30 min at 37°) inhibition of neutrophil degranulation (β-glucuronidase release stimulated by 100 nM FMLP for 10 min at 37°) (A) and on SIN-1 induced increases in cGMP levels in human neutrophils (B). RBCs were added to the incubations 5 min before SIN-1. Data represent the means ± standard error of duplicate experiments with cells from 6 donors. *** ($p < 0.001$), differences from the corresponding value with the same concentration of SIN-1 but without RBCs (□).

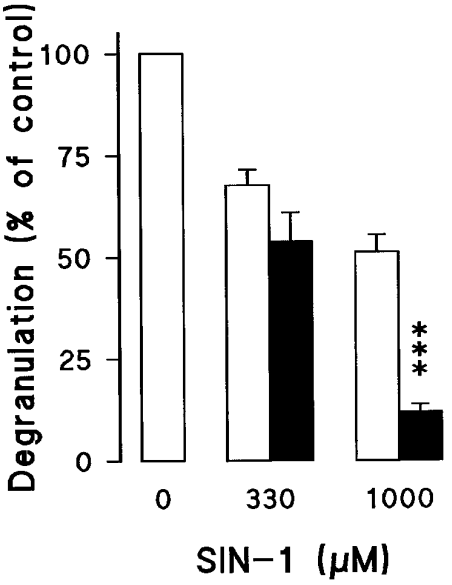

Fig. 4. The effects of equimolar concentrations of carboxy-PTIO (■) on SIN-1–induced (30 min at 37°) inhibition of neutrophil degranulation (β-glucuronidase release stimulated by 100 nM FMLP for 10 min at 37°). Carboxy-PTIO was added to the incubations 5 min before SIN-1. Data represent the means ± standard error of duplicate experiments with cells from 6 donors. *** ($p < 0.001$), difference from the corresponding value with the same concentration of SIN-1 but without carboxy-PTIO.

of neutrophil degranulation by SIN-1 (Fig. 4). In contrast to SIN-1, another NO-donor, (*S*)-nitrosoglutathione (1–1000 μM), did not inhibit human neutrophil degranulation ($n = 5$). The results above suggest that the inhibitory effect of SIN-1 on neutrophil degranulation is not attributable to the NO released.

Effects of peroxynitrite on neutrophil degranulation. SIN-1 is known to release both NO and O_2 (9,10). The reaction of NO and O_2^- produces another reactive compound, peroxynitrite. To find out whether peroxynitrite was responsible for the actions of SIN-1 on neutrophils, we tested the direct effects of chemically synthesized peroxynitrite. Peroxynitrite experiments were done in Dulbecco's phosphate-buffered saline supplemented with 20 mM HEPES (pH 7.40 at 37°) to ensure that the pH remained stable. Peroxynitrite (1–2350 μM; $n = 3$–6) did not reduce neutrophil degranulation, but at high concentrations (1000–2350 μM) potentiated FMLP-induced degranulation up to 1.8–2.2-fold ($p < 0.01$). To further ensure that the actions of SIN-1 were not caused by peroxynitrite formation, we tested the effects of mannitol (a scavenger of hydroxyl radicals formed from peroxynitrite) (10). Inclusion of mannitol (1–20 mM) had no significant effect on the response to 330-1000 μM SIN-1 ($n = 6$). This data suggests that peroxynitrite formed from SIN-1 is not responsible for its action on neutrophil degranulation.

Is the inhibitory action of SIN-1 on neutrophil degranulation attributable to its stable breakdown products? To evaluate the activity of the breakdown products of SIN-1, we first studied the effect of the preincubation time of SIN-1 with neutrophils on degranulation. The inhibitory effect of SIN-1 (1000 μM) on neutrophil degranulation was potentiated by increasing the preincubation time from 10–30 min (37 ± 3 versus 57 ± 3% inhibition, respectively; $n = 6$, $p < 0.01$). This suggested that the active component might be a molecule derived from SIN-1. To find that out we first studied SIN-1 that had been dissolved in phosphate buffer for 7 days earlier and stored at 22°. The decomposed SIN-1 solution still retained almost the same inhibitory action on FMLP-induced degranulation as freshly dissolved SIN-1 ($n = 5$, $p > 0.05$). Our earlier data show that, 40 min after dissolving in phosphate buffer at 22°, about 70% of SIN-1 can already be identified as NO_2^- and NO_3^- by high-performance liquid chromatography (28); therefore, SIN-1 should be almost totally decomposed within a few hours.

To find out whether the inhibitory activity of SIN-1 on neutrophils was attributable to its metabolite formed after NO release (9), SIN-1C was tested. SIN-1C (330–1000 μM) reduced FMLP-induced human neutrophil degranulation in a dose-dependent manner (Fig. 5). To find out whether a similar inhibitory action on neutrophil degranulation also occurs with other NO-deficient sydnonimine metabolites, we

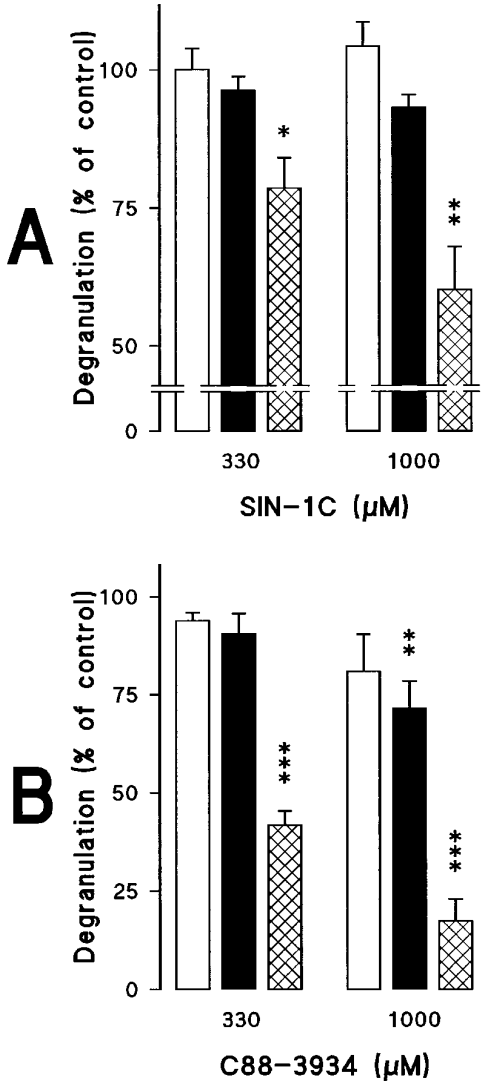

Fig. 5. The effects of SIN-1C (A) and C88–3934 (B) on FMLP- (▨; 100 nM, 10 min), A23187- (■; 1 μM, 10 min), or PMA- (□; 160 nM, 20 min) induced human neutrophil degranulation. SIN-1C or C88–3934 were added to the incubations 10 min before the stimuli. Data represent the means ± standard error of duplicate experiments with cells from 6 donors. * ($p < 0.05$), **, ($p < 0.01$), and *** ($p < 0.001$), differences from the corresponding control value without SIN-1C.

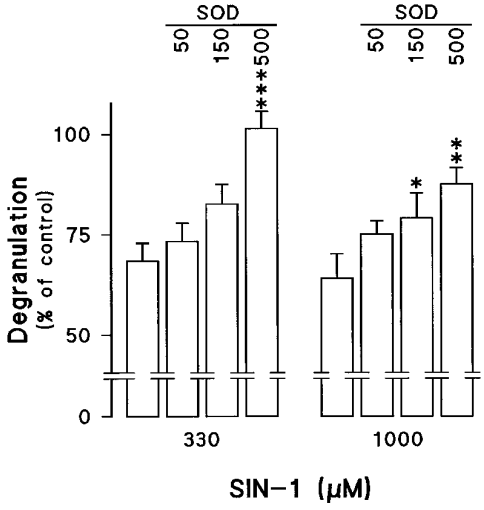

Fig. 6. The effects of SOD (50–500 IU/ml) on SIN-1–induced (30 min at 37°) inhibition of neutrophil degranulation (β-glucuronidase release stimulated by 100 nM FMLP for 10 min at 37°). SOD was added to the incubations 5 min before SIN-1. Data represent the means ± standard error of duplicate experiments with cells from 6 donors. * ($p < 0.05$), **, ($p < 0.01$), and *** ($p < 0.001$), differences from the corresponding value with the same concentration of SIN-1 but without SOD.

tested C88–3934 [(a close NO-deficient chemical analogue of another sydnonimine-class NO-donor, C87–3754) (36) (Fig. 1)]. C88–3934 (330–1000 μM) also inhibited human neutrophil degranulation in a dose-dependent manner (Fig. 5).

SIN-1 is known to decompose by hydrolysis, followed by the release of NO in an oxygen-dependent manner involving the formation of superoxide radical. This reaction is inhibitable by SOD (9). The presence of SOD (50–500 IU/ml) reversed the inhibitory action of SIN-1 on neutrophil degranulation in a dose-dependent manner (Fig. 6). To exclude the possibility that the superoxide anion was the active metabolite and the effect of SOD would be attributable merely to dismutation of the $O_2^{\cdot-}$ and not to inhibition of SIN-1 decomposition, we tested the effects of two superoxide-releasing compounds on neutrophil degranulation. Pyrogallol (1–1000 μM) or $KO_2^{\cdot-}$ (1–1000 μM) did not affect human neutrophil degranulation.

On the mechanism of the inhibitory action of SIN-1C on neutrophil degranulation. To find out the mechanism of the inhibitory action of SIN-1C on neutrophil degranulation, we compared the effects of SIN-1C on neutrophil degranulation induced either by a receptor-mediated stimulus (FMLP), by a protein kinase C activator (PMA) and by a direct calcium elevating agent (calcium ionophore A23187). SIN-1C and C88–3934 inhibited significantly only FMLP-induced degranulation, but not that induced by PMA or A23187 (Fig. 5). To further clarify the mechanism of action of SIN-1C, its effects were studied on FMLP-induced increase in $[Ca^{2+}]_i$. The resting $[Ca^{2+}]_i$ concentration in neutrophils was 72 ± 4 nM and was increased by 232 ± 1 nM after stimulation with FMLP (100 nM). The FMLP-induced increase in $[Ca^{2+}]_i$ was reduced to 162 ± 12 nM in the presence of 1000 μM SIN-1C ($n = 4$, $p < 0.02$). The resting $[Ca^{2+}]_i$ concentration was not affected by SIN-1C.

Discussion

In the present study, we have shown that, surprisingly, the inhibitory action on neutrophil degranulation by SIN-1 is mediated by a cGMP-, NO-, $O_2^{\cdot-}$-, and peroxynitrite-independent mechanism, as a consequence of the effects of its breakdown product SIN-1C on the receptor-mediated signaling cascade in human neutrophils in suspension.

Based on the published data (15, 16, 20), our starting hypothesis was that cGMP could mediate the actions of SIN-1 on neutrophil degranulation. The three cGMP analogues inhibited neutrophil degranulation. Whether this effect is attributable to direct activation of cGMP-dependent protein kinase or cross-activation of cAMP-dependent protein kinase (31, 32) remains to be determined. Although the three different analogues of cGMP mimicked the actions of SIN-1, the unexpected results obtained with an inhibitor of guanylate cyclase (methylene blue) and a phosphodiesterase inhibitor (IBMX) suggested that, according to the Sutherland *et al.* (33) criteria for a second messenger, cGMP does not mediate the actions of SIN-1 on neutrophil degranulation.

NO has been reported to have direct actions on several

components of the cellular signaling cascade in neutrophils and other cells (4, 25–27). Thus a direct action of NO released by SIN-1 was considered. If the actions of SIN-1 were attributable to NO released, removal of NO by oxyhemoglobin (RBCs) or by a novel NO-scavenger, carboxy-PTIO (34, 35), should have reversed the inhibitory effect of SIN-1 on neutrophil function. NO oxidizes oxyhemoglobin (in RBCs) to methemoglobin, which also yields formation of nitrite and nitrate (37); thus, the concentration of free NO is reduced. The reduced biological activity of NO produced by SIN-1 in the presence of RBCs was demonstrated by showing a lowered cGMP response, but RBCs did not affect neutrophil degranulation. A NO-scavenger, carboxy-PTIO, did not diminish but potentiated the inhibitory action of SIN-1 on neutrophil degranulation. Thus the inability of RBCs to alter the inhibitory action of SIN-1 on neutrophil degranulation and the potentiation of the action of SIN-1 by carboxy-PTIO indicate that NO as such is not the active component in this action of SIN-1. Furthermore, another NO-donor, (*S*)-nitrosoglutathione, did not inhibit human neutrophil degranulation.

SIN-1 releases both NO and $O_2^{\cdot-}$, which together form a strong biological oxidant, peroxynitrite, in a reaction with a high rate constant ($k = 6.7 \times 10^9$ $M^{[minus]1}$ sec^{-1}), which subsequently may degrade to hydroxyl radical (10, 11, 38). Peroxynitrite also nitrates tyrosine residues in cells (39) and could thus be the active component of SIN-1 action. Nitration of tyrosine residues in buffer by SIN-1 under similar incubation conditions indeed occurs.[1] However, nitration of tyrosine residues by SIN-1 derived peroxynitrite seems not to be the mechanism of action of SIN-1 in the present experiments, because chemically synthesized peroxynitrite had the opposite action on neutrophil degranulation compared with SIN-1. Furthermore, mannitol, a scavenger of hydroxyl radicals, did not alter the inhibitory effects of SIN-1, which suggests that the $OH^{\cdot}$, probably formed from peroxynitrite (11), does not mediate the action of SIN-1. Thus neither cGMP, NO, nor peroxynitrite were responsible for the actions of SIN-1 in neutrophils. These findings are supported by the earlier report by Ervens and Seifert (40) that *N*-ethoxycarboxy-3-morpholino-sydnonimine-ethyl ester, the prodrug of SIN-1, inhibits neutrophil respiratory burst via a NO-independent mechanism.

The pathway of NO formation from SIN-1 in aqueous milieux has been described (8, 9). In a first step, SIN-1 undergoes hydrolytic ring opening to the nitrosamine *N*-morpholino-*N*-nitrosoaminoacetonitrile and, subsequently, in an oxygen-dependent reaction, to a radical cation and superoxide. The highly unstable radical compound is stabilized by NO release and deprotonation to form SIN-1C (5, 8, 9). An increase in the inhibitory activity of SIN-1, increasing the preincubation time from 10 to 30 min, and retention of the inhibitory activity in an aged SIN-1 solution suggested that the active component is a breakdown product of SIN-1. In the present study SIN-1C, a NO-deficient breakdown product of SIN-1 (5, 9) inhibited human neutrophil degranulation in a dose-dependent manner. C88–3934 is a NO-deficient breakdown product of NO-releasing sydnonimine C87–3754, a methylated analogue of SIN-1 (36). It reduced FMLP-induced neutrophil degranulation in a dose-dependent manner, being more potent than SIN-1 and SIN-1C. Furthermore, inhibition of SIN-1 breakdown to SIN-1C by SOD (9) prevented the inhibitory action of SIN-1. To exclude the superoxide radical as the active constituent of SIN-1 action, we tested the effects of two superoxide generating compounds, $KO_2^{\cdot-}$ and pyrogallol. These compounds had no effect on neutrophil degranulation.

The activation of human neutrophils by chemoattractants via a receptor-mediated mechanism involves the coupling of the agonist/receptor complex with G proteins and activation of phosphoinositide-specific phospholipase C leading to an increase in $[Ca^{2+}]_i$ and activation of protein kinase C (41). SIN-1C did not reduce neutrophil degranulation induced by an activator of protein kinase C (PMA) and a direct calcium elevating agent calcium ionophore A23187. Instead, SIN-1C was shown to inhibit FMLP-induced degranulation and increase in $[Ca^{2+}]_i$. Inhibition of FMLP-triggered increase in $[Ca^{2+}]_i$ by SIN-1C may explain its inhibitory effect on neutrophil degranulation, because inhibitors of receptor-mediated calcium entry [i.e., SK&F 96365 and fenamates (30)] have been reported to reduce degranulation (42). Also, a blockade of FMLP-induced increase in $[Ca^{2+}]_i$ by pertussis toxin is known to reduce the subsequent cell activation (41). Thus, the data suggest that SIN-1C inhibits an early step in the receptor-mediated signaling cascade but does not directly affect the events in the degranulation process taking place after activation of protein kinase C or elevation of intracellular calcium concentration.

With the vastly increased research concerning the pathophysiology and pharmacology of NO, NO-donors are being employed by many investigators because of the instability and inconvenient handling of aqueous solutions of authentic NO (5). SIN-1 was one of the first active chemically synthesized NO-donors available. Our results clearly show that the NO-deficient break-down product(s) of SIN-1 is the active component in inhibiting receptor-mediated activation of human neutrophils in suspension. Thus, for studies assessing the role of $NO/OONO^-$ in physiology and pharmacology, our results demonstrate the crucial importance of the use of relevant control substances in addition to the use of SIN-1 (i.e., other NO-releasing compounds, SIN-1C, chemically synthesized peroxynitrite, and/or $NO/OONO^-$ scavenging compounds) to ensure that the results obtained are attributable to $NO/OONO^-$. This has not been done in all studies with SIN-1, which indicates that the results of those studies should be carefully re-evaluated.

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