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MOLSIDOMINE INHIBITS THE CHEMOATTRACTANT-INDUCED RESPIRATORY BURST IN HUMAN NEUTROPHILS VIA A NO-INDEPENDENT MECHANISM

JÜRGEN ERVENS and ROLAND SEIFERT*
Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69/73, D-1000 Berlin 33, Germany

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Abstract—3-Morpholino-sydnonimine (SIN-1) is a NO-releasing compound which mimics the effects of cGMP through activation of soluble guanylyl cyclase. Its prodrug, molsidomine (SIN-10), does not release NO but does modulate various cell functions. These findings prompted us to study the effects of SIN-10 and SIN-1 on the respiratory burst in human neutrophils. SIN-10 was more effective than SIN-1 in inhibiting superoxide anion (O_2^-) formation induced by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe) and by C5a. The effects of SIN-1 and SIN-10 on O_2^- formation were additive or less than additive, indicating the sydnonimines acted through a common mechanism. The sydnonimines showed no effect on O_2^- formations induced by γ-hexachlorocyclohexane, arachidonic acid and a phorbol ester. They did not inhibit O_2^- formation induced by xanthine oxidase, by autoxidation of pyrogallol and in a cell-free system from HL-60 leukemic cells. Neutrophils did not convert SIN-10 to SIN-1 as assessed by O_2 consumption which accompanies NO release from SIN-1. The cell-permeant analogue of cGMP, N^2,2′-*O*-dibutyryl guanosine 3′: 5′-monophosphate (Bt_2cGMP), and SIN-10 but not SIN-1 inhibited fMet-Leu-Phe-induced O_2 consumption. SIN-1 and SIN-10 slightly enhanced agonist binding to formyl peptide receptors, whereas Bt_2cGMP was inhibitory. The sydnonimines did not affect GTP hydrolysis of heterotrimeric regulatory guanine nucleotide-binding proteins in HL-60 membranes. SIN-1 but not SIN-10 stimulated ADP-ribosylation of a 39-kDa protein in the cytosol of HL-60 cells. SIN-10 reduced fMet-Leu-Phe-induced rises in cytosolic Ca^{2+} concentration in neutrophils. These data suggest that SIN-10 inhibits the respiratory burst via a NO-independent mechanism which may involve inhibition of rises in cytosolic Ca^{2+} concentration.

In human neutrophils, the chemoattractants, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe†) and complement C5a, induce β-glucuronidase release from azurophilic granules and a respiratory burst [1–4]. The latter process is catalysed by NADPH oxidase [EC 1.6.99.6] and is characterized by O_2 consumption and superoxide anion (O_2^-) formation [1–4]. Formyl peptide and C5a receptors interact with heterotrimeric guanine nucleotide-binding proteins (G-proteins), resulting in the stimulation of phospholipase C with subsequent activation of protein kinase C and rises in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [1, 2, 4]. The mechanisms by which chemoattractants activate the respiratory burst are still a matter of debate and may involve activation of G-proteins, low molecular mass GTP-binding proteins, protein kinase C and rises in $[Ca^{2+}]_i$ [1, 2, 4].

Recent data suggest that cGMP plays a role in the regulation of β-glucuronidase release and the respiratory burst, as its cell-permeant analogue, N^2,2′-*O*-dibutyryl guanosine 3′:5′-monophosphate (Bt_2cGMP), inhibits enzyme release and O_2^- formation induced by fMet-Leu-Phe and potentiates those induced by C5a [5–7]. Neutrophils possess a cGMP-forming soluble guanylyl cyclase [8], a cGMP-dependent protein kinase [9] and a cGMP-degrading phosphodiesterase [10]. Guanylyl cyclase is activated by the NO-releasing compounds, sodium nitroprusside (SNP) and 3-morpholino-sydnonimine (SIN-1) [11–13]. In agreement with the above data, SNP and SIN-1 increase cGMP levels in neutrophils [5]. Additionally, NO-releasing compounds activate ADP-ribosylation of a 39-kDa protein and modulate various cell functions in a cGMP-independent manner [14–18]. Similar to other cell types, neutrophils generate NO [19–21], and NO-releasing compounds mimic, in part, the effects of Bt_2cGMP on chemoattractant-induced β-glucuronidase release [5, 7].

SIN-10 is a prodrug and is converted to SIN-1 in the liver [12, 22]. Accordingly, SIN-10 does not increase cGMP levels in neutrophils [5]. Surprisingly, SIN-10 inhibits β-glucuronidase release in neutrophils more effectively than SIN-1 [5, 7]. Interestingly, an analogue of SIN-10 induces vasodilation, indicating that this substance does not only serve as a prodrug for a NO-releasing substance but is a

* Corresponding author. Tel. (49) 30 838 2064; FAX (49) 30 831 5954.

† Abbreviations: Bt_2cAMP, N^6,2′-0-dibutyryl adenosine 3:5′-monophosphate; Bt_2cGMP, N^2,2′-0-dibutyryl guanosine 3′:5′-monophosphate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; G-protein, heterotrimeric regulatory guanine nucleotide-binding protein; HCCH, γ-hexachlorocyclohexane; O_2^-, superoxide anion; PMA, phorbol myristate acetate; SIN-1, 3-morpholino-sydnonimine; SIN-10, molsidomine; SNP, sodium nitroprusside.

pharmacologically active substance *per se* [23]. All these data prompted us to study the effects of SIN-1 and SIN-10 on the respiratory burst in human neutrophils. We report here that SIN-10 inhibits the chemoattractant-induced respiratory burst via a NO-independent mechanism.

MATERIALS AND METHODS

Materials. SIN-1 and SIN-10 were kindly provided by Cassella (Frankfurt/Main, Germany). Stock solutions of SIN-1 (100 mmol/L) and SIN-10 (50 mmol/L) were prepared in 100 and 50 mmol/L Na-acetate, pH 5.0, immediately prior to use under light protection. Xanthine, xanthine oxidase [EC 1.2.3.2] (grade III, from buttermilk), superoxide dismutase (lyophilized powder, from bovine erythrocytes) [EC 1.15.1.1], ferricytochrome *c* (type III, from horse heart), SNP, pyrogallol, antimycin, dithiothreitol and epinephrine were purchased from Sigma Chemie (Deisenhofen, Germany). The preparation of ferricytochrome *c* used for the experiments in the present study contained up to 10% (w/w) ferrocytochrome *c*. Stock solutions of SNP were prepared as described for SIN-1. Pyrogallol (10 mmol/L) was dissolved in 10 mmol/L HCl immediately prior to use. Sources of other materials have been described elsewhere [6, 7, 24–28].

Isolation of human neutrophils. Neutrophils were isolated from buffy coat obtained from the local blood bank or from individual healthy volunteers [6, 7, 24]. Cell preparations consisted of more than 95% viable neutrophils as revealed by Trypan blue dye exclusion and Pappenheim-stained smears.

Cell culture. HL-60 cells were cultured in suspension culture as described [24, 25, 27]. HL-60 cells were differentiated towards neutrophil-like cells upon incubation with 160 mmol/L dimethyl sulfoxide for 120 hr or with 0.2 mmol/L Bt_2cAMP for 48 hr [24, 25, 27]. HL-60 membranes and cytosol were prepared as described [25].

O_2^- formation in intact human neutrophils. O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase [24]. Preliminary experiments revealed that SIN-1 and SIN-10 did not inhibit superoxide dismutase (data not shown). Reaction mixtures (0.5 mL) contained 100 μmol/L ferricytochrome *c* and a buffer consisting of (mmol/L) 138 NaCl, 6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5.5 glucose and 20 Hepes–NaOH, pH 7.4. Neutrophils (2.0×10^6 cells) were suspended in the solution described above and were incubated for 3 min in the presence of various substances at 37°. O_2^- formation was initiated by the addition of stimuli. In the experiments with fMet-Leu-Phe, C5a, HCCH and arachidonic acid, the absolute amounts of O_2^- generated within an incubation period of 5 min were calculated. Within this time, O_2^- formation ceased completely. In the experiments with phorbol myristate acetate (PMA), the maximum rate of O_2^- formation, which was achieved within 5 min of addition of the stimulus, was calculated.

O_2^- formation in a cell-free system from dimethyl sulfoxide-differentiated HL-60 cells. O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase. Reaction mixtures (0.5 mL) contained 50 μg membrane protein, 150 μg cytosolic protein, 10 μmol/L FAD, 500 μmol/L NADPH, 100 μmol/L ferricytochrome *c*, 2 mmol/L $MgCl_2$, 20 mmol/L KH_2PO_4, 40 mmol/L KCl and 20 mmol/L triethanolamine/HCl, pH 7.0. Reaction mixtures were incubated for 3 min at 25° in the presence of various compounds. O_2^- formation was initiated by the addition of arachidonic acid (200 μmol/L) [25]. The maximum rate of O_2^- formation, which was achieved within 5 min of the addition of arachidonic acid, was calculated.

O_2^- formation by xanthine oxidase. O_2^- was generated by xanthine oxidase-catalysed conversion of xanthine to uric acid. O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase [29]. Reaction mixtures (0.5 mL) contained xanthine (50 μmol/L), ferricytochrome *c* (100 μmol/L) and a buffer composed of 50 mmol/L KH_2PO_4 and 0.1 mmol/L EDTA, pH 7.8. Assays were conducted at 25°. Reactions were initiated by the addition of an amount of xanthine oxidase that induced the formation of 1.25 nmol of O_2^-/min. O_2^- formation was monitored for the first 3 min after addition of the enzyme.

O_2^- formation by pyrogallol. O_2^- was generated by autoxidation of pyrogallol [30]. O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase under the conditions used for the determination of O_2^- formation in intact neutrophils. Reaction mixtures were incubated for 3 min in the presence of various substances at 37°. O_2^- formation was initiated by the addition of pyrogallol (30 μmol/L). Pyrogallol induced the formation of 3.5 nmol of O_2^-. Within 5 min, O_2^- formation was terminated.

Effects of SIN-10 and SIN-1 on redox state of cytochrome c. Under the experimental conditions used for measurement of O_2^- formation in intact neutrophils, in a cell-free system from HL-60 cells, by xanthine oxidase and by pyrogallol, SIN-10 and SIN-1 up to 1 mmol/L *per se* did not induce O_2^- formation or oxidation of ferrocytochrome *c* present in the commercial preparation. SIN-10 and SIN-1 (0.1 and 1 mmol/L each) showed no oxidative effects on ferricytochrome *c* reductions induced by dithiothreitol (0.1 mmol/L) or epinephrine (1 mmol/L), regardless of whether sydnonimines were added to reaction mixtures 3 min prior to or 3 min after the reducing substances. In addition, SIN-10 and SIN-1 (0.1 and 1 mmol/L each) did not induced reoxidation of cytochrome *c* that was completely reduced by dithiothreitol or epinephrine.

O_2 consumption in intact human neutrophils. O_2 consumption was measured using a Biometer O_2 electrode (Braun, Melsungen, Germany) according to the protocol described by Absolom [3]. Neutrophils (3×10^7 cells) were suspended at 37° in 3 mL of the buffer used for the determination of O_2^- formation in intact cells supplemented with antimycin (10 μmol/L) and cytochalasin B (1 μg/mL). Cells were stirred at 500 rpm. O_2 consumption was measured in the presence of various compounds.

fMet-Leu-Phe binding assay. fMet-Leu-Phe binding was performed as described [26]. Reaction mixtures (100 μL) contained 40 μg of membrane protein from Bt_2cAMP-differentiated HL-60 cells, bovine serum albumin (0.2%, w/v), 50 mmol/L Tris–HCl, pH 7.3, 1 mmol/L EDTA and 5 mmol/L $MgCl_2$ in the presence of various substances. Incubations were conducted for 30 min at 25° and were initiated by the addition of 3 nmol/L fMet-Leu-[^{3}H]Phe (17 nCi/tube). Reactions were terminated by the rapid filtration technique. Radioactivity on dried filters was determined in a liquid scintillation counter. Unspecific binding was determined in the presence of 10 μmol/L fMet-Leu-Phe and amounted to less than 10% of total binding.

Measurement of GTPase activity. GTP hydrolysis was measured as described [27]. Assay mixtures (100 μL) contained membranes from Bt_2cAMP-differentiated HL-60 cells (7.0 μg of protein/tube), 0.5 μmol/L [γ-^{32}P]GTP (0.1 μCi/tube), 5 mmol/L $MgCl_2$, 0.1 mmol/L EGTA, 0.1 mmol/L ATP, 1 mmol/L adenosine 5′-[β,γ-imido]-triphosphate, 5 mmol/L creatine phosphate, 40 μg of creatine kinase, 1 mmol/L dithiothreitol and 0.2% (w/v) bovine serum albumin in 50 mmol/L triethanolamine–HCl, pH 7.4. Reaction mixtures contained various additional substances. Reactions were initiated by the addition of [γ-^{32}P]GTP and were conducted for 15 min at 25°. Low-affinity GTPase activity was determined in the presence of GTP (50 μmol/L) and amounted to <5% of total GTPase activity.

[^{32}P]ADP-ribosylation of proteins in cytosol from HL-60 cells and gel electrophoresis. [^{32}P]ADP-ribosylation of cytosolic proteins from dimethyl sulfoxide-differentiated HL-60 cells was performed according to Dimmeler and Brüne [15]. Briefly, assay mixtures (60 μL) contained 100 μg of cytosolic proteins, 1 μmol/L [^{32}P]NAD (2 μCi/tube), 2 mmol/L dithiothreitol and 0.2% (w/v) bovine serum albumin in 50 mmol/L Hepes–NaOH, pH 7.5. Assays contained various additional substances. Incubations were conducted for 45 min at 37°. Thereafter, proteins were precipitated by acetone. Precipitates were washed with trichloracetic acid and subsequently with methanol–chloroform (1:2, v/v). Proteins were resolved by SDS–PAGE on gels containing 12.5% (w/v) acrylamide. Staining of dried gels with Coomassie blue and autoradiography were performed as described [28].

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined using the fluorescent dye, Fura-2, as described [7, 26]. Neutrophils were suspended at 5×10^6 cells/mL in a buffer consisting of (mmol/L) 138 NaCl, 6 KCl, 1 $MgSO_4$, 1.1 $CaCl_2$, 0.1 EGTA, 1 Na_2HPO_4, 5 $NaHCO_3$, 5.5 glucose, 20 Hepes–NaOH, pH 7.4, supplemented with bovine serum albumin (0.1%, w/v). Cells were incubated for 1 hr at 37° in the presence of Fura-2–acetoxymethylester (4 μmol/L). Subsequently, cells were diluted with the above buffer to a final concentration of 0.5×10^6 cells/mL and were centrifuged for 10 min at 250 *g* at 20°. Cells were suspended at 1.0×10^6 cells/mL in the above buffer and were kept at 20° until measurement of $[Ca^{2+}]_i$. Fluorescence of neutrophils (1.0×10^6 cells in 2 mL) was determined at 37° under constant stirring at 10^3 rpm using a Ratio II spectrofluorometer (Aminco, Silver Spring, MD, U.S.A.). Cells were incubated for 3 min in the presence of SIN-10 or solvent prior to the addition of fMet-Leu-Phe. The excitation and emission wavelengths were 340 and 500 nm, respectively.

Miscellaneous. Protein was determined according to Lowry *et al.* [31]. [γ-^{32}P]GTP was synthesized according to Johnson and Walseth [32]. [^{32}P]NAD was prepared as described [33]. The effects of sydnonimines were statistically assessed using the Wilcoxon test.

RESULTS

First, the effects of sydnonimines on O_2^- formation in intact human neutrophils were studied. Sydnonimines were employed at concentrations ranging from 3 μmol/L to 1 mmol/L, i.e. concentrations commonly used in various systems [5, 7, 12, 14, 18, 22–34]. SIN-10 and SIN-1 inhibited fMet-Leu-Phe-induced O_2^- formation in a concentration-dependent manner (Fig. 1). SIN-10 was considerably more effective than SIN-1. The solvent Na-acetate did not inhibit O_2^- formation.

The interaction of sydnonimines with fMet-Leu-Phe-induced O_2^- formation was studied (Fig. 2). SIN-1 (1 mmol/L) enhanced additively the inhibitory effect of SIN-10 (0.1 mmol/L) on O_2^- formation. However, under various other conditions, the inhibitory effects of SIN-1 and SIN-10 were not additive.

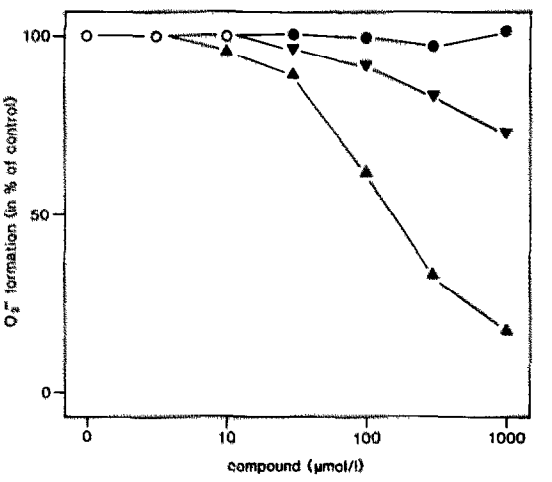

Fig. 1. Concentration dependency of the effects of SIN-10, SIN-1 and Na-acetate on fMet-Leu-Phe-induced O_2^- formation in human neutrophils. Compounds at the indicated concentrations were added to reaction mixtures 3 min prior to fMet-Leu-Phe (1 μmol/L). In the presence of H_2O (control), fMet-Leu-Phe induced the formation of 5.1 ± 0.3 nmol of $O_2^-/10^6$ cells. Data shown are referred to these values and are the means of five independent experiments; the SD values of the data were generally < 15% of the means. The open circles indicate that substances at the indicated concentrations did not inhibit O_2^- formation. (▲) SIN-10; (▼) SIN-1; (●) Na-acetate. The inhibitory effects of SIN-10 and SIN-1 on O_2^- formation versus control were significant at 0.1, 0.3 and 1 mmol/L and at 0.3 and 1 mmol/L, respectively ($P < 0.05$ or $P < 0.01$).

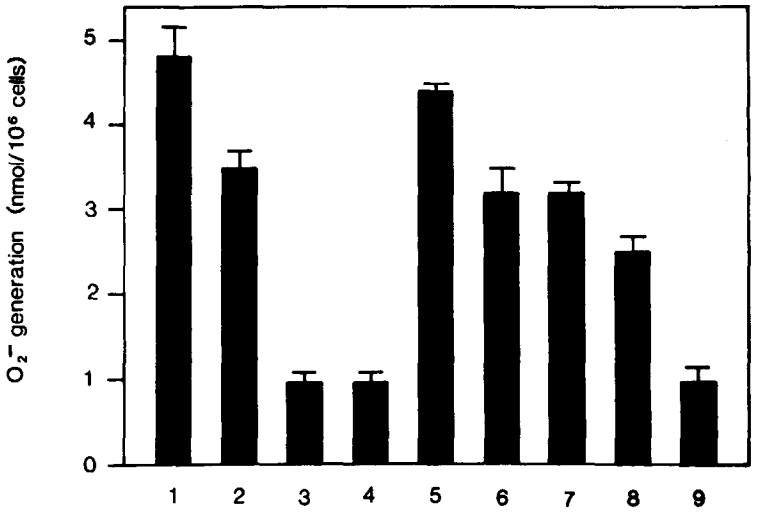

Fig. 2. Interaction of SIN-10 and SIN-1 on fMet-Leu-Phe-induced O_2^- formation in human neutrophils. Compounds at various concentrations were added to reaction mixtures 3 min prior to fMet-Leu-Phe (1 μmol/L). 1, solvent (control); 2, SIN-1 (1 mmol/L); 3, SIN-10 (1 mmol/L); 4, SIN-1 plus SIN-10 (1 mmol/L each); 5, SIN-1 (0.1 mmol/L); 6, SIN-10 (0.1 mmol/L); 7, SIN-1 plus SIN-10 (0.1 mmol/L each); 8, SIN-1 (1 mmol/L) plus SIN-10 (0.1 mmol/L); 9, SIN-1 (0.1 mmol/L) plus SIN-10 (1 mmol/L). Data shown are the means ± SD of four independent experiments.

Table 1 compares the effects of SIN-10 and SIN-1 on O_2^- formation in neutrophils induced by various stimuli. In the presence of fMet-Leu-Phe at a submaximally effective concentration, SIN-10 and SIN-1 inhibited O_2^- formation to similar extents as did fMet-Leu-Phe at a maximally effective concentration. By analogy to fMet-Leu-Phe, SIN-10 was more effective than SIN-1 in inhibiting C5a-induced O_2^- formation. In contrast, SIN-10 and SIN-1 did not affect O_2^- formation induced by HCCH, arachidonic acid and PMA, i.e. stimuli which circumvent receptor stimulation.

In a cell-free system from HL-60 cells, arachidonic acid induced the formation of O_2^- at a rate of 20 nmol of O_2^-/min/mg of membrane protein. Guanosine 5'-*O*-(3-thiotriphosphate) (10 μmol/L), an activator of G-proteins and low molecular mass GTP-binding proteins [2], enhanced this O_2^- formation by about 3.5-fold (data not shown) [25]. SIN-10 and SIN-1 (1 mmol/L each) did not inhibit O_2^- formation in this system (data not shown).

Radical scavenging may explain inhibition by NO of NADPH oxidase-catalysed O_2^- formation [35]. Therefore, the effects of SIN-10 and SIN-1 on O_2^- formation in systems unrelated to NADPH oxidase were assessed. SIN-10 and SIN-1 (1 mmol/L each)

Table 1. Effects of SIN-10 and SIN-1 on O_2^- formation in human neutrophils induced by various stimuli

Stimulus	O_2^- formation (% of control)	
	SIN-10	SIN-1
fMet-Leu-Phe (20 nmol/L)	13†	60†§
fMet-Leu-Phe (1 μmol/L)	20†	73†§
C5a (10 nmol/L)	34†	77†§
HCCH (125 μmol/L)	105*	96*‡
Arachidonic acid (20 μmol/L)	97*	95*‡
PMA (100 ng/mL)	98*	97*‡

SIN-10 or SIN-1 (1 mmol/L each) or solvent (control) was added to reaction mixtures 3 min prior to stimuli. Data shown are referred to O_2^- formations induced by substances in the presence of solvent and are the means of six independent experiments; the SD values of the data were generally < 15% of the means. In the presence of solvent, fMet-Leu-Phe (20 nmol/L) and (1 μmol/L) induced the formation of 1.7 ± 0.3 and 5.3 ± 0.4 nmol of $O_2^-/10^6$ cells, respectively. The corresponding values for C5a, HCCH and arachidonic acid were 1.3 ± 0.1, 1.8 ± 0.2 and 1.9 ± 0.2 nmol of $O_2^-/10^6$ cells, respectively. PMA induced the formation of 10.5 ± 0.2 nmol of O_2^-/min/10^6 cells. Comparison of the effects of compounds versus control: * not significant; † $P < 0.01$. Comparison of the effects of SIN-10 versus SIN-1: ‡ not significant; § $P < 0.01$.

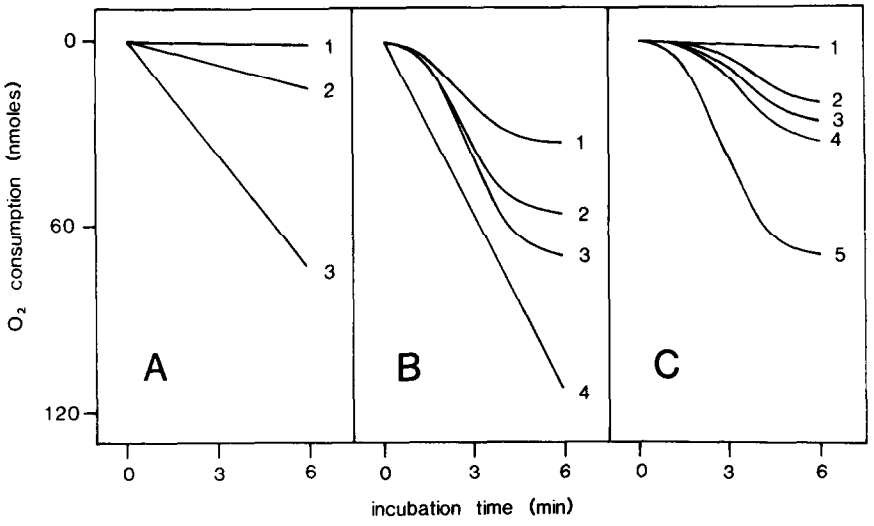

Fig. 3. Effects of SIN-10, SIN-1, Bt_2cAMP and Bt_2cGMP on O_2 consumption in human neutrophils. O_2 consumption was measured at 37° using an O_2 electrode. Assays contained 3×10^7 neutrophils each. In the experiments shown in panel A, sydnonimines were added to cells at $t = 0$ min. In the experiments shown in panels B and C, fMet-Leu-Phe was added to cells at $t = 0$ min; sydnonimines, Bt_2cAMP, Bt_2cGMP or solvent (control) was added to reaction mixtures 3 min prior to fMet-Leu-Phe. Panel A: trace 1, SIN-10 (1 mmol/L); trace 2, SIN-1 (0.1 mmol/L); trace 3, SIN-1 (1 mmol/L). Panel B: trace 1, fMet-Leu-Phe (50 nmol/L) plus solvent (control) or fMet-Leu-Phe (1 μmol/L) plus SIN-10 (1 mmol/L); trace 2, fMet-Leu-Phe (1 μmol/L) plus SIN-10 (0.3 mmol/L); trace 3, fMet-leu-Phe (1 μmol/L) plus solvent (control) or SIN-10 (0.1 mmol/L); trace 4, fMet-Leu-Phe (50 nmol/L) plus SIN-1 (1 mmol/L). Panel C: trace 1, fMet-Leu-Phe (50 nmol/L) plus Bt_2cGMP (1 mmol/L); trace 2, fMet-Leu-Phe (50 nmol/L) plus Bt_2cAMP (1 mmol/L); trace 3, fMet-Leu-Phe (1 μmol/l) plus Bt_2cAMP (1 mmol/L); trace 4, fMet-Leu-Phe (50 nmol/L) plus solvent (control); trace 5, fMet-Leu-Phe (1 μmol/L) plus solvent (control) or Bt_2cGMP (1 mmol/L). Superimposed original tracings from one experiment are shown. Similar results were obtained in five independent experiments.

showed no effect on xanthine oxidase-catalysed O_2^- formation (data not shown). Additionally, sydnonimines (1 mmol/L each) did not inhibit pyrogallol-induced O_2^- formation (data not shown).

Release of NO from SIN-1 is associated with O_2 consumption [22]. In order to answer the question as to whether neutrophils convert SIN-10 to SIN-1, O_2 consumption in the presence of sydnonimines and cells was studied. SIN-10 did not induce O_2 consumption (Fig. 3). By contrast, SIN-1 (0.1 and 1 mmol/L) induced substantial O_2 consumption. SIN-10 (0.3 and 1 mmol/L) significantly inhibited fMet-Leu-Phe-induced O_2 consumption (see Fig. 3). Similar to O_2^- formation, SIN-10 did not inhibit O_2 consumption induced by PMA (100 ng/mL) (see Table 1, data not shown). SIN-1 enhanced fMet-Leu-Phe-induced O_2 consumption in an additive manner. For comparison with sydnonimines, the effects of N^6,2′-*O*-dibutyryl adenosine 3:5′-cyclic monophosphate (Bt_2cAMP) and Bt_2cGMP on fMet-Leu-Phe-induced O_2 consumption were studied (see Fig. 3). As was the case for O_2^- formation [6], Bt_2cGMP was more effective than Bt_2cAMP in inhibiting O_2 consumption stimulated by fMet-Leu-Phe at a submaximally effective concentration. Bt_2cAMP but not Bt_2cGMP inhibited O_2 consumption induced by fMet-Leu-Phe at a maximally effective concentration.

In order to study the mechanism by which sydnonimines inhibit the chemoattractant-induced respiratory burst, their effects on agonist binding to formyl peptide receptors and on high-affinity GTPase activity of G-proteins in HL-60 membranes were studied (Table 2). Sydnonimines enhanced agonist binding by up to 17%, whereas Bt2cGMP inhibited fMet-Leu-Phe binding by 32%. Bt_2cAMP was less inhibitory than Bt_2cGMP. None of the above substances showed an effect on high-affinity GTP hydrolysis (see Table 2).

The effects of sydnonimines and of SNP on ADP-ribosylation of a 39-kDa protein in the cytosol of HL-60 cells are shown in Fig. 4. In agreement with the results of a recent study, we found that SNP stimulates ADP-ribosylation of the 39-kDa substrate in the cytosol of these cells [15]. SIN-1 was more effective than SNP in inducing ADP-ribosylation of this protein. In comparison, SIN-10 induced little, if any ADP-ribosylation of the 39-kDa protein.

Finally, the effects of SIN-10 on $[Ca^{2+}]_i$ were studied. SIN-10 (10 μmol/L) did not affect basal $[Ca^{2+}]_i$ in human neutrophils (Table 3). However, the sydnonimine inhibited rises in $[Ca^{2+}]_i$ induced by fMet-Leu-Phe at a submaximally and maximally effective concentration by more than 30%. Unfortunately, the effects of SIN-10 at higher concentrations on $[Ca^{2+}]_i$ could not be quantitatively assessed as the sydnonimine quenched basal fluorescence signals (data not shown).

Table 2. Effects of SIN-10, SIN-1, Bt_2cAMP and Bt_2cGMP on agonist binding to formyl peptide receptors and on high-affinity GTPase activity in HL-60 membranes

Addition	fMet-Leu-Phe binding (fmol/mg protein)	GTP hydrolysis (pmol P_i/mg/min)
Solvent (control)	598 ± 23	25.6 ± 0.8
SIN-10 (1 mmol/L)	676 ± 38†	25.9 ± 0.3*
SIN-1 (1 mmol/L)	700 ± 42†	26.1 ± 1.0*
Bt_2cAMP (1 mmol/L)	520 ± 18†	25.5 ± 0.9*
Bt_2cGMP (1 mmol/L)	407 ± 19‡	24.8 ± 1.1*

fMet-Leu-Phe binding to formyl peptide receptors and high-affinity GTPase activity of G-proteins in membranes from Bt_2cAMP-differentiated HL-60 cells were measured in the presence of various substances at the indicated concentrations or solvent (control) under the conditions described in Materials and Methods. Data shown are the means ± SD of three experiments performed in quadruplicate each. Comparison of the effects of compounds versus control: * not significant; † $P < 0.05$; ‡ $P < 0.01$.

DISCUSSION

SIN-10 is the prodrug of the NO-releasing substance, SIN-1 [12, 22]. Somewhat unexpectedly, we found that SIN-10 very effectively inhibits the fMet-Leu-Phe-induced respiratory burst in human neutrophils (see Figs 1–3 and Table 1). In agreement with these data, fMet-Leu-Phe-induced β-glucuronidase release is substantially inhibited by SIN-10 [5, 7]. The lack of synergism between SIN-10 and SIN-1 in the inhibition of fMet-Leu-Phe-induced O_2^- formation indicates that sydnonimines act through a common mechanism (see Fig. 2).

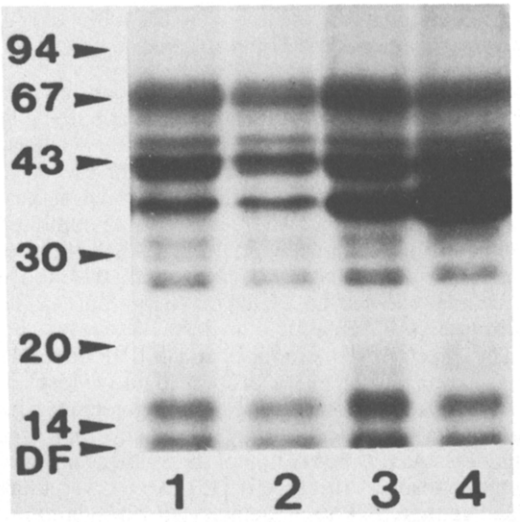

Fig. 4. Effects of SIN-10, SIN-1 and SNP on ADP-ribosylation of a 39-kDa protein in cytosol from dimethyl sulfoxide-differentiated HL-60 cells. Cytosolic proteins from dimethyl sulfoxide-differentiated HL-60 cells (100 μg/tube) were incubated for 45 min in the presence of [^{32}P]-NAD and various substances. Proteins were analysed as described in Materials and Methods. The autoradiogram of a Coomassie blue-stained gel is shown. Lane 1, SIN-10 (1 mmol/L); lane 2, solvent (control); lane 3, SNP (1 mmol/L); lane 4, SIN-1 (1 mmol/L). Numbers on the left represent molecular masses of marker proteins (kDa); DF, dye front. Similar results were obtained in three experiments.

Several data suggest that sydnonimines inhibit the respiratory burst through a mechanism which is independent of NO release with subsequent activation of soluble guanylyl cyclase and increase in cGMP. First, SIN-10 was considerably more effective than SIN-1 in inhibiting fMet-Leu-Phe-induced O_2^- formation (see Figs 1 and 2). Second, SIN-10 substantially inhibited fMet-Leu-Phe-induced O_2 consumption, whereas SIN-1 did not (see Fig. 3). Third, neutrophils did not convert SIN-10 to SIN-1, as the former substance did not induce O_2 consumption, a process which accompanies the release of NO from SIN-1 (see Fig. 3) [22]. In agreement with these data is the finding that SIN-10 does not increase cGMP levels in human neutrophils [5]. Additionally, SIN-10 did not mimic the effects of Bt_2cGMP on the respiratory burst. Specifically, sydnonimines inhibited the effects of fMet-Leu-Phe at a submaximally and maximally effective concentration on the respiratory burst, whereas Bt_2cGMP only inhibited O_2^- formation and O_2 consumption induced by fMet-Leu-Phe at a submaximally effective concentration (see Figs 1–3 and Table 3) [6]. Finally, sydnonimines inhibited C5a-induced O_2^- formation, whereas Bt_2cGMP potentiated the effects of C5a (see Table 1) [6].

What may be the mechanism by which sydnonimines

Table 3. Effects of SIN-10 and SIN-1 on fMet-Leu-Phe-induced rises in $[Ca^{2+}]_i$ in human neutrophils

Stimulus	Increase in $[Ca^{2+}]_i$ (nmol/L)	
	Control	(SIN-10)
fMet-Leu-Phe (1 nmol/L)	487 ± 47	326 ± 50*
fMet-Leu-Phe (10 nmol/L)	955 ± 32	628 ± 38*

Cells were loaded with Fura-2 acetoxymethylester and the increases in $[Ca^{2+}]_i$ induced by fMet-Leu-Phe at the indicated concentrations were determined. SIN-10 (10 μmol/L) or solvent (control) were added to reaction mixtures 3 min prior to fMet-Leu-Phe. Basal $[Ca^{2+}]_i$ in control and SIN-10-treated cells was 149 ± 10 nmol/L and 145 ± 5 nmol/L, respectively. Data shown are the means ± SD of five experiments. Comparison of the effects of SIN-10 versus control: * $P < 0.05$.

inhibit the chemoattractant-induced respiratory burst? Under the conditions employed, the sydnonimines did not interfere with the detection method for O_2^- formation (see Materials and Methods). Additionally, these substances did not reoxidize ferrocytochrome *c* (see Materials and Methods). Moreover, the sydnonimines did not scavenge O_2^- as is supported by their lack of effect on xanthine oxidase-catalysed and pyrogallol-induced O_2^- formation and on receptor-independent activation of O_2^- formation in intact neutrophils and in a cell-free system from HL-60 cells (see Table 1). Furthermore, SIN-10 inhibited not only fMet-Leu-Phe-induced O_2^- formation, but also fMet-Leu-Phe-induced O_2 consumption (see Figs 1 and 3).

As the radical-scavenging properties of sydnonimines are unlikely to account for their inhibitory effects on the respiratory burst, we studied their effects on various steps of the signal transduction cascade in human myeloid cells. Sydnonimines specifically interfered with the chemoattractant-mediated activation of NADPH oxidase (see Figs 1–3 and Table 1). Unlike for Bt_2cAMP and Bt_2cGMP, inhibition of agonist binding to formyl peptide receptors does not contribute to the inhibitory effects of sydnonimines on the respiratory burst as they slightly enhanced binding (see Table 2). Interestingly, interference of Bt_2cGMP with agonist binding was also reported for other peptide receptors [36]. As sydnonimines inhibited the fMet-Leu-Phe- and C5a-induced respiratory bursts to similar extents and as receptors for these agonists couple to G-proteins, we asked the question as to whether sydnonimines could have acted at the level of G-proteins. This is, however, unlikely as they did not inhibit GTP hydrolysis of G-proteins (see Table 2). Additionally, the lack of effect of sydnonimines on potentiation by guanosine 5′-*O*-(3-thiotriphosphate) of O_2^- formation in a cell-free system from HL-60 cells argues against interference of SIN-10 and SIN-1 with G-proteins and/or low molecular mass GTP-binding proteins. Furthermore, the resistance to inhibition by sydnonimines of the PMA-induced respiratory burst suggests that they did not inhibit protein kinase C (see Table 1). Moreover, the lack of effect of sydnonimines on arachidonic acid-induced O_2^- formation in a cell-free system from HL-60 cells argues against interference of these substances with a structural component of NADPH oxidase. Finally, SIN-1 and SNP effectively stimulated ADP-ribosylation of a 39-kDa protein in cytosol of HL-60 cells, but SIN-10 did not (see Fig. 4). This finding is in agreement with the notion that ADP-ribosylation of the 39-kDa protein depends on NO release [14, 15]. Thus, the dissociation of the effects of sydnonimines on the fMet-Leu-Phe-induced respiratory burst on one hand and on ADP-ribosylation of this protein on the other suggests that covalent modification of the 39-kDa substrate is unrelated to their inhibitory effects on O_2^- formation and O_2 consumption (see Figs 1–4 and Table 1).

SIN-1 is known to inhibit osteoclast functions in a cGMP-independent manner, but the effects of SIN-10 were not studied [18]. Recently, Bohn *et al.* [23] reported NO-independent effects of an analogue of SIN-10 on vasodilation and suggested that sydnonimines may interfere with Ca^{2+} influx. Therefore, the effects of sydnonimines on fMet-Leu-Phe-induced rises in $[Ca^{2+}]_i$ in neutrophils were studied. Recently, we reported that SIN-1 up to 0.1 mmol/L did not inhibit fMet-Leu-Phe-induced rises in $[Ca^{2+}]_i$, but the effects of SIN-1 at higher concentrations could not be studied for methodological reasons [7]. In this study, we show that SIN-10 at concentrations as low as 10 μmol/L substantially inhibited rises in $[Ca^{2+}]_i$ induced by fMet-Leu-Phe at a submaximally and maximally effective concentration (see Table 3). Thus, inhibition of rises in $[Ca^{2+}]_i$may explain, at least in part, the inhibitory effects of SIN-10 on the chemoattractant-induced respiratory burst. This notion is supported by the finding that the sydnonimines did not inhibit the PMA-induced respiratory burst, a process which is independent of rises in $[Ca^{2+}]_i$ (see Table 1) [1, 2, 4]. Also in line with our findings are data showing that SIN-10 (10 μmol/L) effectively inhibits thrombin-induced rises in $[Ca^{2+}]_i$ in human platelets [34]. Undoubtedly, the concentrations of SIN-10 required to inhibit the chemoattractant-induced respiratory burst are rather high (see Figs 1–3 and Table 1). However, there is ample evidence for the assumption that regulation of the respiratory burst *in vitro* and *in vivo* is quite different (for review see Ref. 2). Thus, it cannot be ruled out that the effects of sydnonimines are of clinical relevance. Similarly, the clinical importance of the effects of SIN-10 on other cell functions is not yet known [5, 7, 23, 34].

In conclusion, SIN-10 effectively inhibits the chemoattractant-induced respiratory burst in human neutrophils via a mechanism which is independent of NO release. Our present results are in accordance with other recent reports on NO-independent effects of sydnonimines [5, 7, 23, 34]. Thus, future studies dealing with the pharmacological effects of SIN-1 should include experiments with the so-called "prodrug" SIN-10. This sydnonimine is not inert but may modulate various cell functions even more effectively than its so-called "active metabolite", SIN-1.

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