Background: Sodium nitroprusside (SNP) and molsidomine are used in the treatment of coronary heart disease. Since the neutrophils play a pathological role in ischaemic heart disease, it is important to understand the direct action of nitrovasodilators on their function.

Aim: We examined the effects of SNP and 3-morpholinosydnonimine (SIN-1, molsidomine metabolite) on the respiratory burst of human neutrophils and their adhesion in vitro. The influence of nitric oxide (NO) donors on the activity of protein kinases, which are involved in the NADPH oxidase activation, was also investigated.

Methods: The respiratory burst of neutrophils was determined by chemiluminescence and fluorescence methods, while the adhesion was assayed by adherence of neutrophils to the plastic surface.

Results: NO donors decreased the oxidative burst of activated neutrophils. However, the effects of SNP and SIN-1 strongly depended on the treatment time of neutrophils and on the stimulus employed to cells activation. Protein tyrosine kinase inhibitor did not affect the action of SNP, but diminished the inhibitory effect of SIN-1 on fMLP-stimulated but not on PMA-stimulated oxidative burst of neutrophils. This suggests that SNP action is mainly associated with protein kinase C, while SIN-1 is associated with protein tyrosine kinase activity. We also found that SIN-1 but not SNP diminished the adhesive activity of neutrophils.

Conclusions: Our data show that SIN-1 biological effect on some neutrophils activity is different from both spermine NONOate and SNP, and mainly depends on ONOO⁻, while SNP action is mediated by NO.

Key words: Nitrovasodilators, Neutrophils, Respiratory burst, Protein kinases, Adherence

Introduction

Neutrophils play a key role in the pathogenesis of ischaemic heart disease as the mediators of myocardial ischaemia reperfusion injury. Activated neutrophils produce and release a variety of powerful inflammatory mediators such as reactive oxygen species, enzymes and cytokines that are responsible for cell and tissue damage and serious complications associated with heart diseases.1–3

The reactive oxygen species are produced by neutrophils as result of NADPH oxidase activation (reactions named ‘respiratory burst’). Various protein kinases are involved in the NADPH oxidase activation including protein kinase C (PKC), mitogen-activated protein kinases, protein kinase A, and protein tyrosine kinase (PKT).4–10 NADPH oxidase can be activated by various stimuli such as chemotactic peptide (formylo-methionylo-leucylo-phenylalanine (fMLP)), phorbol 12-myristate 13-acetate (PMA) or opsonized zymosan. Stimulation of neutrophils with fMLP, a receptor-dependent stimulator, activates G-proteins and various downstream proteins including PKC, PKT, and mitogen-activated protein kinase. PMA as a receptor-independent stimulator directly activates PKC without G-proteins participation. It also indirectly activates mitogen-activated protein kinase and probably PKT.6,9

Nitric oxide (NO) donors such as sodium nitroprusside (SNP) and molsidomine are used as nitrovasodilators in the treatment of patients with coronary heart disease.12–14 Besides their effects on the vascular smooth muscle cells, NO-releasing compounds may affect the circulating white blood cells.
cells; for example, inhibit the adhesion, aggregation and chemotaxis of neutrophils.\textsuperscript{15} The recognition of the pathway by which the nitrovasodilators affect neutrophils function seems to be significant. The aim of this study was to examine the effects of SNP and 3-morpholinosydnonimine (SIN-1, molsidomine metabolite) on the respiratory burst of human neutrophils and their adhesion \textit{in vitro}. The influence of these compounds on the activity of kinases (PKT and PKC) that are involved in the NADPH oxidase activation was investigated.

**Materials and methods**

**Chemical reagents**

Polymorphprep was obtained from Nycomed (Oslo, Norway). Hanks balanced salt solution (HBSS), SNP, SIN-1, PMA, spermine NONOate, genistein, herbimicin A, 1-(5-isouquinoliniumsulfonyl)-2-methylpipерazine (H-7), NPC 15437, fMLP, tumour necrosis factor-alpha (TNF-\( \alpha \)), human recombinant, 2',7'-dichlorofluorescein (DCFH) diacetate, 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium nitrite, sulfanilamide, naphthylethenediamine dihydrochloride, and phosphoric acid were purchased from Sigma (USA). Luminol was a product of Serva (Germany). Phosphate-buffered saline (PBS) was purchased from BIOMED-Lublin (Poland).

**Isolation of neutrophils**

Heparinized peripheral blood was obtained from healthy volunteers and the neutrophils were isolated by centrifugation of blood sample on Polymorphprep as described previously.\textsuperscript{16} The Regional Commission approved the protocol of these studies for Ethics in Research.

**Measurement of the generation of nitric oxide from NO donor compounds**

NO donors (10–1000 \( \mu \)M) were added to HBSS (cell free system) or to neutrophils (1 \( \times \) 10\(^5\) cells) in HBSS for 30 or 60 min at 37\(^{\circ}\)C. The generation of NO was measured using Griess reagent as described previously.\textsuperscript{16}

**Respiratory burst of neutrophils**

Two assays were used to assess the respiratory burst because a single technique could be insufficient to determine the effect of different NO donors on the neutrophil function.\textsuperscript{16} Luminol-enhanced chemiluminescence measures the intracellularly and extracellularly released NADPH-derived and myeloperoxidase-derived oxygen metabolites, while DCFH oxidation assay measures the NADPH-derived oxygen species generated mainly intracellularly.\textsuperscript{17,18}

Chemiluminescence (CL) was measured in a 96-well plate Fluoroscan Ascent FL fluorometer (Labystem, Helsinki, Finland). Neutrophils (1 \( \times \) 10\(^5\) cells/well in HBSS) were untreated or pre-treated with 1, 10, 100 or 1000 \( \mu \)M of SNP or SIN-1 for 1–2 min or 30 min at 37\(^{\circ}\)C with 5% CO\(_2\) as indicated in the figures. In control experiments, the neutrophils were pre-treated with 10 \( \mu \)M or 100 \( \mu \)M of spermine NONOate for 1–2 min or 30 min at 37\(^{\circ}\)C with 5% CO\(_2\). Neutrophils were then removed. PMA, fMLP and luminol were then added as previously. The CL reading for all experiments was recorded for 30 min at 2 min intervals. The CL intensity was given in relative light units (RLU). The data were expressed as the area under the curve of CL versus time (RLU total).

The DCFH oxidation assay was measured in a 96-well plate Fluoroscan Ascent FL fluorometer (Labystem). Briefly, the neutrophils (2 \( \times \) 10\(^6\) cells/ml) were loaded with DCFH diacetate (100 \( \mu \)g/ml) in HBSS for 30 min at 37\(^{\circ}\)C, with 5% CO\(_2\). Cells were then washed twice with PBS, resuspended in fresh HBSS and distributed into a 96-well plate (1 \( \times \) 10\(^5\) cells/well). Neutrophils were untreated or pre-treated with SNP or SIN-1 at concentrations of 1, 10, 100 or 1000 \( \mu \)M for 1–2 min or 30 min at 37\(^{\circ}\)C with 5% CO\(_2\). The plate was placed into fluorometer and the intensity of fluorescence was determined at 485 nm excitation and 530 nm emission wavelengths (\( T_0 \)). Neutrophils were then stimulated with PMA (0.1 \( \mu \)g/ml) or fMLP (10\(^{-6}\) M) for the next 30 min at 37\(^{\circ}\)C with 5% CO\(_2\). After that time, the intensity of fluorescence was measured once more as described earlier (\( T_0 \)). In some experiments the neutrophils were untreated or pre-treated with 100 \( \mu \)M or 1000 \( \mu \)M of SNP or SIN-1 for 30 min at 37\(^{\circ}\)C with 5% CO\(_2\). Cells were then washed to remove NO-releasing drugs. The intensity of fluorescence at \( T_0 \) and after stimulation with PMA or fMLP at \( T_{30} \) was measured. The percentage of increase in the intensity of fluorescence was calculated from the formula \([(FT_{30} – FT_0)/FT_0] \times 100.\textsuperscript{19}

**Determination of the role of PKT and PKC activity in the NO donor-mediated respiratory burst**

Neutrophils (1 \( \times \) 10\(^5\) cells/well) were pre-treated with PKT inhibitors genistein (10 \( \mu \)M) and herbimicin A (1 \( \mu \)M) or PKC inhibitors H-7 (100 \( \mu \)M) and NPC (1 \( \mu \)M) for 15 min at 37\(^{\circ}\)C with 5% CO\(_2\). Neutrophils
were then incubated with SNP or SIN-1 (100 μM and 1000 μM) or with NONOate (10 μM and 100 μM) for 30 min. PMA, fMLP and luminol were then added as already described. The data were expressed as the mediated by NO donors’ per cent inhibition of PMA-induced or fMLP-induced respiratory burst of neutrophils calculated by the following formulae:

A. \[(\text{RLU}_{\text{total}} \text{ cells treated with SNP or SIN} + \text{stimulated with PMA or fMLP}) - (\text{RLU}_{\text{total}} \text{ cells treated with SNP or SIN-1})/\text{RLU}_{\text{total}} \text{ cells stimulated with PMA or fMLP}) - (\text{RLU}_{\text{total}} \text{ of untreated (control) cells}) \times 100\%

B. \[(\text{RLU}_{\text{total}} \text{ cells pretreated with PKC or PKT inhibitor} \text{ and SNP or SIN-1} \text{+ stimulated with PMA or fMLP}) - (\text{RLU}_{\text{total}} \text{ cells treated with SNP or SIN-1} \text{+ stimulated with PKC or PKT})/\text{RLU}_{\text{total}} \text{ of cells treated with PKC or PKT inhibitor} \text{+ stimulated with PMA or fMLP}) - (\text{RLU}_{\text{total}} \text{ of cells treated with PKC or PKT inhibitor}) \times 100\%.

Measurement of neutrophil adhesion

The adhesion was assayed by adherence of neutrophils to the plastic surface. This is a simple quantitative technique to examine the ability of human neutrophils to active adherence in vitro. Briefly, neutrophils were treated with SNP and SIN-1 or with 20 ng/ml of TNF-α (as indicated later in Table 2). Non-adherent cells were removed by carefully washing the wells’ surface with PBS and adherent neutrophils were exposed to MTT (2 mg/ml of PBS) containing 10 ng/ml of PMA for 30 min. Formazan produced by adherent cells was dissolved in 500 μl of 2-propanol. The absorbance at 560 nm and 630 nm dual wavelength was measured in an ELISA reader Multiscan RC (Labsystem). The absorption of formazan is directly related to the number of adherent neutrophils. The amount of adherent cells was also controlled microscopically by independent researcher. The loss of cells treated with SIN-1 was observed under the microscope.

Statistical analysis

Data are presented as the mean ± standard deviation. Statistical analysis was performed with Wilcoxon’s singed rank test. Statistical significance was defined as \(p \leq 0.05\).

Results

Oxidative response of neutrophils to nitrovasodilators

The generation of nitrite (a stable metabolite of NO) from NO donors in the presence of neutrophils or in the cell free system is presented in Table 1.

In pilot experiments, we noticed that 1 min or 30 min pre-treatment of neutrophils with SNP or SIN-1 at 1 μM and 10 μM had no significant effects on oxygen radical generation by stimulated cells (data not shown). Therefore, the concentrations of 100 μM and 1000 μM were selected for further experiments.

We observed that 1–2 min pre-incubation with either SNP or SIN-1 decreased the respiratory burst of PMA-stimulated neutrophils as it was measured with CL and DCFH oxidation assays. The effect of pre-incubation with SNP and SIN-1 on the respiratory burst of fMLP-stimulated neutrophils was not seen when CL measurement was used. However, different effects of SNP and SIN-1 on fMLP-stimulated neutrophils were observed when DCFH oxidation assay was used. SNP increased, while SIN-1 decreased the fMLP-stimulated respiratory burst of neutrophils (Fig. 1). Figure 2 demonstrates that 30 min pre-incubation with both nitrovasodilators decreased the respiratory burst of PMA-stimulated and fMLP-stimulated neutrophils as it was measured with CL and DCFH oxidation assays. As shown in Fig. 3, the removal of SNP from the cells environment cancelled its down-regulating effect on the oxidative burst of

Table 1. Nitrite generation from NO donors in the presence of neutrophils and in the cell free system

| Incubation (min) | SNP (cell free system) | SIN-1 (cell free system) | Spermine NONOate (cell fee system) |
|------------------|------------------------|--------------------------|-----------------------------------|
|                  | 1000 μM 100 μM         | 1000 μM 100 μM           | 1000 μM 100 μM 10 μM              |
| 30               | 1.2 ± 0.1 0.1 ± 0.1    | 48.4 ± 15.3 9.4 ± 3.1    | 288.1 ± 32.2 137.8 ± 3.1 15.5 ± 0.5 |
| 60               | 2.3 ± 0.4 0.7 ± 0.4    | 187 ± 20.3 13.8 ± 2.5    | 288.0 ± 10.1 125.2 ± 7.9 13.3 ± 1.6 |
| SNP + neutrophils| 36.1 ± 8.7 4.1 ± 0.8   | 104.3 ± 12.7 19.3 ± 9.6  | 308.0 ± 16.9 132.2 ± 12.2 13.7 ± 7.8 |
| SIN-1 + neutrophils| 12.3 ± 1.7 2.0 ± 0.9 | 200.3 ± 11.9 118.5 ± 9.4 | 300.9 ± 11.9 120.3 ± 1.2 |

SNP (100 μM and 1000 μM) or SIN-1 (100 μM and 1000 μM) or spermine NONOate (10–1000 μM) were added to HBSS (cell free system) or to neutrophils (1 × 10⁶ cells) in HBSS for 30 or 60 min at 37°C with 5% CO₂. The generation of nitrite from NO donors was measured using Griess reagents. Data are expressed as mean ± standard deviation of three independent experiments.
FIG. 1. Effect of brief pre-treatment with nitrovasodilators on the respiratory burst of stimulated neutrophils in vitro. Neutrophils (1 × 10^6 cells) were untreated or pre-treated with nitrovasodilators at the concentrations of 100 μM and 1000 μM for 1–2 min, and then stimulated with PMA (closed bars) or with fMLP (hatched bars) or were unstimulated (open bars). See details in Materials and methods. Data are expressed as means of RLU total or per cent increase of fluorescence ± standard deviation of six independent experiments carried out with neutrophils from different individuals. * Statistically significant decrease: neutrophils versus neutrophils with nitrovasodilators (p ≤ 0.02). # Statistically significant increase: neutrophils versus neutrophils with SNP (p ≤ 0.02).

FIG. 2. Effect of 30 min pre-treatment with nitrovasodilators on the respiratory burst of activated neutrophils in vitro. Neutrophils (1 × 10^6 cells) were untreated or pre-treated with nitrovasodilators at the concentrations of 100 μM and 1000 μM for 30 min and then stimulated with PMA (closed bars) or with fMLP (hatched bars) or were unstimulated (open bars). See details in Materials and methods. Data are expressed as means of RLU total or per cent increase of fluorescence ± standard deviation of six independent experiments carried out with neutrophils from different individuals. * Statistically significant decrease: neutrophils versus neutrophils with nitrovasodilators (p ≤ 0.02).
fMLP-stimulated but not PMA-stimulated neutrophils, whereas the inhibitory effects of SIN-1 were not reversed after its withdrawal.

Data presented in Fig. 4 demonstrate that 1-2 min pre-incubation with spermine NONOate decreased the respiratory burst of PMA-stimulated but not of fMLP-stimulated neutrophils. Thirty minutes' pre-incubation with this NO donor decreased the oxidative burst of PMA-activated and fMLP-activated cells. It was not possible to perform experiments with NONOate at concentrations higher than 100 μM because that caused cell damage.

It should be stressed that both nitrovasodilators at the concentrations used did not interfere with luminescence assay
nol or DCFH diacetate and did not produce any signal in the cell free system as was verified in both assays.

Involvement of PKC and PKT pathways in the NO donor-mediated decrease of respiratory burst of activated neutrophils

We examined the effect of blocking the PKC or PKT activity on the nitrovasodilator-mediated decrease of respiratory burst of stimulated neutrophils. As shown in Fig. 5, PKC inhibitors (H-7 and NPC) at the used doses had no effect on fMLP-induced neutrophil oxidative burst, but when added prior to the induction of this process by PMA they caused a significant reduction of it. These data indicate that fMLP activates neutrophils’ oxidative metabolism through the PKC-independent mechanism. PKT inhibitors (genistein and herbimicin A) caused a significant reduction of respiratory burst of PMA-stimulated and fMLP-stimulated neutrophils (Fig. 5). These results indicate that PKT participates in the induction of PMA-activated neutrophils’ respiratory burst. Data presented in Fig. 6 demonstrate that PKC inhibitor H-7, administered prior to the addition of SNP and PMA or fMLP, partially but significantly diminished the inhibitory effect of the NO donor, whereas H-7 did not affect the action of SIN-1. The similar results we observed with SIN-1 (data not shown). As shown in Fig. 7, the PKT inhibitor genistein did not affect the inhibitory effect of SNP. However, genistein attenuated the inhibitory effect of SIN-1 on fMLP-stimulated but not on PMA-stimulated neutrophils. Similar results were observed with herbimicin A (data not shown).

Genistein did not affect the inhibitory effect of spermine NONOate but H-7 significantly diminished the inhibitory effect of spermine NONOate on respiratory burst of activated neutrophils. The percentage of inhibition was as follow: neutrophils + NONOate + fMLP, 22.2 ± 11.2 versus neutrophils + H-7 + NONOate + fMLP, 10.7 ± 9.4 (p ≤ 0.05, n = 5); neutrophils + NONOate + PMA, 23.9 ± 11.4 versus neutrophils + H-7 + NONOate + PMA, 15 ± 7.2 (p ≤ 0.05, n = 5).

Adhesive activity of nitrovasodilator-treated neutrophils

TNF-α enhanced the adhesion of neutrophils after 30 min of incubation with cells (from optical density (OD) = 0.070 ± 0.029 in the control sample up to OD = 0.130 ± 0.052 in the presence of cytokine; p ≤ 0.04, n = 5). The data in Table 2 demonstrate the different effect of SNP and SIN-1 on the neutrophil
FIG. 6. Effect of PKC inhibitor on the SNP-mediated and SIN-1-mediated oxidative burst of activated neutrophils. Neutrophils (1 × 10⁶) were first treated with H-7 (100 μM) for 15 min. Then, the cells were incubated with 1000 μM of SNP or 1000 μM of SIN-1 for 30 min and next stimulated with 0.1 μg/ml of PMA or 10⁻⁸ M of fMLP. The CL reading was recorded for 30 min at 2 min intervals. The data are expressed as means of per cent inhibition of respiratory burst ± standard deviation of six independent experiments carried out with neutrophils from different individuals. Statistical significance: * SNP + PMA versus SNP + PMA + H-7, p ≤ 0.02; ** SNP + fMLP versus SNP + fMLP + H-7, p ≤ 0.02.

FIG. 7. Effect of PKT inhibitor on the SNP-mediated and SIN-1-mediated oxidative burst of activated neutrophils. Neutrophils (1 × 10⁶) were first treated with genistein (10 μM) for 15 min. Then, the cells were incubated with 1000 μM of SNP or 1000 μM of SIN-1 for 30 min and next stimulated with 0.1 μg/ml of PMA or 10⁻⁸ M of fMLP. The CL reading was recorded for 30 min at 2 min intervals. The data are expressed as means of per cent inhibition of respiratory burst ± standard deviation of six independent experiments carried out with neutrophils from different individuals. Statistical significance: * SIN-1 + fMLP versus SIN-1 + fMLP + genistein, p ≤ 0.02.
adhesion. Neutrophil adhesion was reduced after treatment of cells with 100 μM of SIN-1 for 60 min and with 1000 μM of SIN-1 for 30 and 60 min. In contrast, SNP did not affect their adhesion to the plastic surface. Spermine NONOate did not influence on the adherence of neutrophils (data not shown).

Discussion

The mechanism of NO generation from SNP is controversial. Some authors have shown that NO could be spontaneously generated from SNP,13,21 but others have demonstrated that NO formation required the presence of vascular tissue12 and/or the visible light.22 We have shown here that SNP released NO in the cell free system; however, the presence of neutrophils enhanced its generation (Table 1). SIN-1, the metabolite of molsidomine, produced NO spontaneously without any cofactors.12–14

The results of our experiments are in accordance with observations of others21–24 that SNP and SIN-1 at concentrations above 50 μM decrease the reactive oxygen species production by neutrophils. However, it should be also emphasized that biological effects of SNP and SIN-1 strongly depended on the treatment time of neutrophils and on the stimulus employed to activate the cells. Our data indicate that SNP and SIN-1 should act on neutrophils for longer than 1–2 min to exert their modulatory effects, when the receptor-dependent activator is used (Figs. 1 and 2). We may speculate that the different oxidative response of short-time nitrovasodilator pre-treated neutrophils arise from different kinetics of the respiratory burst induced by PMA and by fMLP. The kinetics of CL responses show that the peak of the PMA-induced oxidative response occurs 10–15 min after activation of cells while the peak of the fMLP-induced respiratory burst of neutrophils occurs 1–5 min after neutrophil activation.25 Our results and those of others22 suggest that SNP and SIN-1 may affect the respiratory burst of neutrophils by various signalling pathways (Fig. 3). The inhibition of the PMA-dependent respiratory burst of neutrophils by SNP and SIN-1 indicates that NO donors exert effects independent of G-proteins. However, SIN-1 can also exert some effect via or close to G-proteins since it decreased the fMLP-mediated neutrophils’ oxidative burst.25

In the course of NADPH oxidase activation distinct signal transduction pathways can be activated.4,7,11 The signal transduction through receptors with tyrosine kinase activity occurs upon interaction of fMLP with its receptor; however, the other signalling pathway involves PKC via activation of phospholipase C. PMA directly activates PKC, and indirectly PKT.11,26 The role of protein kinases in the action of NO donors on the respiratory burst of neutrophils is not clear and it was not examined intensively. In this paper we show that SNP action is mainly associated with PKC, while SIN-1 is associated with PKT activity (Figs. 6 and 7). We suppose that the influence of NO donors on PKC, PKT and/or G-protein activities can be responsible for the decrease of respiratory burst of activated neutrophils.

It was demonstrated by others that the effect of SNP and SIN-1 on the neutrophil functions was mediated by NO.21,23,24 Our results suggest that NO donors can also affect neutrophils in the NO-independent pathway. Feelisch13 maintained that a relatively small amount of NO released from SNP in vitro was not sufficient for activating soluble guanylate cyclase and that biological effects of SNP were largely independent of NO. It was also suggested that SNP might mediate effects by a NO-independent and soluble guanylate cyclase-independent mechanism.14 It should be also considered that during decomposition of SNP a cyanide is released. However, Forslund and Sundqvist22 demonstrated that the effect of SNP on the respiratory burst of neutrophils was mediated by NO not by cyanide. Our data show that SNP, similar to spermine NONOate, decreases PMA-induced respiratory burst of neutrophils and affects the PKC activity. It can suggest that the action of SNP may depend on NO. Spontaneous decomposition of SIN-1 leads to the generation of equimolar quantities of NO and O2, which interact almost instantly to produce peroxynitrite anion (ONOO−).12 However, it should be emphasized that formation of ONOO− during decomposition of SIN-1 in vitro strongly depends on the oxygen concentration in the reaction mixture.27,28

Table 2. Effect of nitrovasodilators on the adhesive activity of neutrophils

| Time (min) | Control | SNP | SIN-1 |
|-----------|---------|-----|-------|
|           | OD (560 nm and 630 nm) |       |       |
| 30        | 0.070 ± 0.023 | 0.068 ± 0.010 | 0.075 ± 0.018 |
| 60        | 0.258 ± 0.039 | 0.215 ± 0.043 | 0.260 ± 0.043 |

Neutrophils (1 x 10⁶ cells) were distributed into 24-well plates and were incubated without or with nitrovasodilators (100 μM or 1000 μM) for 30 and 60 min at 37°C, with 5% of CO2. Non-adherent cells were removed, and adherent cells were exposed for MTT solution as described in Materials and methods. Data are expressed as means of optical densities ± standard deviation of eight independent experiments carried out with neutrophils from different individuals. * Statistical significance: neutrophils versus neutrophils with SIN-1 (p ≤ 0.05).
Our data show that the SIN-1 biological effect on some neutrophils’ activity is different from both spermine NONOate and SNP, and mainly depends on ONOO\textsuperscript{−}, while SNP action is mediated by NO.

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