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Enhancing and inhibitory effects of nitric oxide on superoxide anion generation in human polymorphonuclear leukocytes

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1 The effects of sodium nitroprusside (SNP, a nitric oxide donor) and authentic nitric oxide (NO) on superoxide anion (O_2^-) generation were investigated in human polymorphonuclear leukocytes (PMNs).

2 Neither SNP (10 nM to 10 μM) nor NO (40 nM to 40 μM) alone induced O_2^- generation or change of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human PMNs.

3 Pretreatment with SNP or NO at the concentrations used (SNP, 10 nM to 10 μM: NO, 40 nM to 40 μM) showed a biphasic concentration-dependent effect on O_2^- generation induced by f-methionyl-leucyl-phenylalanine (FMLP). Low concentrations of SNP (10 nM to 100 nM) and NO (400 nM) did not affect either basal cyclic GMP levels or cyclic GMP levels stimulated by FMLP, but enhanced FMLP-induced O_2^- generation and $[Ca^{2+}]_i$ elevation. On the other hand, high concentrations of SNP (10 μM) and NO (40 μM) alone elevated cyclic GMP levels and inhibited FMLP-induced O_2^- generation and $[Ca^{2+}]_i$ elevation.

4 8-Bromo-cyclic GMP (8-Br-cyclic GMP) at concentrations ranging from 1 μM to 1 mM did not induce O_2^- generation on its own and had little effect on FMLP-induced O_2^- generation and $[Ca^{2+}]_i$ elevation.

5 Addition of a high concentration of NO (40 μM) decreased authentic O_2^- formation by pyrogallol in a cell-free system, but a low concentration of NO (400 nM) had no effect on this. On the other hand, addition of SNP in the concentration-ranges used had no effect on authentic O_2^- formation by pyrogallol.

6 In this study, we have shown that SNP and NO have dual effects (enhancement and inhibition) on O_2^- generation induced by FMLP in human peripheral PMNs. The results suggest that the enhancement observed with SNP and NO at low concentrations is not mediated by activation of the guanylate cyclase-cyclic GMP pathway. The suppressive effect of SNP and NO at higher concentrations is mediated by the NO-induced O_2^--scavenging effect and activation of the guanylate cyclase-cyclic GMP pathway.

Keywords: Nitric oxide (NO); sodium nitroprusside (SNP); superoxide anion (O_2^-) generation; human polymorphonuclear leukocytes (PMNs)

Introduction

Nitric oxide (NO) production is shown in a variety of cells, and NO has been recognized as an important mediator of various cellular activities. For example, NO regulates vascular tone and blood flow and in sepsis, excess production of NO by macrophages and other cells is thought to induce massive vasodilatation and shock. Polymorphonuclear leukocytes (PMNs) have been found to produce NO (McCall *et al.*, 1989; Wright *et al.*, 1989; Yui *et al.*, 1991). However, the role of NO generated by PMNs is still insufficiently understood. The effects of NO on monocytes/macrophages and PMNs include the stimulation of chemotaxis (Renz *et al.*, 1988; Kubes *et al.*, 1991) and vimentin phosphorylation (Wyatt *et al.*, 1991), and the NO/cyclic GMP-mediated blockade of f-methionyl-leucyl-phenylalanine (FMLP)-triggered elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Lopez *et al.*, 1991; Riesco *et al.*, 1993). Some reports have indicated that NO or NO donors (at high concentrations, $>10\mu$M) can inhibit PMN functions: chemotaxis, superoxide anion (O_2^-) generation and degranulation (Rubanyi *et al.*, 1991; Clancy *et al.*, 1992; Moilanen *et al.*, 1993; Rengasamy & Johns, 1993). O_2^- generation is a key function of PMNs required for killing of bacteria. On the other hand, O_2^- is a cytotoxic free radical that, if released in large quantities, can cause tissue injury (Fridovich, 1983). In the present study, we examined the effects of sodium nitroprusside (SNP), authentic NO and 8-bromo-cyclic GMP (8-Br-cyclic GMP) on FMLP-induced O_2^- generation, and found that SNP and NO have dual effects (enhancement and inhibition) on O_2^- generation induced by FMLP in human PMNs.

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Methods

Preparation of PMNs

Human blood was obtained from healthy donors who had abstained from taking any drugs for at least 14 days before sampling. PMNs were separated by the dextran sedimentation and centrifugation on a Ficoll-Paque density gradient. After lysing erythrocytes in the PMNs suspension, PMNs were resuspended in Hank's balanced salt solution (pH 7.4, HBSS) as described previously (Morikawa *et al.*, 1990). After preparation, the PMNs suspension consisted of approximately 95% PMNs and the viability of cells was >97% as determined by trypan blue exclusion.

Determination of superoxide generation

The PMNs suspension was preincubated with SNP (1 nM to 10 μM) or NO (40 nM to 40 μM) for 10 min and then stimulated with FMLP for 10 min at 37°C. The concentration of generated O_2^- was measured as superoxide dismutase-inhibitable reduction of ferricytochrome c (Morikawa *et al.*, 1992). The effects of SNP and NO on authentic O_2^- formation in a cell-free system were analysed by using pyrogallol (5 μM) instead of FMLP and other procedures were similar to those used for the PMNs suspension.

Determination of cyclic GMP levels

The reaction of PMNs suspension with FMLP was stopped by addition of ice-cold trichloroacetic acid solution and the suspension was centrifuged at 10,000 *g* for 10 min. The supernatant was washed three times with water-saturated ethyl ether, and guanidine 3' : 5'-cyclic monophosphate (cyclic GMP)

content was measured by radioimmunoassay as described previously (Morikawa *et al.*, 1992).

Measurement of intracellular Ca^{2+} concentration

The PMNs suspension was recentrifuged and the PMNs obtained were resuspended in Ca^{2+}-free HEPES buffer of the following composition (mM); NaCl 140, KCl 5, glucose 5.6, $MgCl_2$ 0.5, HEPES 20, pH 7.4 and loaded with Fura 2-AM 3 μM for 30 min at 37°C. After dilution 2 fold with Ca^{2+}-free HEPES buffer the PMNs suspension was centrifuged and the pellets were resuspended in HBSS. The changes in fluorescence ratio of excitation wavelengths (Ex = 340 nm and 380 nm, Em = 500 nm) induced by FMLP were measured in Fura 2-loaded PMNs by using a Ca^{2+} analyser (CAF-100, JASCO). The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the method of Grynkiewicz *et al.* (1985).

Statistical analysis

Data are presented as the means ± s.e. Statistical analysis of data was performed with Student's *t* test or Analysis of Variance followed by Dunnett's test. Differences between control and test groups were evaluated with $P<0.05$ as the level of significance.

Drugs

Preparation of authentic NO solution: HBSS (50 ml) in a vial was bubbled with Argon gas (Touyou Sanso, Tokyo) for 30 min, and then NO gas (99.0%, Nippon Sanso, Tokyo) for 20 min at room temperature. The vial containing saturated NO solution was sealed with a rubber stopper and stored in a refrigerator. The NO concentration was measured by h.p.l.c. combinated with diazotization using $NaNO_2$ as a standard. The following agents were used: f-methionyl-leucyl-phenylalanine (FMLP), cytochrome c, superoxide dismutase, pyrogallol, sodium nitroprusside (SNP), 8-bromoguanosine 3′ : 5′-cyclic monophosphate (8-Br-cyclic GMP), and haemoglobin (Hb) (all Sigma); 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid, pentaacetoxymethyl ester (Fura 2-AM, Dojin Laboratories); 2-O-propoxyl-phenyl-8-azopurine-6-one (M&B 22,948, May & Baker); Dextran T-500 and Ficoll-Paque (Pharmacia).

Results

Effects of SNP and NO on O_2^- generation in PMNs

The effects of SNP and NO on O_2^- generation in human PMNs were examined. Neither SNP (1 nM to 10 μM) nor NO (40 nM to 40 μM) alone induced O_2^- generation. Addition of SNP and NO had no effect on absorption of cytochrome c at 550 nm.

FMLP stimulated O_2^- generation in a concentration-dependent manner, and the effect reached a plateau at 100 nM (Figure 1). Preincubation with SNP (1 nM to 10 μM) or NO (40 nM to 40 μM) showed effects on FMLP-induced O_2^- generation. Figure 1 shows the effect of 10 nM SNP on the concentration-response curve for FMLP-induced O_2^- generation. Pretreatment with 10 nM SNP enhanced O_2^- generation induced by FMLP (1 nM to 100 nM), but did not affect that induced by 1 μM FMLP.

Figure 2 shows the effect of SNP on 10 nM FMLP-induced O_2^- generation. Low concentrations of SNP (10 nM and 100 nM) enhanced FMLP (10 nM)-induced O_2^- generation (without SNP: 0.238 ± 0.024 nmol/10^5 PMNs, $n=11$; with 10 nM SNP: 0.404 ± 0.076 nmol/10^5 PMNs, $n=6$; with 100 nM SNP: 0.346 ± 0.042 nmol/10^5 PMNs, $n=10$). On the other hand, a high concentration of SNP (10 μM) showed an inhibitory effect on 10 nM FMLP-induced O_2^- generation (with 10 μM SNP: 0.138 ± 0.022 nmol/10^5 PMNs, $n=6$).

As shown in Figure 3, NO like SNP, showed a dual effect on FMLP (10 nM)-induced O_2^- generation. At 400 nM, NO enhanced FMLP-induced O_2^- generation (without NO: 0.238 ± 0.024 nmol/10^5 PMNs, $n=11$; with 400 nM NO: 0.388 ± 0.056 nmol/10^5 PMNs, $n=6$), but had an inhibitory effect at 40 μM (with 40 μM NO: 0.164 ± 0.048 nmol/10^5 PMNs, $n=6$).

Haemoglobin (Hb), a known NO scavenger, alone did not affect FMLP-induced O_2^- generation (without Hb: 0.258 ± 0.059 nmol/10^5 PMNs, $n=6$; with 0.5 μM Hb: 0.243 ± 0.052 nmol/10^5 PMNs, $n=6$). But the enhancing effects of SNP (10 nM and 100 nM) and NO (400 nM) on O_2^- generation induced by FMLP were reversed by addition of 0.5 μM Hb.

Effects of SNP and NO on O_2^- formed by pyrogallol

Pyrogallol induced authentic O_2^- formation in a cell-free system in a concentration-dependent manner (1 μM to 50 μM). Low concentrations of NO (<400 nM) had no effect on au-

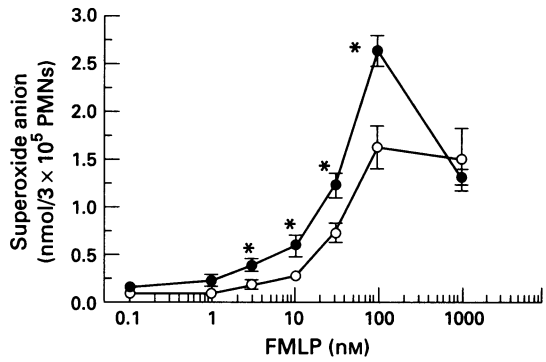

Figure 1 Effect of 10 nM sodium nitroprusside (SNP) on the concentration-response curve for FMLP-induced superoxide anion generation in human PMNs. PMNs were preincubated with (●) or without (○) 10 nM SNP for 10 min, and then stimulated with FMLP. Mean ± s.e. ($n=6-12$), *$P<0.05$ vs. the value for PMNs without SNP.

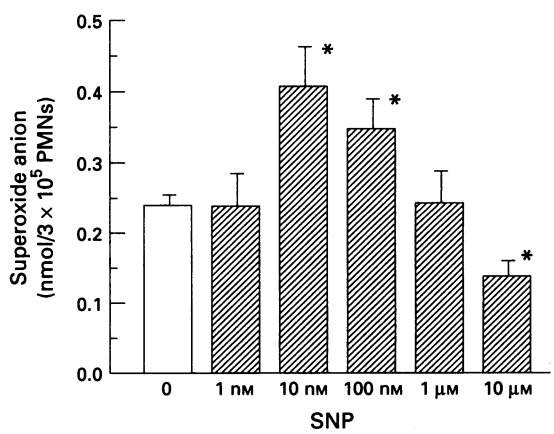

Figure 2 Effect of sodium nitroprusside (SNP) on 10 nM FMLP-induced superoxide anion generation in human PMNs. PMNs were preincubated with (hatched columns) or without (open column) SNP for 10 min, and then stimulated with 10 nM FMLP. Mean ± s.e. ($n=6-11$) *$P<0.05$ vs. the value for PMNs without SNP.

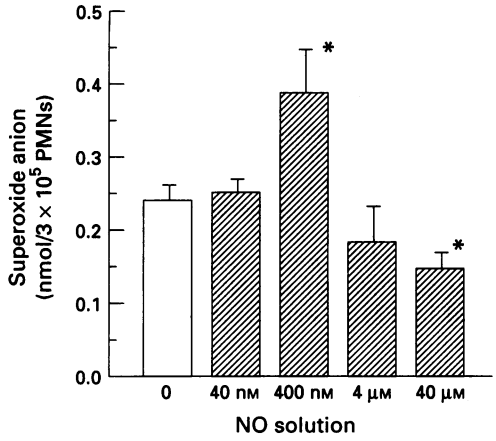

Figure 3 Effect of NO solution on FMLP-induced superoxide anion generation in human PMNs. PMNs were preincubated with (hatched columns) or without (open column) NO solution for 10 min, and then stimulated with 10 nM FMLP. Mean ± s.e. ($n = 6-11$), $^*P < 0.05$ vs. the value for PMNs without NO solution.

thentic O_2^- formation by 5 μM pyrogallol, but high concentrations of NO (40 μM) decreased it (without NO: 0.289 ± 0.032 nmol; with 400 nM NO: 0.259 ± 0.025 nmol; with 40 μM NO: 0.137 ± 0.015 nmol). However, high concentration of NO did not affect O_2^- formation by high concentrations of pyrogallol (> 10 μM). On the other hand, SNP in the concentration-ranges used in this study had no effect on authentic O_2^- formation by pyrogallol.

Effects of 8-Br-cyclic GMP on O_2^- generation in PMNs

It is widely known that NO produces an increase in the second messenger cyclic GMP by directly activating guanylate cyclase. To examine the cause of the biphasic effects of SNP and NO, the effect of 8-Br-cyclic GMP on FMLP-induced O_2^- generation in human PMNs was studied. 8-Br-cyclic GMP (1 μM to 1 mM) did not induce O_2^- generation by itself, and showed little effect on that induced by 10 nM FMLP (without 8-Br-cyclic GMP: 0.219 ± 0.014 nmol/10^5 PMNs, $n = 5$; with 1 μM 8-Br-cyclic GMP: 0.201 ± 0.030 nmol/10^5 PMNs, $n = 5$; with 1 mM 8-Br-cyclic GMP: 0.216 ± 0.056 nmol/10^5 PMNs, $n = 5$). The effect of 8-Br-cyclic GMP on FMLP-induced O_2^- generation was not influenced by the presence of 100 mM M&B 22,948, a specific cyclic GMP phosphodiesterase inhibitor (10 nM FMLP without 8-Br-cyclic GMP: 0.184 ± 0.031 nmol/10^5 PMNs, $n = 5$; 10 nM FMLP with 1 mM 8-Br-cyclic GMP: 0.224 ± 0.0034 nmol/10^5 PMNs, $n = 5$).

Effects of SNP and NO on intracellular cyclic GMP levels

The effects of SNP and NO on the intracellular cyclic GMP levels in human PMNs were examined and the results are shown in Table 1. The basal cyclic GMP level in human PMNs was 135 ± 35 fmol/10^7 PMNs, $n = 5$. Low concentrations of SNP (10 nM to 100 nM) and NO (400 nM) alone did not change the cyclic GMP level, but high concentrations of SNP (10 μM) and NO (40 μM) produced an elevation of cyclic GMP levels in the presence of 100 nM M&B 22,948.

In the concentration-ranges (1 nM to 1 μM) used in this study, FMLP showed no significant effect on the cyclic GMP level (10 nM FMLP: 138 ± 40 fmol/10^7 PMNs, $n = 5$; 100 nM FMLP: 137 ± 26 fmol/10^7 PMNs, $n = 5$; 1 μM FMLP: 140 ± 28 fmol/10^7 PMNs, $n = 5$). Preincubation with SNP (1 nM to 10 μM) and NO (40 nM to 40 μM) had little effect on the cyclic GMP level stimulated by FMLP in the presence of 100 mM M&B 22,948.

Table 1 Effects of sodium nitroprusside (SNP) and NO on cyclic GMP levels in FMLP-stimulated human PMNs

	Cyclic GMP levels (fmol/10^7 PMNs) FMLP 0	FMLP 10 nM
None	135 ± 35	138 ± 40
SNP 10 nM	140 ± 28	145 ± 32
SNP 10 μM	310 ± 45*	327 ± 30*
NO 400 nM	142 ± 39	156 ± 56
NO 40 μM	388 ± 68*	401 ± 77*

PMNs were incubated with or without SNP or NO for 10 min, and then stimulated with FMLP. Mean ± s.e. ($n = 5$), $^*P < 0.05$ vs. the baseline value.

Table 2 Effects of sodium nitroprusside (SNP) and NO on FMLP (10 nM)-induced $[Ca^{2+}]_i$ elevation in human PMNs

		$\Delta[Ca^{2+}]_i$ (nM)
FMLP 10 nM	None	410.1 ± 48.1
	SNP + 1 nM	480.2 ± 119.1
	SNP + 10 nM	796.3 ± 87.1*
	NO + 40 nM	418.6 ± 40.5
	NO + 400 nM	556.3 ± 50.1*
	NO + 4 μM	443.4 ± 1.76
	NO + 40 μM	367.3 ± 16.0

PMNs were incubated with SNP or NO for 5 min, and then stimulated with 10 nM FMLP. Mean ± s.e. ($n = 6-11$), $^*P < 0.05$ vs. the value for PMNs stimulated with 10 nM FMLP alone.

Effects of SNP and NO on FMLP-induced $[Ca^{2+}]_i$ elevation

SNP (< 10 nM) and NO (40 nM to 40 μM) alone showed no effect on $[Ca^{2+}]_i$ in human PMNs. In the $[Ca^{2+}]_i$ determination experiment, we could not use SNP at concentrations above 10 nM because of its fluorescence.

FMLP induced a $[Ca^{2+}]_i$ elevation in a concentration-dependent manner (1 nM FMLP: 87.2 ± 9.8 nM; 10 nM FMLP: 410.1 ± 48.5 nM; 100 nM FMLP: 558.6 ± 87.5 nM; $n = 11$). Table 2 shows the effects of SNP and NO on 10 nM FMLP-induced $[Ca^{2+}]_i$ elevation. Low concentrations of SNP (10 nM) and NO (400 nM) enhanced FMLP (10 nM)-induced $[Ca^{2+}]_i$ elevation, but a high concentration of NO (40 μM) had an inhibitory effect.

Discussion

Some recent reports have indicated that at concentrations greater than 10 μM, authentic NO and NO-releasing compounds can inhibit neutrophil functions (Rubanyi *et al.*, 1991; Moilanen *et al.*, 1993). NO reacts directly with O_2^- in aqueous solution and has been reported to form peroxynitrite (ONOO) (Radi *et al.*, 1993). Clancy *et al.* (1992) showed that NO (10 μM to 100 μM) reduced O_2^- generation induced by FMLP in human PMNs and 30 μM NO transiently inhibited O_2^- production by hypoxanthine-xanthine oxidase in a cell-free system, consistent with O_2^- -scavenging of NO. It was also reported that SNP and NO had biphasic concentration-dependent effects, consisting of inhibition and excitation of contraction in oesophageal longitudinal muscle (Saha *et al.*, 1993), or protection and destruction in the central nervous system (Dawson *et al.*, 1992).

In this study we examined the effects of SNP and NO on O_2^- generation induced by FMLP in human PMNs. Neither

SNP (1 nM to 10 μM) nor NO (40 nM to 40 μM) alone induced O_2^- generation in PMNs. Low concentrations of SNP (10 nM to 100 nM) and NO (400 nM) enhanced FMLP-induced O_2^- generation, but both SNP (10 μM) and NO (40 μM) at higher concentrations had suppressive effects (Figures 1–3).

A low concentration of NO (400 nM) had no effect on authentic O_2^- formation by pyrogallol in a cell-free system, but a high concentration of NO (40 μM) decreased it. It is probable that NO at higher concentrations acts as a pure O_2^- scavenger, as at higher concentrations NO decreased O_2^- generation of PMNs and authentic O_2^- formation by pyrogallol in a cell-free system. The addition of SNP at the concentration-ranges used in this study had no effect on authentic O_2^- formation by pyrogallol in a cell-free system, because SNP does not release NO in a cell-free system. These results suggest that the suppressive effects of high concentrations of NO and SNP on FMLP-induced O_2^- generation relate to the O_2^- scavenging effect of NO.

The biological functions of NO, regulation of vasodilatation (Furchgott, 1983) and inhibition of platelet aggregation (Radomski *et al.*, 1987), are believed to be mediated by activation of soluble guanylate cyclase and the consequent increase in intracellular cyclic GMP concentration (Moncada *et al.*, 1991). It was reported that cyclic GMP inhibits PMN functions (Riesco *et al.*, 1993; Moilanen *et al.*, 1993). However, in the present study, 8-Br-cyclic GMP did not itself induce O_2^- generation and showed little effect on FMLP-triggered O_2^- generation. Low concentrations of SNP (10 nM to 100 nM) and NO (400 nM) evoked no change in basal cyclic GMP levels or that stimulated by FMLP in the presence of M&B 22,948. On the other hand, at higher concentrations both SNP (10 μM) and NO (40 μM) alone increased cyclic GMP levels, but showed no effect on that stimulated by FMLP. These results show that the enhancing effects of low concentrations of SNP (10 nM to 100 nM) and NO (400 nM) on FMLP-induced O_2^- generation are cyclic GMP-independent, and that a part of the inhibitory effects of high concentrations of SNP (10 μM) and NO (40 μM) is cyclic GMP-dependent. In agreement with other studies (Schroder *et al.*, 1990; Molilanen *et al.*, 1993), we have shown that NO at high concentrations elicits an inhibitory effect on PMNs functions. The inhibitory effect of NO at higher concentrations can be attributed in part to O_2^--scavenging activity and in part to involve cyclic GMP; however, a direct effect of NO as a scavenger of O_2^- radical cannot be excluded (Rubanyi *et al.*, 1991; Clancy *et al.*, 1992).

It is reported that stimulation by FMLP induces an increase of $[Ca^{2+}]_i$ and triggers O_2^- generation in human PMNs. Part of the O_2^- production evoked by FMLP is dependent on $[Ca^{2+}]_i$ elevation. Therefore, we also determined the effect of SNP and NO on $[Ca^{2+}]_i$ changes in response to FMLP, to evaluate whether this effect contributed to the enhancement of the FMLP-induced O_2^- generation. Low concentrations of SNP (10 nM) and NO (400 nM) enhanced FMLP-induced $[Ca^{2+}]_i$ elevation. The elevation of cyclic GMP induced by SNP and NO may contribute to the inhibitory effects of SNP and NO at higher concentrations. Clancy and co-workers (1993) reported that NO stimulates the ADP-ribosylation of a 43 kD protein in human PMNs and Galione (1992) reported that cyclic ADP-ribose may open a Ca^{2+}-induced Ca^{2+}-releasing mechanism mediated by a ryanodine receptor. Our results suggest that the enhancing effects of SNP and NO at low concentrations on FMLP-induced $[Ca^{2+}]_i$ elevation may be caused by the activation of a Ca^{2+}-cyclic ADP-ribose-dependent pathway induced by NO.

PMNs release NO when activated by agonists (McCall *et al.*, 1989; Salvemini *et al.*, 1989; Wright *et al.*, 1989) but the physiological and pathophysiological functions of endogenous NO are not well known. In the brain, NO is clearly a unique biological effector molecule under conditions of normal release, but with excessive release, NO may function as a cytotoxic molecule mediating macrophage-induced cell death as well as being involved in several neurodegenerative processes (Dawson *et al.*, 1992).

In this study, we investigated the effects of NO and SNP on O_2^- generation in human PMNs. Our findings suggest that NO and SNP have dual effects (enhancement and inhibition) on FMLP-induced O_2^- generation, and the enhancement effect of NO and SNP at low concentrations is cyclic GMP-independent. The enhancement effect of NO or NO donors on O_2^- generation induced by FMLP in human PMNs has not been described previously. The results of the present study suggest that a low concentration of PMN-derived NO induces vasodilatation and inhibition of platelet aggregation, and thus, could enhance PMN function (chemotaxis, O_2^- generation etc.), while a high concentration of NO could act as a negative feedback signal for the process of PMN activation (excessive O_2^- production causes cell injury).

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