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Effects of Respiratory Burst Inhibitors on Nitric Oxide Production by Human Neutrophils

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Human neutrophils (PMN) activated by N-formyl-methionyl-leucyl-phenylalanine (fMLP) simultaneously release nitric oxide (·NO), superoxide anion ($O_2^{\bullet-}$) and its dismutation product, hydrogen peroxide (H_2O_2). To assess whether ·NO production shares common steps with the activation of the NADPH oxidase, PMN were treated with inhibitors and antagonists of intracellular signaling pathways and subsequently stimulated either with fMLP or with a phorbol ester (PMA). The G-protein inhibitor, pertussis toxin (1–10 μg/ml) decreased H_2O_2 yield without significantly changing ·NO production in fMLP-stimulated neutrophils; no effects were observed in PMA-activated cells. The inhibition of tyrosine kinases by genistein (1–25 μg/ml) completely abolished H_2O_2 release by fMLP-activated neutrophils; conversely, ·NO production increased about 1.5- and 3-fold with fMLP and PMA, respectively. Accordingly, orthovanadate, an inhibitor of phosphotyrosine phosphatase, markedly decreased ·NO production and increased $O_2^{\bullet-}$ release. On the other hand, inhibition of protein kinase C with staurosporine and the use of burst antagonists like adenosine, cholera toxin or dibutyryl-cAMP diminished both H_2O_2 and ·NO production. The results suggest that the activation of the tyrosine kinase pathway in stimulated human neutrophils controls positively $O_2^{\bullet-}$ and H_2O_2 generation and simultaneously maintains ·NO production in low levels. In contrast, activation of protein kinase C is a positive modulator for $O_2^{\bullet-}$ and ·NO production.

Keywords: Neutrophils, nitric oxide, superoxide anion, hydrogen peroxide, respiratory burst, tyrosine kinase

INTRODUCTION

Neutrophil (PMN) activation in response to chemotactic agents, phagocytic stimuli and other endogenous regulators is a complex process involving migration, phagocytosis, degranulation and generation of active oxygen species. Formyl-peptides (fMLP) are among the best-known stimuli of circulating neutrophils. They are released from bacteria at the site of infection and diffuse into the blood vessels located in close proximity. They bind to specific surface receptors of circulating neutrophils promoting the chemotactic response.[1] The fMLP-dependent neutrophil activation elicits a mechanism of signal transduction, which involves receptor interaction with guanine

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nucleotide binding proteins (G-proteins), phospholipases activation, protein-tyrosine phosphorylation, calcium mobilization and kinases activation. Phorbol esters exert their action predominantly through a direct effect on protein kinase C (PKC) (Fig. 1).[2-6] Recently, PMN-produced nitric oxide (•NO) has been suggested as a second messenger involved in chemotaxis and degranulation[7,8] and we have shown that human neutrophils activated with phorbol esters generate •NO and superoxide anion ($O_2^{\bullet-}$) yielding peroxynitrite.[9] The signaling pathways involved in the activation of NADPH oxidase with the consequent generation of $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) through dismutation are not completely understood. However, there is evidence indicating the importance of protein-tyrosine phosphorylation and protein kinase C activation in the regulation of neutrophil activation.[10-18]

Despite growing evidence showing that •NO has roles in regulating PMN activities and in peroxynitrite formation during the respiratory burst there are no reports about •NO synthase (NOS) signaling pathways.

The aim of this study is to evaluate the linkage between the intracellular signaling for NADPH oxidase and •NO synthase by using inhibitors of the signaling pathways of the respiratory burst of human neutrophils.

MATERIALS AND METHODS

Preparation of Human Neutrophils

Heparinized venous blood was drawn from healthy volunteers. Human neutrophils were isolated by Ficoll-Hypaque gradient centrifugation,

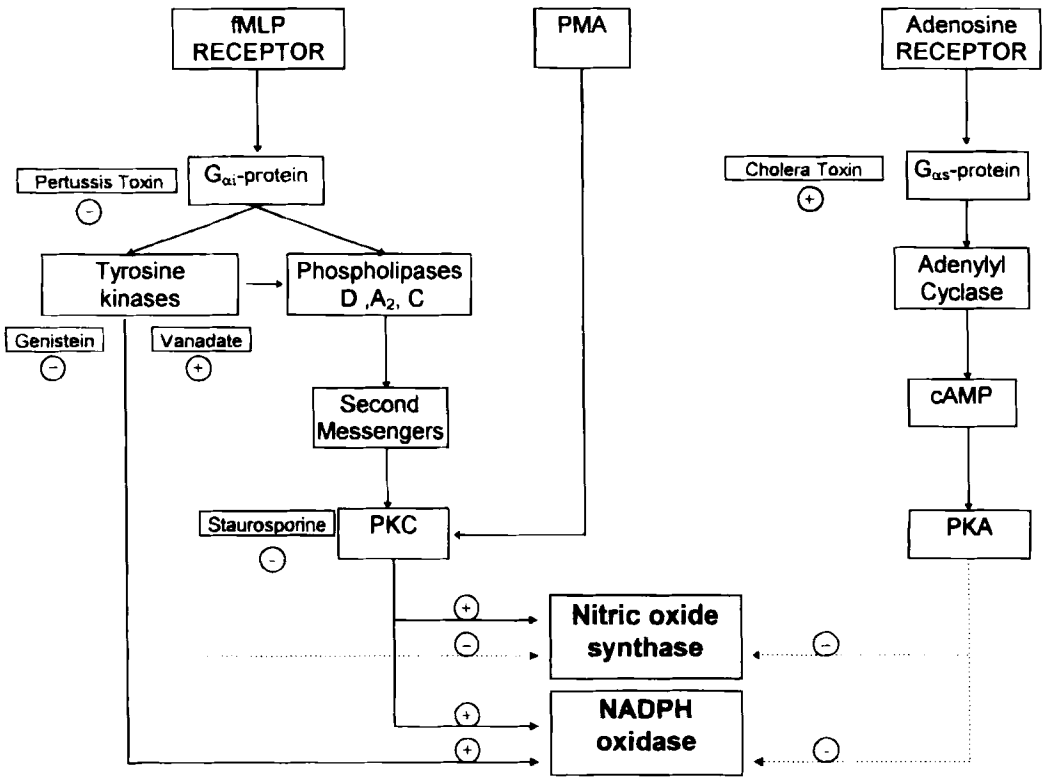

FIGURE 1 Scheme of the modulation (activation, solid lines; inhibition, dashed lines) of NADPH oxidase and •NO synthase of human polymorphonuclear cells.

dextran sedimentation and hypotonic lysis of contaminant erythrocytes at room temperature.[19] The cells were resuspended in 120 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 20 mM HEPES and 5.5 mM glucose, pH 7.4 (HBSS) at a concentration of 3×10^7 cells/ml. Cell preparations consisted of about 98% of viable neutrophils (Trypan blue test). Samples were preincubated with respiratory burst inhibitors at 37°C during the described times. The assays were done in HBSS buffer with the addition of 1 mM $CaCl_2$ and 1.3 mM $MgCl_2$ (HBSS-Ca-Mg).

Hydrogen Peroxide Generation

Hydrogen peroxide was measured by the horseradish peroxidase (HRP)/p-hydroxyphenyl acetic acid (4-HPA) assay at 37°C with a Hitachi F-2000 spectrofluorometer at 315–425 nm.[20] The reaction medium contained HBSS-Ca-Mg, 0.5 mM 4-HPA, 12 U/ml HRP and 10^6 cells/ml. Fluorescence was continuously recorded for about 1 min before the addition of 1 µM fMLP or 0.1 µg/ml PMA and for about 9 min after the addition of the stimuli. A linear rate of fluorescence was observed immediately after fMLP addition for about 30 sec and with a lag time of about 60 sec after PMA addition for about 2 minutes. Hydrogen peroxide generation was calculated from a standard curve made with titrated H_2O_2 solutions and was expressed in nmol/min · 10^6 cells.

Nitric Oxide Production

·NO production was measured by the oxidation of oxymyoglobin (oxyMb) to metmyoglobin (metMb) as previously described,[21] but modified and adapted by us for neutrophil suspensions.[9,22] The absorbance difference at 581–592 nm was continuously monitored at 37°C in a double beam-double wavelength 356 Perkin Elmer spectrophotometer and the rate of ·NO formation was calculated using the extinction coefficient 11.6 $mM^{-1}.cm^{-1}$ and expressed as nmol/min · 10^6 cells. The reaction medium consisted of HBSS-Ca-Mg, 20 µM oxyMb and 10^6 cells/ml. The reaction was recorded before and after the addition of the stimuli for 10 minutes, a linear production rate was observed for about 2 min with fMLP and 4 min with PMA. Oxymyoglobin was prepared by reduction of metMb with sodium hydrosulphite followed by gel filtration in a prepacked Sephadex G-25 column previously equilibrated with 50 mM potassium phosphate buffer at pH 7.4. The concentration of oxyMb was determined spectrophotometrically.

The eventual contribution of hydrogen peroxide to oxyMb oxidation was assayed in the presence of non-stimulated PMN and glucose-glucose oxidase system and was not significant.

Electrochemical Detection of Nitric Oxide

Nitric oxide release was continuously measured before and after the addition of the stimulus by a ·NO sensitive electrode from World Precision Instruments, Inc. (Sarasota, Fl, USA).[22] The cells (3×10^6 PMN/ml) were placed in a thermostated chamber under very gentle stirring. The electrode was calibrated with $NaNO_2$ in acid solution.

Superoxide Anion Generation

$O_2^{\bullet -}$ production was continuously recorded by monitoring the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm using a Hitachi U 1100 spectrophotometer at 37°C, as previously described.[19]

Materials

All chemicals were purchased from Sigma Chemical Co.

Statistical Analysis

Statistical differences were assessed with one-way analysis of variance (ANOVA) and Dunnett's test.

All differences were considered significant when $P < 0.05$.

RESULTS

Nitric Oxide and Hydrogen Peroxide Production by fMLP-Stimulated Human Neutrophils

Production of •NO and H_2O_2 by human neutrophils was increased by addition of fMLP to the maximal rates of 0.21 ± 0.01 nmol •NO/min • 10^6 cells and 0.45 ± 0.02 nmol H_2O_2/min • 10^6 cells at about 1μM fMLP (Fig. 2). Half-maximal effects were observed at about 10^{-7} M fMLP for both •NO and H_2O_2 production. Production of $O_2^{\bullet-}$ the stoichiometric precursor of H_2O_2, was also measured at all fMLP concentrations and the inset of Figure 2 shows the ratios of $H_2O_2/O_2^{\bullet-}$ and $\bullet NO/O_2^{\bullet-}$ production rates as a function of fMLP concentration. The $H_2O_2/O_2^{\bullet-}$ ratio remains constant at 0.26–0.28 while the ratio of $\bullet NO/O_2^{\bullet-}$ decreases in a dose-dependent manner from an almost equimolar relationship at low fMLP levels to a value of 0.10 at high fMLP levels.

Effect of the Inhibition of G_i-Proteins By Pertussis Toxin

Preincubation of PMN for 60 min with pertussis toxin, which catalyzes the ADP-ribosylation of the α-subunit of G_i-proteins preventing the interaction of the complex with the receptors,[23] abolished H_2O_2 production in fMLP-stimulated neutrophils (from 1.70 ± 0.20 to 0.17 ± 0.03 nmol/min • 10^6 cells at 10 μg/ml toxin, $P < 0.05$) while •NO production was not affected (0.21 ± 0.02 to 0.18 ± 0.02 nmol/min • 10^6 cells at 10 μg/ml toxin) (Fig. 3A). In contrast, preincubation with the toxin did not affect either H_2O_2 (3.93 ± 0.48 vs 3.91 ± 0.63) or •NO release (0.73 ± 0.12 vs 0.70 ± 0.14) when neutrophils were stimulated with PMA (Fig. 3B).

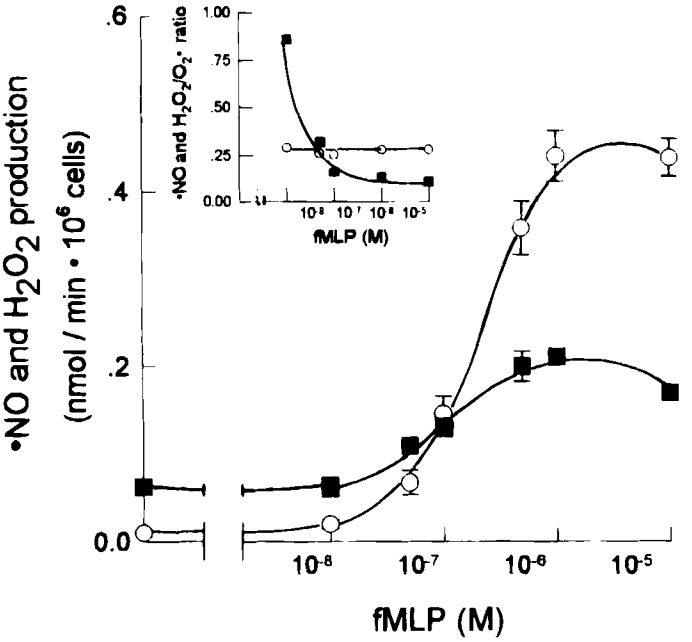

FIGURE 2 Production of •NO (■) and H_2O_2 (○) by human neutrophils stimulated by fMLP. Values are mean ± S.E.M. from 4–6 samples. Inset: Ratios $\bullet NO/O_2^{\bullet-}$ (■) and $H_2O_2/O_2^{\bullet-}$ (○) production rates.

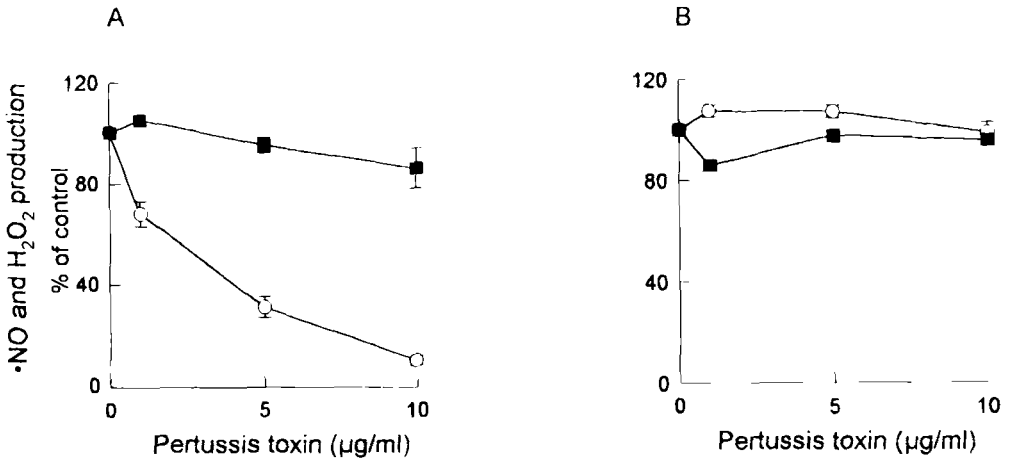

FIGURE 3 Effect of pertussis toxin on •NO (■) and H_2O_2 (○) production by human neutrophils. Cells were preincubated for 60 min with pertussis toxin at 37°C and then stimulated with 1μM fMLP (A) or 0.1 μg/ml PMA (B).

Effect of Protein Kinase C Inhibition

Preincubation of PMN for 5 min with staurosporine, a PKC inhibitor, decreased •NO production while highly increased H_2O_2 production in fMLP-stimulated neutrophils (Fig. 4A). In contrast, preincubation with staurosporine markedly inhibited both •NO and H_2O_2 production (up to 73 and 98%, respectively) when the cells were stimulated with PMA (from 0.68 ± 0.02 to 0.18 ± 0.01 nmol •NO/min • 10^6 cells, $P < 0.05$ and from 3.11 ± 0.39 to 0.05 ± 0.01 nmol H_2O_2/min • 10^6 cells, $P < 0.05$) (Fig. 4B).

Effect of Tyrosine Kinase Inhibition

Preincubation for 30 min with genistein, a tyrosine kinase inhibitor, markedly inhibited H_2O_2 production by neutrophils stimulated with fMLP (from 0.87 ± 0.11 to 0.02 ± 0.01 nmol/min • 10^6 cells, $P < 0.05$) and slightly decreased H_2O_2 pro-

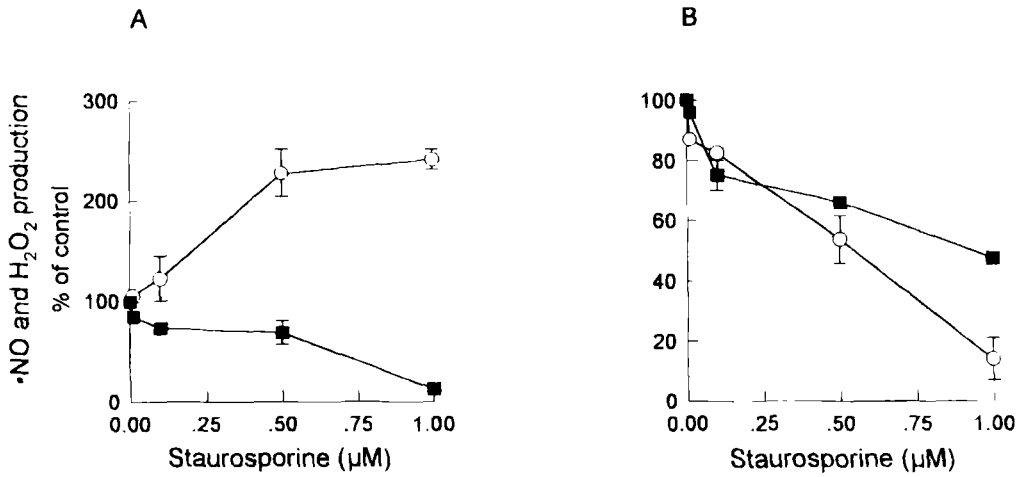

FIGURE 4 Effect of protein kinase C inhibition on •NO (■) and H_2O_2 (○) production by human neutrophils. The cells were preincubated for 5 min with staurosporine at 37°C and then stimulated with 1 μM fMLP (A) or with 0.1 μg/ml PMA (B).

duction by PMN activated by PMA (from 3.31 ± 0.20 to 2.65 ± 0.18 nmol/min · 10^6 cells). In contrast, genistein preincubation increased ·NO production PMN activated by fMLP (from 0.23 ± 0.03 to 0.60 ± 0.09 nmol/min · 10^6 cells, $P < 0.05$) and by PMA; in this latter case with a marked increase from 0.66 ± 0.02 to 1.90 ± 0.09 nmol/min · 10^6 cells ($P < 0.05$) (Fig. 5A and 5B). Preincubation for 30 min with orthovanadate, a phosphotyrosine phosphatase inhibitor, decreased 40% ·NO production (from 0.25 ± 0.03 to 0.10 ± 0.02 nmol/min · 10^6 cells, $P < 0.05$) while slightly increasing $O_2^{\bullet-}$ generation from 4.19 ± 0.35 to 5.33 ± 0.27 nmol/min · 10^6 cells (Fig. 5C). In addition, the association of orthovanadate with staurosporine practically abolished ·NO production by PMN stimulated with fMLP (0.04 ± 0.02 nmol/min · 10^6 cells; data not shown).

In PMA-stimulated neutrophils vanadate inhibited ·NO production from 0.70 ± 0.09 to 0.29 ± 0.05 nmol/min · 10^6 cells while $O_2^{\bullet-}$ generation was not affected (Fig. 5D).

Effect of the Activation of Adenylyl Cyclase

Adenosine (an antagonist of the respiratory burst), cholera toxin (that ADP-ribosylates the GTPase subunit of G_s-proteins resulting in accumulation of cAMP) and dibutyryl cAMP (DBcAMP), an analogue of cAMP,[24,26] were

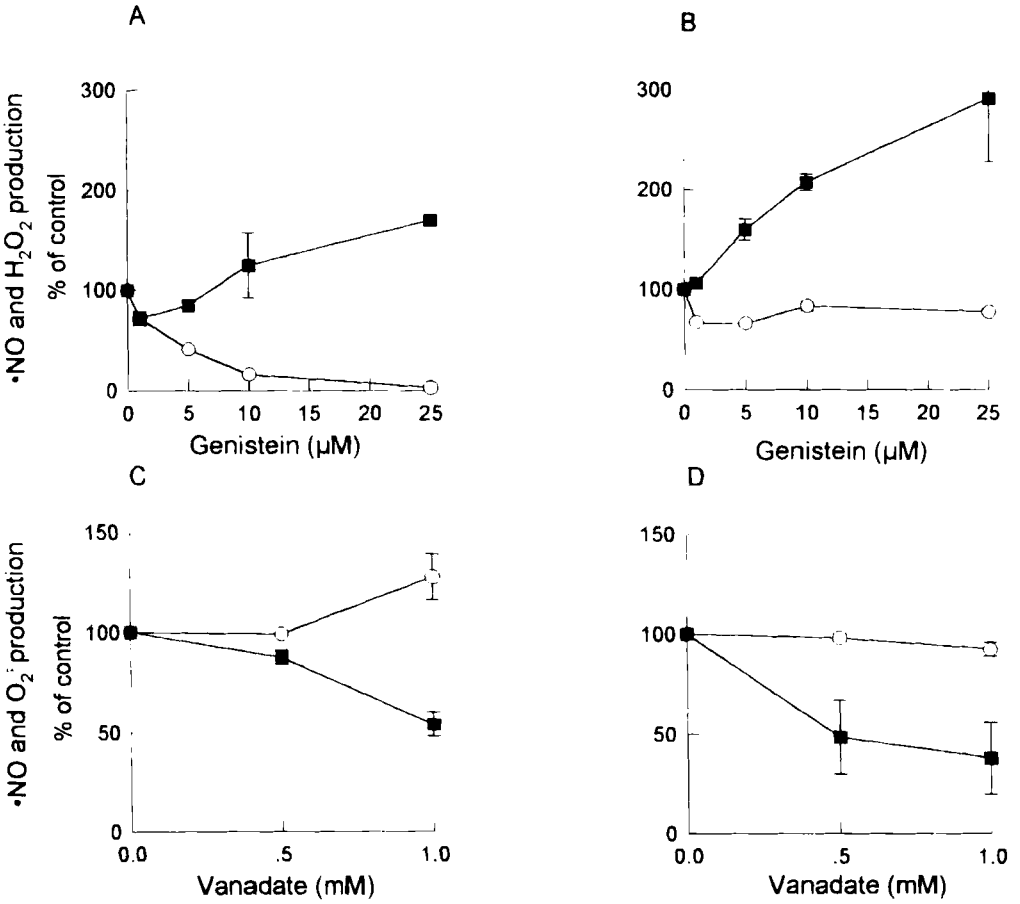

FIGURE 5 Effect of tyrosine kinase and phosphotyrosine phosphatase inhibitors on ·NO (■) and H_2O_2 or O_2^- (○) production by human neutrophils. Neutrophils preincubated 30 min with genistein were stimulated with 1μM fMLP (A) or with 0.1 μg/ml PMA (B). Neutrophils preincubated 30 minutes with orthovanadate were stimulated with 1μM fMLP (C) or with 0.1 μg/ml PMA (D).

added to PMN and the production of H_2O_2 and •NO assayed to evaluate the effect of cAMP accumulation (Table I). Preincubation with adenosine decreased H_2O_2 production up to 80% and •NO production up to 50%. Cholera toxin also diminished H_2O_2 and •NO production up to 98% and 56%, respectively. Treatment with DB-cAMP decreased both H_2O_2 and •NO production up to 77% and 70%, respectively.

Electrochemical Measurements of •NO

Nitric oxide is electrochemically undetectable in fMLP-stimulated neutrophils (Fig. 6A) unless the $O_2^{\bullet-}$ steady-state concentration in the reaction medium is markedly decreased by adding superoxide dismutase and subtracting Ca^{2+} (Fig. 6B) or by preincubation with pertussis toxin in the presence of superoxide dismutase (Fig. 6C).

After preincubation with genistein there is a marked increase in basal •NO production, noticeable even in the absence of added SOD, suggesting a basal inhibition of NOS by constitutive tyrosine kinase activity. Addition of fMLP produces a large response of •NO generation.

DISCUSSION

This study shows that intracellular signaling modulates •NO production occurring during the respiratory burst of human neutrophils, as we have previously reported with PMA-activated neutrophils.[9] The results also provide evidence that cell signaling pathways for NADPH oxidase activation modulate NOS activity either positively or negatively (Fig. 1).

The simultaneous generation of $O_2^{\bullet-}$ and H_2O_2 by NADPH oxidase activation and of •NO by NOS activation in either fMLP- or PMA-stimulated neutrophils suggests that some of the activation signals are common for both enzymes. In this sense, the previously reported PMA-stimulation of •NO and $O_2^{\bullet-}$ production by human neutrophils and the effects of staurosporine, a PKC inhibitor, in •NO and H_2O_2 production in PMA-stimulated neutrophils seem to indicate that PKC activation is a common step for both NOS and NADPH oxidase stimulation.

In contrast, the other NADPH oxidase activating pathway, the tyrosine kinase route (Fig. 1), has an inhibitory effect on NOS activity; for example, the inhibition of $G_{\alpha i}$-protein activating pathway by pertussis toxin did not change •NO generation but suppressed NADPH oxidase activation. In addition, genistein that decreases tyrosine phosphorylation, increased •NO production while orthovanadate, with opposite effects, inhibited NOS activity. In accord with these results, the production of •NO by neutrophils should be inversely and closely related to phosphotyrosine levels.

TABLE I Effect of respiratory burst inhibitors on H_2O_2 and •NO production by human neutrophils. Neutrophils were preincubated with adenosine or DBcAMP for 5 min and with cholera toxin for 30 min and then stimulated with 1 μM fMLP. Values are mean ± S.E.M. from 3 experiments and are expressed in nmol/min •10^6 cells and as % of controls. *$P < 0.05$

Treatment	Concentration	H_2O_2	•NO
Control 5 min		0.75 ± 0.07	0.23 ± 0.02
Adenosine (μM)	10	64 ± 5%*	70 ± 4%*
	100	37 ± 1%*	57 ± 4%*
	500	23 ± 1%*	48 ± 9%*
DBcAMP (mM)	0.25	85 ± 7%	87 ± 13%
	0.50	44 ± 3%*	65 ± 4%*
	1.0	23 ± 3%*	30 ± 4%*
Control 30 min		0.83 ± 0.09	0.25 ± 0.03
Cholera toxin (μg/ml)	1	93 ± 10%	76 ± 8%
	10	57 ± 4%*	36 ± 4%*
	50	2 ± 1%*	44 ± 4%*

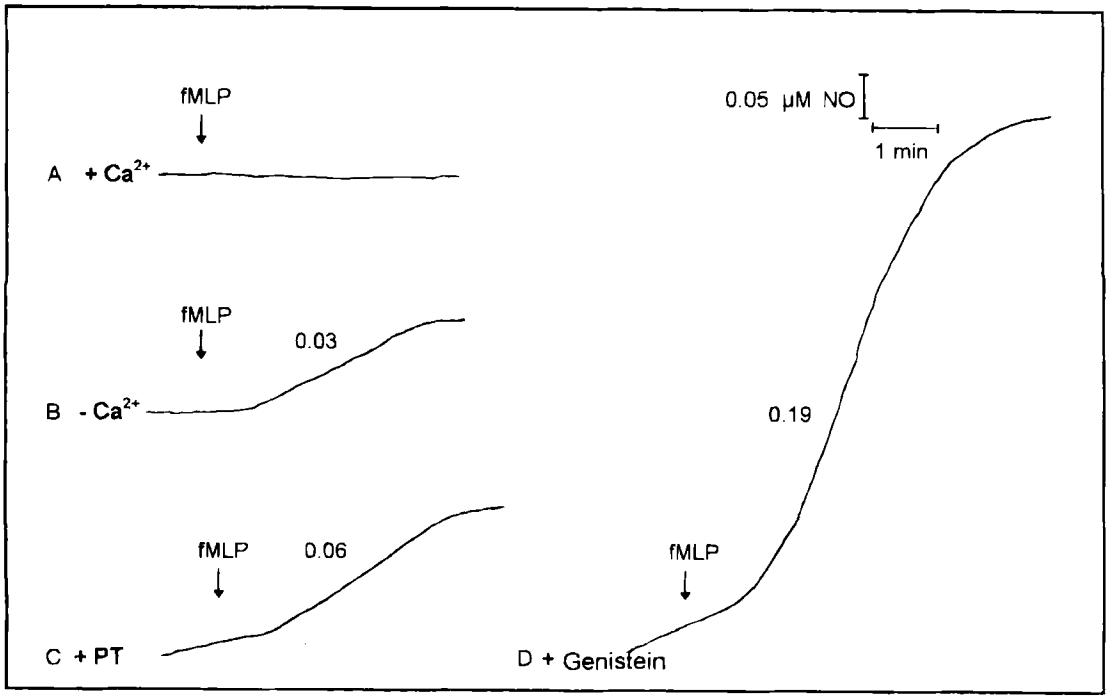

FIGURE 6 Electrochemical traces of •NO production by fMLP-stimulated human neutrophils. A: with Ca^{2+}, B: without Ca^{2+} and with 3000 U SOD/ml, C: preincubated with 10 μg/ml pertussis toxin and added with 3000 U SOD/ml and D: preincubated with 25 μg/ml genistein. The numbers near the traces indicate nmol •NO/min • 10^6 cells.

This study shows that NADPH oxidase activation is not required for NOS activation in PMN; the opposite situation cannot reliably be affirmed. Nitric oxide has been implicated in the production of $O_2^{\bullet-}$ in other tissues[27] and the effect of NOS inhibitors on $O_2^{\bullet-}$ and H_2O_2 production has been reported before.[9] The addition of •NO or •NO donors to human polymorphonuclear leukocytes has promoted both enhancing and inhibitory effects on $O_2^{\bullet-}$ release depending on •NO concentrations.[28] In this case the inhibitory effects at higher •NO concentrations were related to cGMP increase and also to the $O_2^{\bullet-}$ scavenging power of •NO in itself. In addition, neutrophil oxygen free radicals can increase the levels of phosphorylated tyrosine by either activating some tyrosine kinases and inhibiting phosphotyrosine phosphatases.[29] A dissociation between •NO and $O_2^{\bullet-}$ productions has been observed in cases of chronic granulomatous disease[30] while in other clinical situations the production of •NO and H_2O_2 by human neutrophils decreases or increases in parallel.[31-33] Experimental and "ex vivo" studies converge to the fact that a "cross-talk" exists between the signal transducing messengers but also between the products of NOS and NADPH oxidase pathways.

The use of antagonists of the respiratory burst like cholera toxin and adenosine could indicate that activation of the adenylyl cyclase pathway through PKA-dependent phosphorylation is inhibitory for •NO as well as for $O_2^{\bullet-}$ formation.

Recent reports have identified the constitutive neuronal isoenzyme type in human neutrophils.[34] and inhibition of neuronal NOS isoenzyme was observed after its phosphorylation by cAMP-dependent protein kinase but also with PKC.[35] However, a 40% increase of neuronal NOS activity by PKC phosphorylation was also reported.[36] The inhibitory effects of phorbol

esters on purified NOS[37] or cell homogenates contrast with the increase in constitutive NOS activity after PMA addition to human neutrophils as reported here. The discrepancies could be accounted by the use of homogenates or isolated purified NOS instead of intact cells where other enzymes could also be targets of PKC activity. Accordingly, the results presented here suggest that activation of PKC by phorbol esters exerts a positive control of NOS activity; this activation could be related to some direct action of PKC on NOS but also to stimulation of phosphotyrosine phosphatases, as suggested by the comparative effects of staurosporine and vanadate. Also, the different responses to PKC inhibition with fMLP or with PMA suggest that in intact cells, PKC exerts some kind of inhibition on phosphorylated tyrosine levels.

The physiological or pathological significance of the reported regulatory signals remains to be defined. In this way, inhibition of tyrosine kinases with genistein has been related to neutrophil apoptosis; in opposite, a direct (not G-protein-related) activation of tyrosine kinase activity by the granulocyte/macrophage-colony stimulating factor receptor, leads to increased cell survival.[38] From our results, it is apparent that both tyrosine kinase activity and ·NO levels play an integrated role in the process of neutrophil cell apoptosis or survival in addition to its microbicidal properties. In this sense, it has been shown the participation of ·NO in macrophage and neuronal apoptosis.[39,40]

In sum, the activation of NADPH oxidase and NOS appears to share common steps in the intracellular signal transducing pathways of activated human neutrophils, while others have opposite effects in both systems.

Acknowledgements

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