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Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils

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Abstract

Nitric oxide (•NO) release, oxygen uptake and hydrogen peroxide (H_2O_2) production elicited by increasing phorbol 12-myristate 13-acetate (PMA) concentrations were measured in human neutrophils. Half-maximal activities were sequentially elicited at about 0.0001–0.001 μg PMA/ml (•NO) and 0.001–0.01 μg PMA/ml (H_2O_2). At saturated PMA concentrations, •NO production, oxygen uptake and H_2O_2 release were 0.56 ± 0.04, 3.32 ± 0.52 and 1.19 ± 0.17 nmol · min^{-1} · 10^6 $cells^{-1}$. •NO production accounts for about 30% of the total oxygen uptake. Luminol-dependent chemiluminescence, reported to detect NO reactions in other inflammatory cells, was also half-maximally activated at about 0.001–0.01 μg PMA/ml. Preincubation with N^G-monomethyl-L-arginine (L-NMMA) decreased O_2 uptake and •NO release but increased H_2O_2 production, while superoxide dismutase (SOD) increased •NO detection by 30%. Chemiluminescence was also reduced by preincubation with L-NMMA and/or SOD. The results indicate that •NO release is part of the integrated response of stimulated human neutrophils and that, in these cells, kinetics of •NO and $O_2^{\bullet-}$ release favour the formation of other oxidants like peroxynitrite.

Key words: Neutrophil; Nitric oxide; Peroxynitrite; Superoxide anion; Hydrogen peroxide; Respiratory burst

1. Introduction

Human neutrophils (PMN) exposed to soluble or particulate stimuli undergo a large increase in cyanide-insensitive oxygen consumption termed the respiratory burst [1,2]. Several authors demonstrated that superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are produced and that these compounds are apparently responsible for the microbicidal activity of PMN [3,4].

It has recently been shown that circulating human neutrophils release nitric oxide (•NO) when they are exposed to appropriate stimuli by activation of a constitutive nitric oxide synthase (cNOS), while inflammatory (emigrated) neutrophils express an inducible nitric oxide synthase [5–8]. Several reports suggest that the nitric oxide pathway interacts with the respiratory burst of activated rat peritoneal neutrophils and other inflammatory cells [9,10]. Simultaneous production of •NO and $O_2^{\bullet-}$ may form peroxynitrite which could mediate nitric oxide-dependent microbe killing by phagocytic cells, as was recently described for rat macrophages [11,12]; it remains to be seen whether this interaction can be reproduced by activated human neutrophils.

In this report, we describe the kinetics of nitric oxide release during respiratory burst of human neutrophils stimulated with PMA, and relate it to the simultaneous production of reactive oxygen species in order to assess possible interactions between these radicals.

2. Materials and methods

2.1. Preparation of human neutrophils

Heparinized venous blood was drawn from healthy volunteers. Human neutrophils were isolated by Ficoll–Hypaque gradient centrifugation, dextran sedimentation and hypotonic lysis of contaminant erythrocytes [13]. The cells were resuspended in 120 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM $Na_2HPO_4 \cdot 12H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 1 mM $MgCl_2$, 1 mM$CaCl_2$, 4.2 mM $NaHCO_3$, 20 mM HEPES and 5.5 mM glucose, pH 7.4 (HBSS). Cell preparations consisted of about 98% of viable neutrophils (Trypan blue test).

2.2. Oxygen consumption

Oxygen consumption was measured with a Clark type oxygen electrode at 30°C [14]. PMN were placed in a thermostatted chamber at 2×10^6 cells/ml in HBSS. The rate of oxygen consumption was calculated from the linear trace initiated by addition of PMA to the incubation medium and expressed in nmol $O_2 \cdot min^{-1} \cdot 10^6$ $cells^{-1}$.

2.3. Hydrogen peroxide generation

Hydrogen peroxide was continuously measured by the horseradish peroxidase (HRP)/*p*-hydroxyphenyl acetic acid (pHPA) assay at 30°C with a Hitachi F 3010 spectrofluorimeter [15]. The reaction medium contained HBSS, 5 U/ml HRP, 40 μM pHPA and 10^6 cells. Hydrogen peroxide generation was calculated from a standard curve made with titrated H_2O_2 solutions and was expressed in nmol · min^{-1} · 10^6 $cells^{-1}$.

2.4. Nitric oxide production

•NO production was measured by the oxidation of oxymyoglobin (oxyMb) to metmyoglobin (metMb) in a double beam-double wavelength 356 Perkin-Elmer spectrophotometer [16]. The absorbance dif-

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ference at 581–593 nm was continuously monitored at 30°C and the rate of •NO formation was calculated by using the extinction coefficient 11.6 $mM^{-1} \cdot cm^{-1}$ and expressed as nmol $\cdot min^{-1} \cdot 10^6$ $cells^{-1}$. The reaction medium contained 20 μM oxyMb, HBSS and 10^6 cells/ml. 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 [17].

The eventual contribution of hydrogen peroxide to oxyMb decay was assayed in the presence of non-stimulated PMN and glucose oxidase/glucose producing 1 nmol H_2O_2/min. OxyMb decay accounted for less than 10% of the rate of H_2O_2 production.

To study possible radical interactions, •NO and H_2O_2 release were measured after preincubation with 1 mM L-NMMA for 30 min.

2.5. Luminol-dependent chemiluminescence

Luminol-dependent chemiluminescence was measured with a C-660 computerized Thorn EMI (UK) photon counter at 30°C [18]. The reaction medium contained HBSS, 5 μM luminol and 5×10^5 cells/ml. To assess a possible contribution of •NO reactions on chemiluminescence, the experiments were repeated with 100 U/ml SOD and 1 mM L-NMMA.

2.6. Materials

All chemicals were purchased from Sigma Chemical Co.

2.7. Statistical analysis

Statistical differences were assessed with paired Student's *t*-test; all differences were considered significant when $P < 0.05$.

3. Results

3.1. Oxygen consumption and hydrogen peroxide production

Upon exposure of human neutrophils to several PMA concentrations, oxygen uptake and H_2O_2 release increased in a dose-dependent manner up to 0.1–1 μg PMA/ml. Oxygen consumption rates increased from the basal unstimulated value (0.56 ± 0.03 nmol $O_2 \cdot min^{-1} \cdot 10^6$ $cells^{-1}$) up to maximal plateau rates at 0.1–1 μg PMA/ml (3.32 ± 0.52 nmol $O_2 \cdot min^{-1} \cdot 10^6$ $cells^{-1}$; Fig. 1).

Hydrogen peroxide production was detected at 0.001 μg PMA/ml and increased up to 1.19 ± 0.17 nmol $H_2O_2 \cdot min^{-1} \cdot 10^6$ $cells^{-1}$ at 0.1–1 μg PMA/ml (Fig. 2). The half-maximal stimulatory effects of oxygen uptake and hydrogen peroxide production were observed between 0.001 and 0.01 μg PMA/ml. Preincubation with or without 1 mM L-NMMA for 30 min at 37°C depressed oxygen uptake but, conversely, increased the H_2O_2 generation rate, $P < 0.05$ (Table 1).

3.2. Nitric oxide production

•NO production by human neutrophils was increased by addition of PMA; the effect was noticeable at very low PMA concentrations (0.0001 μg PMA/ml), and increased up to a maximal rate of 0.56 ± 0.08 nmol NO $\cdot min^{-1} \cdot 10^6$ $cells^{-1}$ at 0.01 μg PMA/ml. The half-maximal effect was observed between 0.0001 and 0.001 μg PMA/ml (Fig. 2). Preincubation with L-NMMA diminished •NO production by about 50%: $P < 0.005$ (Table 1). The addition of 100 U/ml SOD produced an increase in •NO detection of about 30% at 0.1 μg PMA/ml, $P < 0.01$ (Fig. 3).

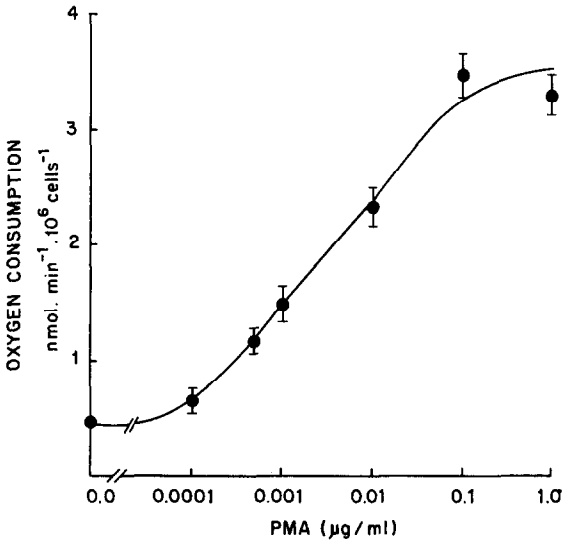

Fig. 1. Oxygen consumption by human neutrophils stimulated with PMA. Values are mean ± S.E.M. from 6 experiments.

3.3. Luminol-induced chemiluminescence

Addition of PMA also increased the luminol-dependent chemiluminescence, reaching a half-maximal effect between 0.001 and 0.01 μg PMA/ml and a maximal rate at 0.1–1 μg PMA/ml (Fig. 2, inset). Preincubation with L-NMMA decreased chemiluminescence yield by 50% (Table 1) and the addition of 100 U SOD/ml further diminished it by 70% (control 111 ± 13 vs. SOD 36 ± 5 cps $\cdot 10^6$ $cells^{-1}$: $P < 0.01$), thus suggesting that the NOS pathway contributed to luminol-sensitized chemiluminescence of activated human neutrophils.

4. Discussion

According to these results, nitric oxide is generated together with superoxide anion and hydrogen peroxide during the respiratory burst of polymorphonuclear leucocytes. However, some differences between the kinetics of •NO and H_2O_2 generation are observed. First, •NO release starts at PMA concentrations lower than those that elicited detectable H_2O_2 increase, with half-maximal effects at about 0.001 μg PMA/ml for •NO and 0.01 μg PMA/ml for oxygen uptake and H_2O_2 production. Thus, it appears to be an early and sensitive indicator of triggering of the respiratory burst by phorbol esters. Additionally, the rate of •NO production and probably the activity of cNOS in neutrophils reached a 'plateau' earlier than NADPH oxidase. There was no further increase at PMA concentrations above 0.01 μg/ml. This strongly suggests an organized and sequential pathway for activa-

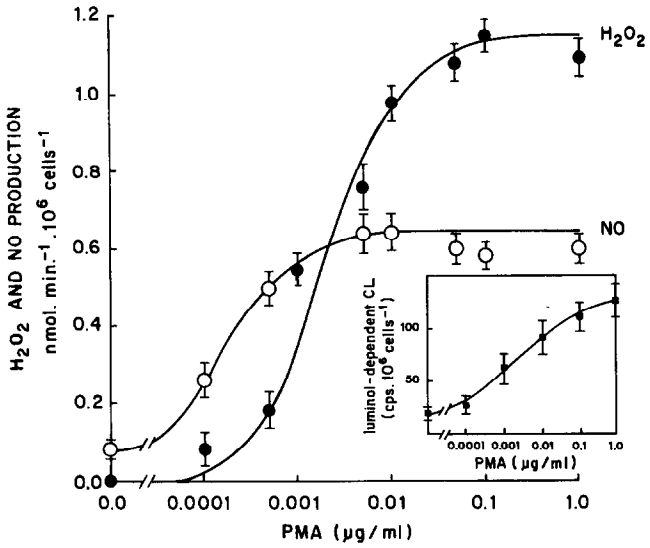

Fig. 2. Nitric oxide and hydrogen peroxide production by human neutrophils stimulated by PMA. Values are mean ± S.E.M. from 5–7 samples. Inset: luminol-dependent chemiluminescence (CL) by human neutrophils stimulated with PMA.

tion of cNOS and NADPH oxidase by protein kinase C stimulators like PMA.

In our experimental conditions, •NO production accounts for about 30% of the total oxygen uptake of maximally stimulated neutrophils. In this line, Ischiroupoulos et al. [11] reported that inhibition of nitric oxide synthesis decreased oxygen uptake by 20% in stimulated macrophages.

Nitric oxide and superoxide radicals can react in a second-order reaction:

$$\text{•NO} + O_2^{\bullet-} \rightarrow ONOO^- \quad (1)$$

In our case, the data seem to indicate that •NO and $O_2^{\bullet-}$ generated simultaneously during the respiratory burst, are kinetically able to react to form peroxynitrite. In agreement with this view, the incubation of PMN with SOD showed that at least 0.2 nmol $\text{•NO} \cdot \text{min}^{-1} \cdot 10^6$ cells^{-1} are converted to peroxynitrite at 0.1 μg PMA/ml (Fig. 3); in the same way, L-NMMA increased H_2O_2 production by about 0.3 nmol $\cdot$ min^{-1} $\cdot$ 10^6 cells^{-1} (Table 1). This rate of peroxynitrite formation (0.2 nmol $\cdot$ min^{-1} $\cdot$ 10^6 cells^{-1}) similar to that reported by Ischiroupoulos et al. for rat alveolar macrophages [11].

Luminol-dependent chemiluminescence by PMN reflects a complex co-oxidative process; excitation of luminol is currently attributed to hypochlorous acid–H_2O_2 [18], but recently, it was shown that $OONO^-$ might contribute to light emission [19,20]. In this system, neither •NO nor $O_2^{\bullet-}$ alone is capable of directly inducing significant chemiluminescence yield [19]. The •NO derived intermediates contribute to luminol chemiluminescence in other cells like PMA-activated Kupffer cells and rat macrophages and may react via $ONOO^-$ [20,21]. In this work, the dependence of chemiluminescence on PMA concentrations (Fig. 2, inset) and, the fact that it was inhibited by L-NMMA and SOD suggest $OONO^-$ formation in the human PMN respiratory burst. In contrast, HOCl should be the main determinant of luminol chemiluminescence in other cells like rat granulocytes where NO is released in minor quantities, if at all [21].

The reaction of $O_2^{\bullet-}$ with •NO yielding peroxynitrite (reaction 1) has been reported to proceed in aqueous solution at a constant rate of 3.7×10^7 $M^{-1} \cdot s^{-1}$ [22]; however, recent evidence suggests that this rate constant might be 200-fold higher, almost diffusion controlled, and similar to that of the gas phase reaction of $HO_2^{\bullet}$ with •NO (6.7×10^9 $M^{-1} \cdot s^{-1}$) [23]. Considering this value, it is apparent that all •NO generated by neutrophils should be converted to $ONOO^-$ so that, from 0.01 μg PMA/ml, $O_2^{\bullet-}$ and H_2O_2 production rates should determine the maximal $ONOO^-$ levels. Nevertheless, it is possible that the rate constant for $ONOO^-$ formation is somewhat lower in biological systems; indeed, SOD catalyzed dismutation of $O_2^{\bullet-}$ to H_2O_2 with a rate constant of 2×10^9 $M^{-1} \cdot s^{-1}$ [24] competes with reaction 1 (Fig. 3). Possible discrepancies between total •NO and $ONOO^-$ formation with human neutrophils could be due to the prompt conversion of $ONOO^-$ to •NO_2 and •OH or to NO_3^-; Radi et al. reported rapid exponential decay of $ONOO^-$ induced chemiluminescence following first-order kinetics [19]. Other reactions such as conversion of •NO to NO^- by SOD or NADPH oxidase could also affect final $ONOO^-$ levels [11,16].

The biological significance of the simultaneous production of $O_2^{\bullet-}$ and •NO in PMN is not clear at the present time. However, Malawista et al. [25] showed that reactive nitrogen intermediates participate in the killing of staphylococci by human neutrophil cytoplasts; Marletta et al.

Table 1
Effect of L-NMMA on oxygen consumption, •NO and H_2O_2 production and luminol-dependent chemiluminescence by PMA-stimulated human neutrophils

	PMA (μg/ml)	− L-NMMA	+ 1 mM L-NMMA
Oxygen consumption (nmol/min/10^6 cells)	–	0.56 ± 0.03	0.50 ± 0.05
	0.1	3.98 ± 0.27	2.97 ± 0.28*
NO production (nmol/min/10^6 cells)	–	0.08 ± 0.01	0.05 ± 0.01
	0.1	0.58 ± 0.04	0.28 ± 0.04*
H_2O_2 production (nmol/min/10^6 cells)	–	ND	ND
	0.1	1.19 ± 0.17	1.52 ± 0.19*
Luminol-dependent chemiluminescence (cps/10^6 cells)	–	21 ± 2	12 ± 2
	0.1	111 ± 13	59 ± 5***

The PMN were preincubated for 30 min at 37°C with or without 1 mM L-NMMA. Values are mean ± S.E.M. of 4–6 experiments. ND, not detectable.
*$P < 0.05$; **$P < 0.005$; ***$P < 0.025$.

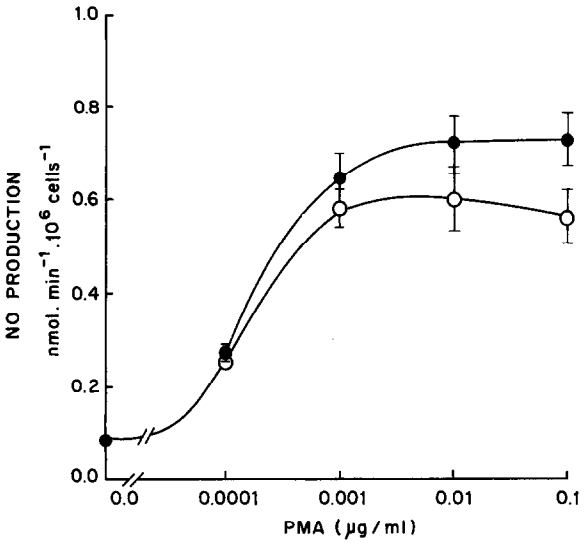

Fig. 3. Effect of superoxide dismutase on nitric oxide detection by PMA stimulated human neutrophils. Values are mean ± S.E.M. from 3–4 samples with (filled circles) or without 100 U SOD/ml (open circles).

[26] related •NO production to microbicidal and tumoricidal activity of macrophages; Zhu et al. [12] showed the bactericidal activity of peroxynitrite on *Escherichia coli* cultures and Radi et al. [27] demonstrated the ability of peroxynitrite to kill *T. cruzi*. Moreover, Mulligan et al. [28–30] showed that inhibition of nitric oxide synthesis with L-NMMA reduced inmune complex-induced injury.

Finally, we suggest that •NO production by cNOS is a part of the integrated neutrophil response and that the kinetics of simultaneous •NO and $O_2^{\bullet-}$ production favour the formation of peroxynitrite. This process, a general feature of the chemistry of phagocytes, can be involved either in defence mechanisms or in neutrophil-mediated tissue injury.

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