

Activated human neutrophils rapidly break down nitric oxide

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Received 8 September 1997; revised version received 8 October 1997

Abstract Isolated human neutrophils produced no detectable (<10 nM) nitric oxide (NO) before or after activation with phorbol 12-myristate 13-acetate (PMA) or a chemotactic peptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine. Physiological levels of NO (1 μ M) added before or after neutrophil activation had no effect on their respiratory burst oxygen consumption. Neutrophils activated with PMA caused very rapid breakdown of exogenously added NO. NO breakdown rates recorded at 250 nM NO were 0.09 ± 0.02 and 3.77 ± 0.23 nmol NO/min/ 10^6 cells ($n=3$) before and after activation respectively and addition of copper-zinc superoxide dismutase during activation significantly decreased this rate (1.06 ± 0.09 nmol NO/min/ 10^6 cells ($n=3$)), suggesting that superoxide (O_2^-) production was mainly responsible for the NO breakdown. These results suggest that activation of human neutrophils *in vivo* will dramatically decrease surrounding NO levels, potentially causing vasoconstriction, platelet aggregation and adhesion and peroxynitrite ($ONOO^-$) formation.

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Key words: Neutrophils; Nitric oxide; Respiratory burst; Superoxide

1. Introduction

Neutrophils are by far the most abundant leukocytes ($>70\%$) in the human immune system. It has been well documented that human neutrophils can be activated by very low levels of various stimuli such as phorbol 12-myristate 13-acetate (PMA) [1] or chemotactic peptides such as *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) [2]. During activation of human neutrophils, cellular oxygen consumption increases severalfold [3,4], known as the respiratory burst, and results in the production of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) [5].

Recently it has been suggested that during the respiratory burst of human neutrophils, nitric oxide (NO) is produced via activation of a constitutive nitric oxide synthase (cNOS) [1]. However, there are conflicting reports concerning NO production in that some studies have not been able to detect any NOS activity in human neutrophils, including NO production from inducible nitric oxide synthase [6,7]. NO has also been reported to inhibit the respiratory burst [8]. Since activated neutrophils produce high levels of O_2^- , which reacts rapidly with NO to give peroxynitrite ($ONOO^-$) [9], it is possible that activated neutrophils may decrease the ambient level of NO rather than increase it.

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Abbreviations: cNOS, constitutive nitric oxide synthase; Cu/Zn-SOD, copper/zinc superoxide dismutase; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PMA, phorbol 12-myristate 13-acetate

The present work aims to determine: (a) whether human neutrophils produce detectable levels of NO, (b) whether NO inhibits the respiratory burst, and (c) whether activated neutrophils can cause significant levels of NO breakdown.

2. Materials and methods

2.1. Preparation of human neutrophils

Venous blood (18 ml) was drawn from a healthy male volunteer and mixed with 2 ml trisodium citrate (3.8%) and 400 μ l T500 dextran (6%). Erythrocytes were allowed to sediment for 45 min and the serum (approximately 8 ml) removed. The serum was diluted to 20 ml with a calcium and magnesium free buffer consisting of: 5.0 mM EDTA, 5.6 mM glucose, 10 mM HEPES, 5.4 mM KCl, 137 mM NaCl, pH 7.4 (CMF-E). The diluted serum was then centrifuged at $400 \times g$ for 5 min. The pellet was resuspended in 10 ml CMF-E and layered on a Percoll gradient (6 ml 54% Percoll and 4 ml 78% Percoll) and centrifuged at $400 \times g$ for 30 min. 100% Percoll contained 150 mM NaCl. Neutrophils were isolated as a distinct band at the 54% Percoll:78% Percoll interface. Neutrophils were washed twice with CMF-E to remove Percoll and then stained with either Leishman's or Wright's stain. Cell preparations consisted of about 95% of viable neutrophils (trypan blue test). Neutrophils were stored at room temperature in an aerated vessel containing CMF-E at approximately 2×10^7 cells/ml.

2.2. Preparation of NO

NO-saturated water was prepared by purging a gas-tight glass vial filled with distilled de-ionised water with nitrogen for approximately 20 min and then bubbling pure NO gas through the deoxygenated vial for a further 15 min or until saturation. The concentration of NO in NO-saturated water was taken as 2.0 mM at 20°C [10].

2.3. NO and oxygen consumption measurements

Measurement of NO levels were performed using a Clark-type NO electrode (World Precision Instruments) (based on [11]) inserted through the top of a Clark-type oxygen electrode, permitting simultaneous measurement of NO and oxygen. Both electrodes were connected to a chart recorder. Experiments were performed in a Krebs-HEPES buffer consisting of: 1.5 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.1 mM $MgSO_4$, 118 mM NaCl, pH 7.4 at 37°C, stirred continuously at a constant speed.

Neutrophils (approximately 1×10^6 cells) were activated with either PMA (10 ng/ml) or FMLP (4 μ g/ml) and the rate of oxygen consumption was calculated before and after the addition of PMA to the oxygen electrode vessel chamber and expressed in nmol O_2 /min/ 10^6 cells.

Aliquots of 1 μ M NO were added by injecting an aliquot of NO-saturated water (see above) into the incubation chamber in triplicate before and after neutrophil stimulation by PMA or FMLP and also after Cu/Zn-SOD (10 μ g/ml) addition. NO breakdown rate was calculated in units of nmol NO/min. The effect of a steady-state level of 1 μ M NO for 10 min (achieved by adding small aliquots of NO to the incubation chamber – measured by the NO electrode) before neutrophil activation on respiratory burst oxygen consumption was also tested.

2.4. Materials

Percoll and T500 dextran were purchased from Pharmacia. NO gas was from MG Gas Products. Cu/Zn-SOD was from bovine kidney, suspended in 3.8 M $(NH_4)_2SO_4$, pH 7.0 and bought from Sigma Chemical Co. All other chemicals were from Sigma Chemical Co.

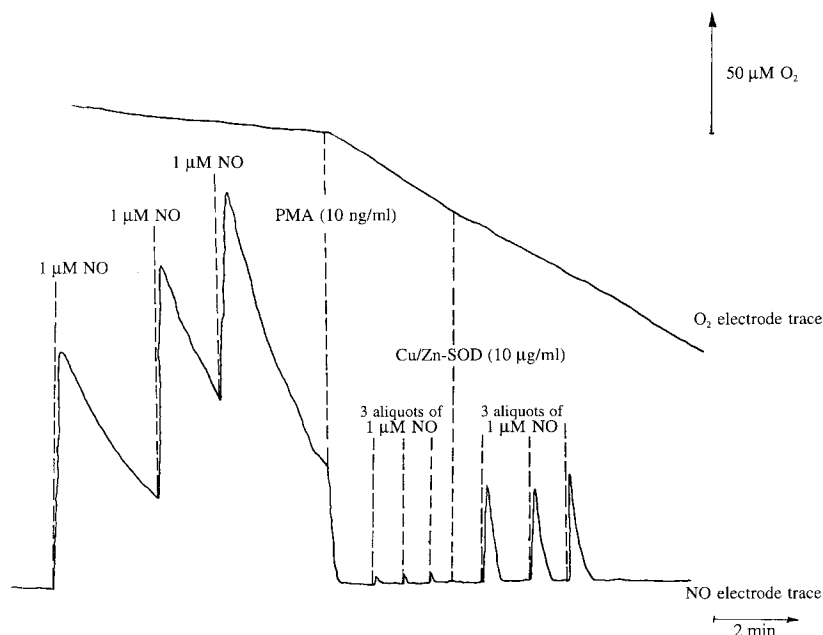


Fig. 1. Activated human neutrophils cause rapid NO breakdown. Neutrophils (approximately 1×10^6 cells/ml) were incubated with an oxygen electrode (upper trace) and NO electrode (lower trace) to simultaneously measure oxygen and NO levels. Aliquots of $1 \mu\text{M}$ NO were added followed by 10 ng/ml PMA, followed by three aliquots of $1 \mu\text{M}$ NO, followed by $10 \mu\text{g/ml}$ Cu/Zn-SOD, followed by another three $1 \mu\text{M}$ NO aliquots. NO breakdown (lower trace) is greatly increased by PMA (10 ng/ml) addition to neutrophils and this fast rate is decreased by the presence of Cu/Zn-SOD ($10 \mu\text{g/ml}$). PMA also causes an increase in the oxygen consumption (upper trace) of neutrophils (known as the respiratory burst). Experiments were performed using approximately 10^6 cells/ml in Krebs-HEPES medium, pH 7.4 at 37°C .

3. Results

3.1. NO production by neutrophils

NO was not detected before or after PMA ($1\text{--}10 \text{ ng/ml}$) stimulation of neutrophils ($1\text{--}2 \times 10^6$ cells) in the presence of 5 mM L-arginine. Cu/Zn-SOD ($10 \mu\text{g/ml}$) was also added to activated neutrophils to reduce the number of free O_2^- radicals, and also $0.1 \mu\text{M}$ catalase in order to break down any H_2O_2 produced, but no NO production was detected. Neutrophils were also treated with a calcium ionophore, A23187 ($0.1\text{--}1 \mu\text{M}$), in order to increase intracellular calcium levels. A23187 addition before or after neutrophil activation with PMA did not cause measurable NO production. Also, A23187 had no effect on the oxygen consumption of neutrophils before or after activation. Staurosporine ($10\text{--}100 \text{ nM}$), a protein kinase inhibitor, was also added to neutrophils before or after activation. Protein kinases can cause inhibition of NOS by phosphorylation however including staurosporine did not permit measurable NO production. NO production was not detected either when FMLP ($4 \mu\text{g/ml}$) was used to activate neutrophils.

3.2. Neutrophil oxygen consumption and the effect of NO on the neutrophil respiratory burst

PMA (10 ng/ml) was found to increase oxygen consumption of human neutrophils (approximately 1×10^6 cells) from a basal level of $1.35 \pm 0.01 \text{ nmol O}_2/\text{min}/10^6$ cells to $6.80 \pm 0.36 \text{ nmol O}_2/\text{min}/10^6$ cells ($n=6$). The increase in oxygen consumption was observed within the response time of the oxygen electrode (about 5 s) (Fig. 1). PMA (1 ng/ml) did not cause a measurable increase in oxygen consumption (result not shown). In order to investigate whether NO inhibits the respiratory burst, NO was added either before or during the burst, and the oxygen consumption rate measured during the burst. Prior to activation of the neutrophils, the NO level in the reaction medium was maintained at close to $1 \mu\text{M}$ NO for 10 min by adding aliquots of NO to the vessel (similar to Fig. 1). A steady-state level of $1 \mu\text{M}$ NO for 10 min prior to activation did not affect the oxygen consumption during the respiratory burst of neutrophils (approximately 1×10^6 cells) activated with PMA (10 ng/ml). Respiratory burst oxygen consumption rates were $6.88 \pm 0.36 \text{ nmol O}_2/\text{min}/10^6$ cells after treatment of neutrophils with $1 \mu\text{M}$ NO for 10 min .

Table 1

Rate of NO decay before neutrophil activation with PMA (10 ng/ml) and after activation and also in the presence of Cu/Zn-SOD ($10 \mu\text{g/ml}$)

	NO decay rate at 250 nM NO ($\text{nmol NO}/\text{min}/10^6$ cells)	S.D.	<i>n</i>
Before PMA addition	0.09	0.02	3
After PMA addition	3.77	0.23	3
After PMA and SOD addition	1.06	0.09	3

Experiments were performed using approximately 1×10^6 cells/ml in Krebs-HEPES medium, pH 7.4 at 37°C . Results expressed as mean \pm S.D. where n = number of experiments.

and 6.74 ± 0.44 nmol O_2 /min/ 10^6 cells for control neutrophils ($n=3$). Also, addition of up to five $1 \mu M$ aliquots of NO during the respiratory burst had no effect on the oxygen consumption rate of activated neutrophils (Fig. 1).

3.3. NO breakdown by neutrophils

We tested whether neutrophils could cause a significant rate of NO breakdown, either before or after activation, by adding an aliquot of NO to the medium and following its rate of breakdown with the NO electrode. Prior to activation, neutrophils did not significantly increase the rate of NO breakdown compared to medium alone. Activated human neutrophils caused rapid breakdown of exogenously added NO (Fig. 1). Rapid NO decay was observed immediately after adding PMA (10 ng/ml) or FMLP (4 μg /ml) to neutrophils. All NO additions were made at approximately 150–200 μM O_2 . The decay rate of NO added before and after neutrophil stimulation by PMA and also after Cu/Zn-SOD (10 μg /ml) addition was calculated at a level of 250 nM NO and expressed in terms of nmol NO/min/ml (Table 1). Cu/Zn-SOD (10 μg /ml) decreased the NO breakdown rate during the respiratory burst of neutrophils. PMA itself (in the absence of neutrophils) did not affect the breakdown rate of added NO. When FMLP (4 μg /ml) was used instead of PMA as an activator of the respiratory burst, it caused a similar increase in the rate of NO breakdown by neutrophils (result not shown). NO reacts with O_2^- to yield $ONOO^-$ [9] and $ONOO^-$ is known to react with other biomolecules to generate NO donors [12]. However, this reaction is likely to be slow and delayed and because of the high levels of O_2^- produced by activated neutrophils, it is unlikely that we would have detected any secondary NO production.

4. Discussion

NO production from macrophages is well documented (reviewed in [13]) and has recently been reported in human macrophages [14,15]. However, production of NO from neutrophils, the main leukocyte type and certainly the major cell type at early stages of inflammation, is less clearly understood. Certainly inducible NOS (i-NOS) activity has been observed in rodent neutrophils by activating the cells with various agents such as interferon- γ , tumour necrosis factor α and lipopolysaccharide [16,17]. NO production from i-NOS in human neutrophils is generally unclear, although recently it has been demonstrated in neutrophils from the human urinary tract [18]. Some studies have failed to show any NO production even after cytokine treatment of human neutrophils [6,7]. Yet recently, it has been reported that NO production from cNOS was detected during the respiratory burst of human neutrophils activated with PMA [1]. At saturating PMA concentrations, NO production was 0.56 ± 0.04 nmol/min/ 10^6 cells, measured by the oxidation of oxymyoglobin to metmyoglobin.

In the present work, we were unable to detect measurable levels of NO from human neutrophils, before or after activation with PMA. We used an NO electrode for NO assay, which can detect NO levels above about 10 nM. Half-maximal NO production was observed in [1] at about 1 ng PMA/ml; however, we did not observe any NO over the range 1–10 ng PMA/ml. NO was not measurable even in the presence of Cu/Zn-SOD and low levels of catalase; these were included be-

cause it has also been shown that O_2^- and H_2O_2 are produced during the respiratory burst of human neutrophils [5,19]. However, it remains possible that we did not detect NO with the electrode in the presence of PMA because the rate of NO breakdown is so high, even in the presence of SOD. Thus we tried to stimulate cNOS in the absence of PMA activation, by adding the calcium ionophore A23187 and the protein kinase inhibitor staurosporine, both of which have been reported to stimulate cNOS activity in cells [20]. However, neither agent caused detectable NO release, thus if human neutrophils do produce NO, the rate must be low.

Recent work indicates that NO reversibly inhibits the respiratory burst of PMA-activated neutrophils [8], so we tested the effect of a sustained level of $1 \mu M$ NO for 10 min on the respiratory burst of human neutrophils. This treatment had no effect on the neutrophil respiratory burst. In [8], neutrophils were treated with high micromolar aliquots of NO. These levels of NO will not exist for more than a fraction of a second because in an oxygenated medium, the NO will rapidly react with oxygen to give NO_2 and N_2O_3 [21] and these strongly oxidising species might have been responsible for the inhibition observed in [8]. We used a level of NO (1 μM) which is at the top end of the physiological or pathological range, but which does not give rise to significant levels of NO_2 or N_2O_3 . We did not see any inhibition of the respiratory burst under these conditions and we conclude that such inhibition is unlikely to occur in vivo.

We found that neutrophils activated with PMA cause very rapid breakdown of exogenously added NO. The fact that Cu/Zn-SOD caused a large decrease in the NO breakdown rate by activated neutrophils suggests that NO rapidly reacts with O_2^- radicals produced during activation. If all the extra oxygen consumption during activation (5.45 nmol O_2 /min/ 10^6 cells) was used to produce O_2^- , this rate would be sufficient to quantitatively account for the activated rate of NO breakdown (3.77 nmol NO/min/ 10^6 cells).

If neutrophils do not produce significant levels of NO, but rather activated neutrophils cause rapid breakdown of exogenous NO, then activation of neutrophils in vivo may significantly decrease tissue NO levels. This decrease in NO levels would promote vasoconstriction and platelet aggregation and adhesion, and inhibit inflammation. This regulatory role might be a significant physiological function of the respiratory burst O_2^- production. During inflammation, high levels of activated neutrophils enter the inflamed tissue, due to local vasodilation and increased endothelial permeability, caused in part by increased NO production [22]. The increased NO breakdown due to the presence of activated neutrophils might function as a feedback to inhibit further inflammation. The production of $ONOO^-$ accompanying NO breakdown might contribute to the cytotoxicity of neutrophils [1].

In conclusion, the present study indicates that activated human neutrophils cause rapid breakdown of added NO, probably via the reaction of NO with O_2^- produced during the respiratory burst. This suggests the possibility that human neutrophil activation might significantly decrease in vivo NO levels and thus might regulate vasodilation, inflammation and other NO-regulated functions.

Acknowledgements: Thanks to Dr H. Harris (Department of Biochemistry, Cambridge) for help in the isolation of neutrophils and for the gift of T500 dextran. Also to Miss G.E. Browne (Department of Pathology, Cambridge) for technical assistance in taking blood

samples and to Mrs G.A. King (Department of Anatomy, Cambridge) for advice on neutrophil staining. This work was sponsored by the Biotechnology and Biological Sciences Research Council and the Royal Society (London).

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