

Nitrous Oxide Impairs the Neutrophil Oxidative Response

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Background: Nitrous oxide has been shown inconsistently to impair the oxidative function of neutrophils. The choice of the stimulus, receptor agonists, or stimuli acting independent of receptors seems to determine whether nitrous oxide impairs the oxidative functions, suggesting an interference with the cytosolic signaling of neutrophils.

Methods: Production of hydrogen peroxide by neutrophils was assessed using flow cytometric analysis. N-formyl-methionyl-leucyl-phenylalanine (FMLP), C5a, dioctanoylglycerol, and phorbol-12-myristate-13-acetate were used as stimuli. In addition, the expression of receptors for FMLP and the cytosolic-free calcium response of cells were measured.

Results: Nitrous oxide depresses C5a- or FMLP-induced generation of reactive oxygen derivatives in a concentration-dependent manner. However, the response with direct activation of protein kinase C was unaffected. Further, the number of FMLP receptors and the cytosolic calcium response were unaffected.

Inhibition of the oxidative response was not reversible within the observation period of 4 h.

Conclusions: Nitrous oxide inhibited the intracellular signaling of the investigated G-protein-coupled receptors for chemotactic peptides. No interference of nitrous oxide with reduced nicotinamide adenine dinucleotide phosphate oxidase, the oxidative enzyme system of neutrophils, nor with its activation through protein kinase C was detected. (Key words: C5a; N-formyl-methionyl-leucyl-phenylalanine; respiratory burst; second messengers.)

POLYMORPHONUCLEAR neutrophils are a major component of the nonspecific cell-mediated immune response.¹ A crucial mechanism of defense is the generation of reactive oxygen derivatives.

Data on the effects of nitrous oxide (N₂O) on the motility and microbicidal oxidative activity of neutrophils are controversial. Some authors, using receptor agonists,^{2,3} observed the inhibitory effects of N₂O, whereas others, using intracellularly acting stimuli,^{4,5} have not. Presumably, the choice of the stimulus used to activate neutrophils determines whether N₂O exerts an inhibitory effect. This suggests an interference of N₂O with neutrophil signal reception or transduction.

To characterize the mechanism of interference, this *in vitro* study investigated the effect of 30% and 70% N₂O on the generation of reactive oxygen derivatives in human neutrophils with respect to the signaling pathways used. The flow cytometric assay used for this study allows the quantification of the oxidative response to stimulation of multiple neutrophils at the cellular level.⁶

To localize potential sites of the N₂O interactions, several stimuli were used to initiate neutrophil responses. The bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) and a product of the complement cascade, C5a, both potent chemoattractants for neutrophils, were used to induce hydrogen peroxide (H₂O₂) generation by neutrophils.⁷ Both substances are agonists of specific G-protein-coupled receptors and share the same second messenger pathway (fig.

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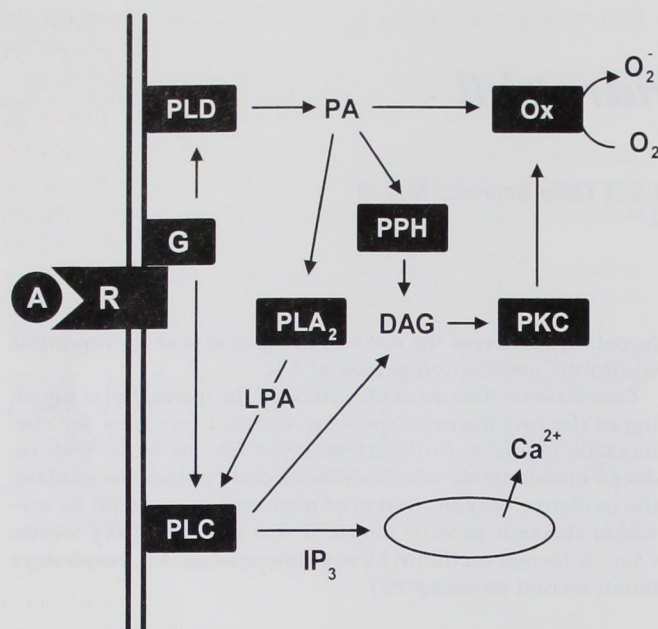


Fig. 1. Signaling pathway of neutrophils linking G-protein-coupled receptors to the enzyme system generating reactive oxygen products. The agonist (A), in this study C5a or FMLP, binds to the specific receptor (R), which transduces the signal via the coupled G protein (G) to phospholipase C (PLC) and phospholipase D (PLD). PLC generates the second messengers, inositol-trisphosphate (IP₃) and diacylglycerol (DAG), from phosphatidylinositolbisphosphate. IP₃ liberates Ca²⁺ from intracellular stores. PLD generates phosphatidic acid (PA). PA activates NADPH-oxidase (Ox). PA is transformed by phosphatidatephosphohydrolase (PPH) to DAG and by phospholipase A₂ (PLA₂) to lysophosphatidic acid. DAG activates protein kinase C (PKC), leading finally to the initialization of the superoxide anion production by Ox. Lysophosphatidic acid can initiate the generation of IP₃ and DAG by activating PLC.

1). Binding of a fluorescent receptor ligand to FMLP receptors was used to analyze changes in receptor numbers. The increase in cytosolic-free Ca²⁺ [Ca²⁺]_i, after stimulation with FMLP or C5a, was assessed for further analysis of receptor function.⁸ Finally, the receptors and parts of the second messenger cascade were bypassed through induction of an oxidative response by membrane-permeable dioctanoylglycerol (DiC8-DAG) and phorbol-13-myristate-12-acetate (PMA).^{9,10} Both DiC8-DAG and PMA are synthetic analogs of diacylglycerol (DAG), a compound of the signaling pathway, and induce the oxidative response by direct activation of protein kinase C, an enzyme in the neutrophil signaling chain downstream of receptors and second messengers but upstream of the enzyme system responsible for the generation of reactive oxygen products.⁷

Materials and Methods

Leukocyte Preparation

This study was approved by the local ethical board of the University of Regensburg Medical School. After informed consent, venous blood was drawn from healthy donors with no history of infections 2 weeks before the experiments. The mean age of the donors (n = 6) was 32 yr, with a range of 27–38 yr. Average leukocyte counts were 6,300 ± 1,200/μl (mean ± SD), and hemoglobin levels were 15.1 ± 1.3 g/dl. The differential leukocyte counts were as follows: neutrophils, 53 ± 8%; lymphocytes, 32 ± 7%; monocytes, 8 ± 3%; eosinophils, 3 ± 2%; and basophils, 1 ± 1%. The complete blood count and the differential count were determined before each experiment using a Technicon H*3 counter (Bayer, Tarrytown, NY). No donors had to be excluded because of abnormal blood counts.

Leukocytes were isolated by a sedimentation of erythrocytes on Ficoll-Paque (Pharmacia, Upsalla, Sweden). Heparinized (10 U/ml) whole blood (3 ml) was layered on 3 ml lymphocyte separation medium (Ficoll; density, 1.077 g/ml). Erythrocytes aggregated at the interface and settled at room temperature without centrifugation. After 40 min, the upper 800 μl of the supernatant leukocyte-rich plasma was withdrawn, with care taken to avoid contact with that part of the plasma close to the interface with the separation medium. To avoid artifactual activation of cells, the isolation process did not involve lysis, centrifugation, or washing procedures.

Intracellular Hydrogen Peroxide Production

Nitrous oxide (Linde, Unterschleißheim, Germany) was tested *in vitro* with neutrophils from each of the six donors. The oxygen content of fresh gas was maintained at 30% in all samples (control, 30 vol%, and 70 vol%). The assay for the intracellular H₂O₂ production was done in an airtight glovebox with a volume of about 75 l. Fresh gas was prepared using a gas-mixing unit (Draeger, Lübeck, Germany). Fresh gas flow, after equilibration of the atmosphere inside the box using high flow rates, was 5 l/min during the experiments. Inside the glovebox, the samples were kept at 37°C. To keep the amount of evaporated water from the samples as low as possible, the tubes were sealed with gas-permeable parafilm during incubation. Nitrous oxide delivery under the hood was monitored continually using a multigas-analyzer (Capnomac Ultima; Datex, Helsinki, Finland).

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Before the propylene-tubes were filled and the leukocytes added, Dulbecco's PBS (Gibco-Life Technologies, Heidelberg, Germany) was equilibrated with N_2O inside the glovebox by bubbling fresh gas through for 30 min at 37°C. The supernatant leukocyte-rich plasma was suspended 1:50 (20 μ l leukocytes and 980 μ l PBS) in pre-equilibrated Dulbecco's PBS and incubated for 20 min at 37°C. The leukocytes were loaded in the presence of N_2O with the fluorogenic substrates dihydrorhodamine 123 and carboxy-seminaphthorhodafluor-1-acetoxymethylester (SNARF1/AM) for 10 min (both dyes from Molecular Probes, Eugene, OR). The overall exposure time to N_2O before stimulation was 30 min. The final concentrations were 1 μ M for dihydrorhodamine 123 and 0.1 μ M for SNARF1/AM. Next FMLP, C5a, DiC8-DAG, or PMA, respectively, were added to stimulate the H_2O_2 production of neutrophils (all from Sigma Chemical Co., Deisenhofen, Germany). After 15 min of incubation at 37°C, the reaction was stopped on ice. Dead cells were counterstained with propidium iodide (PI; Serva, Heidelberg, Germany) at a final concentration of 30 μ M. The specimens were stored on ice in complete darkness and measured within 1 h. To evaluate the recovery of neutrophil function, samples of unexposed and exposed cells (70% N_2O) were allowed to recover for varying periods of time before initial stimulation: during exposure, 1 h, 2 h, and finally 4 h after the end of exposure.

For analysis, we used a FACScan flow cytometer (Becton Dickinson, San Jose, CA) with argon ion laser excitation at 488 nm, with 10,000 cells of each stained sample measured. Data were acquired and processed using LYSIS-II software. After calibration with standard dye beads (Quantum 26; Flow Cytometry Standards Europe, Leiden, The Netherlands), the results of the cellular fluorescence study were expressed in molecule equivalents of soluble fluorochrome. These units were used for the absolute quantification of cellular fluorescence and allowed interassay and interlaboratory comparison of data. The dead cells were assessed by their lack of esterase activity and their propidium iodide fluorescence (>600 nm). Leukocyte esterase activity was determined based on SNARF1-related orange fluorescence.⁶ The SNARF1/AM is cleaved in vital leukocytes by esterases to SNARF1. Neutrophils were identified by their typical side-scatter and forward scatter light patterns as well as their esterase activity. Side scatter depends primarily on the granularity of cells, whereas forward scatter is related to cell size.

Expression of Receptors for FMLP

We used the assay established by Allen *et al.*¹¹ without modification. Briefly, leukocyte-rich plasma suspended in Dulbecco's PBS (1:50) was incubated for 30 min in the presence of N_2O at 37°C under the same conditions as described for the H_2O_2 assay. Subsequently, the samples were cooled to 4°C to avoid further activation of neutrophils. For staining of FMLP receptors, fluorescein-labeled formyl-Nle-Leu-Phe-Nle-Tyr-Lys was added in a final concentration of 100 nM, incubated for 10 min, and washed twice. Samples were kept in the N_2O atmosphere during the staining and measuring procedure. Formyl-Nle-Leu-Phe-Nle-Tyr-Lys, a highly specific binding analog of FMLP, emitting light at 520 nm when excited at 488 nm (Molecular Probes). Neutrophils were identified based on side scatter. Nonspecific binding of formyl-Nle-Leu-Phe-Nle-Tyr-Lys was determined by adding excess FMLP (10^{-4} M) in a parallel set of tubes.

Increase in Cytosolic-free Calcium

The increase in $[Ca^{2+}]_i$ was assessed using flow cytometric analysis combined with the fluorescent calcium indicator fluo-3/acetoxymethylester (fluo3/AM; Molecular Probes).¹² The fluorescence of this 488-nm excitable indicator is enhanced in the presence of Ca^{2+} . Leukocytes were suspended in N_2O -equilibrated Dulbecco's PBS containing 2 μ M fluo-3/AM and incubated for 25 min in the glovebox described previously. Separate samples were prepared for controls and different concentrations of N_2O . Thereafter, the tubes were sealed with gas-tight tape and immediately connected to the flow cytometer. After 60 s of recording, the stimuli, FMLP (100 nM), or C5a (200 pM) were added, and the measurement was continued for 240 s. Subsequently, ionomycin (2 μ M) was added. Ionomycin (Calbiochem, Bad Soden, Germany), a calcium ionophore, was used to saturate the intracellular indicator with Ca^{2+} to standardize the analysis, independent of dye loading. The resulting increased fluorescence was recorded for 1 min.

Gas Chromatography and Mass Spectrometry

The volatility of the investigated agent may make the maintenance of stable, reproducible drug concentrations in the fluid phase of the experiment difficult. In a separate set of experiments, concentrations of N_2O in the fluid and gas phases were analyzed by gas chromatography and high-resolution mass spectrometry, using as gas chromatograph the HRGC Mega 2 (Fisons Instruments SpA, Rodano, Italy). The molecular ion of N_2O

was recorded by single-ion monitoring, using the molecular ion of carbon dioxide as a reference mass; the cycle time was 0.2 s. Quantification was based on signal areas in the mass chromatograms. The gas chromatography and high-resolution mass spectrometry measurements showed that the equilibration with N₂O of the buffer solution was complete after 5 min with a buffer/gas partition coefficient of 0.423 and a concentration of 13.4 ± 1.2 mM in the fluid with 70% N₂O at 37°C. The concentrations of N₂O during experiments were stable. Ten minutes after withdrawal of N₂O, the concentration decreased to <2% of the initial values.

Statistical Analysis

All experimental data are presented as mean values with standard deviations. Lilliefors' test was used to examine normal distribution, Levene's test to check homogeneity of variance, and Student's *t* test to check differences between N₂O-exposed and control samples assessed in parallel. To test differences between different N₂O concentrations, analysis of variance and the Tukey B test were computed. Statistical significance was assumed when probability values were <0.05.

Results

Hydrogen Peroxide Production of Neutrophils

The small amounts of intracellular H₂O₂ generated after stimulation could be assessed by quantifying the intracellular oxidation of the indicator dye dihydrorhodamine 123 to rhodamine 123. The former is a nonfluorescent and membrane-permeable fluorogenic substrate, whereas the latter oxidation product emits a green light (510–530 nm) on excitation. This method has several advantages over those previously used to quantify neutrophil oxidative function. Purification of neutrophils, which often leads to artifactual activation of cells, is unnecessary. Flow cytometric results are not affected by variations in the concentration of neutrophils in the assay because the oxidative response is analyzed on the single-cell level. Flow cytometric analysis revealed a heterogeneous oxidative response after receptor-dependent stimulation with either FMLP (100 nM) or C5a (200 pM). Only approximately one half of all neutrophils produced reactive oxygen products, such as H₂O₂, at this degree of stimulation (fig. 2, table 1).

In the presence of N₂O, the oxidative response to either FMLP or C5a stimulation decreased in a concen-

tration-dependent manner (fig. 3 and table 1). This effect was characterized by a dose-dependent reduction in the number of neutrophils recruited for the oxidative response, in close correlation with a decrease of the total oxidative activity (fig. 3, table 1).

The protein kinase C (PKC) activators, DiC8-DAG and PMA, were used to bypass the receptor and the downstream intracellular signaling. Both substances elicit the H₂O₂ production through a direct activation of PKC. The reaction of neutrophils in response to both stimuli was homogeneous. DiC8-DAG (7.5 μM) induced a mean oxidative response intensity comparable with that of either FMLP (100 nM) or C5a (200 pM), whereas PMA produced a much higher response. The presence of N₂O had no effect on the oxidative response after direct activation of PKC with either DiC8-DAG or PMA. The percentage of dead cells was always less than 0.1% independent of drug exposure or stimulation. Less than 5% of neutrophils in control samples displayed a spontaneous oxidative response, indicating a low degree of artifactual activation of neutrophils by the leukocyte preparation and by the assay procedures.

Expression of Receptors for FMLP

To exclude changes in receptor number, the FMLP receptors of neutrophils were stained in the presence of N₂O using a fluorescent ligand. The exposure time of neutrophils to N₂O for these experiments was similar to that of the H₂O₂ assays, and N₂O was present during the staining procedure to exclude potential competition of N₂O and the fluorescent receptor ligand, formyl-Nle-Leu-Phe-Nle-Tyr-Lys, at the binding site of the FMLP receptor. Under these conditions, N₂O had no effect on the expression of FMLP receptors on the cell surface. Neutrophils exposed to N₂O expressed $39,200 \pm 3,200$ receptors (*n* = 6) for FMLP per cell compared with $37,500 \pm 3,300$ receptors (*n* = 6) on neutrophils of the control samples, indicating no significant interference of N₂O with receptor expression.

Cytosolic-free Calcium Increase

Early steps of signaling, leading to the induction of the oxidative response, were further analyzed based on the increase of cytosolic-free calcium [Ca²⁺]_i on stimulation. Both tested receptor agonists, C5a (200 pM) and FMLP (100 nM), induced a similar [Ca²⁺]_i response (table 2). Cytosolic-free Ca²⁺ was maximal after 30.3 ± 3.1 s of stimulation in N₂O exposed cells and after 33 ± 2.7 s in control neutrophils. The time course of this reaction

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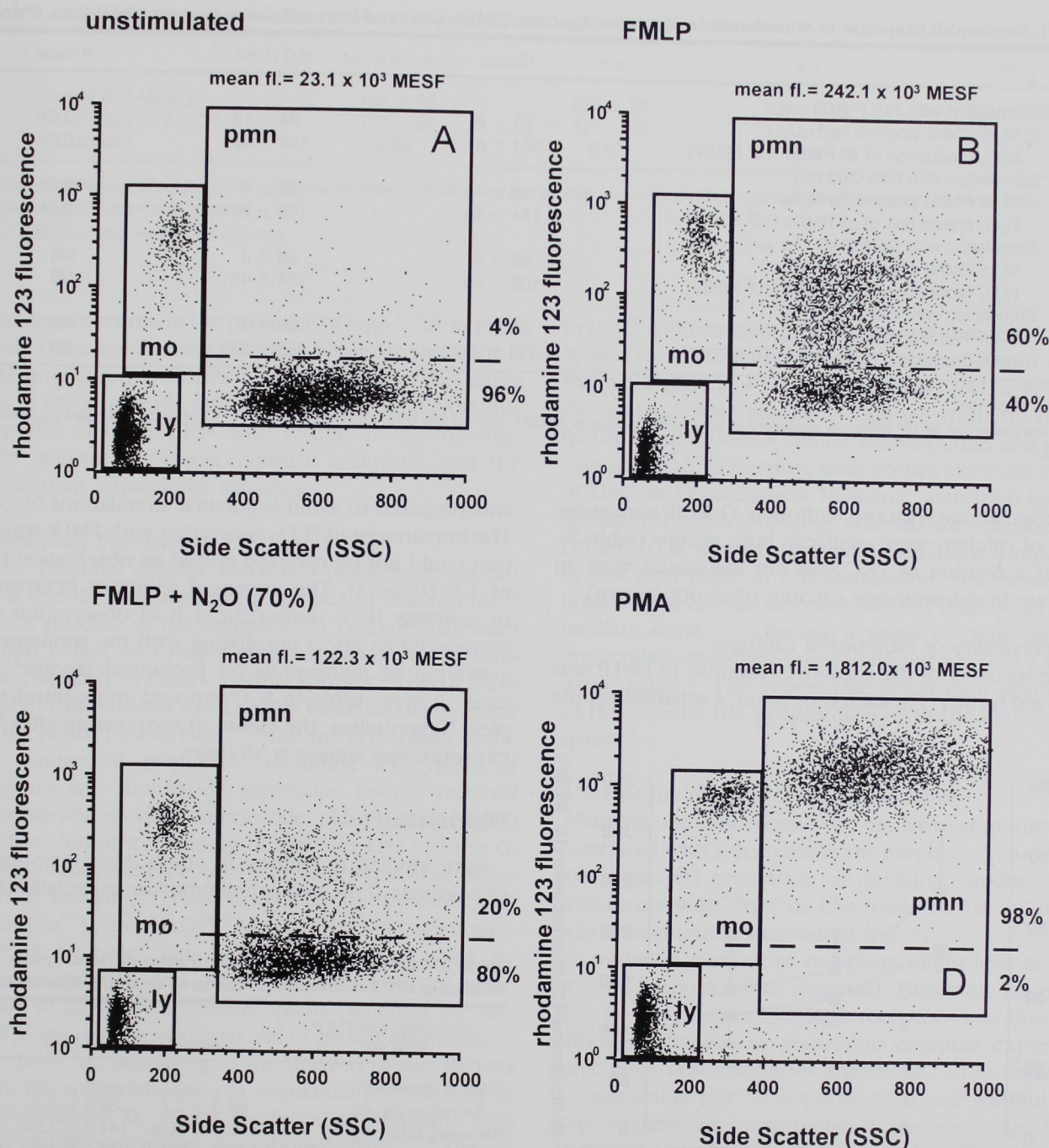


Fig. 2. Flow cytometric analysis of the oxidative burst in 10,000 leukocytes of a single donor using different stimuli in the presence of nitrous oxide (N_2O). The y axis represents the hydrogen peroxide (H_2O_2) generation (rhodamine-123 fluorescence) in arbitrary units. The x axis indicates the side scatter of leukocytes as a marker of cellular granularity: neutrophils (pmn), monocytes (mo), lymphocytes (ly). (A) Unstimulated leukocytes. Neutrophils in B and C were stimulated with N-formyl-methionyl-leucyl-phenylalanine (FMLP) (100 nM), the latter in the presence of N_2O (70%). Therefore, B serves as a control for C. The response to FMLP was heterogeneous; only a subset of neutrophils showed a generation of oxidative product (60%). The percentage of nonresponsive cells, as given on the right of each dot plot, was higher in the presence of N_2O (80%) compared with the control percentage (40%). The reaction of neutrophils after PMA stimulation was homogeneous (D). In conclusion, the fluorescence of reacting neutrophils was not lowered, but the percentage of reacting neutrophils decreased in the presence of N_2O . This correlated to an overall reduction of the mean fluorescence value as given on top of each dot plot.

Table 1. Neutrophil Response to Stimulation by Receptor Agonists (FMLP, C5a) and Intracellular Activators (DiC8-DAG, PMA)

	Control	N ₂ O (70%)	P Value
Stimulation with FMLP (100 nM)			
% of PMNs positive for H ₂ O ₂	53 ± 6	34 ± 13	<0.01
H ₂ O ₂ production of all PMNs (10 ³ MESF)	244 ± 61	142 ± 52	<0.01
Stimulation with C5a (200 pM)			
% of PMNs positive for H ₂ O ₂	55 ± 15	35 ± 6	<0.01
H ₂ O ₂ production of all PMNs (10 ³ MESF)	174 ± 46	103 ± 26	<0.01
Stimulation with DiC8-DAG (7.5 μM)			
% of PMNs positive for H ₂ O ₂	95 ± 2	96 ± 4	NS
H ₂ O ₂ production of all PMNs (10 ³ MESF)	102 ± 46	104 ± 48	NS
Stimulation with PMA (100 nM)			
% of PMNs positive for H ₂ O ₂	98 ± 1	99 ± 1	NS
H ₂ O ₂ production of all PMNs (10 ³ MESF)	1,613 ± 938	1,458 ± 986	NS

NS = not significant.

Values are presented as 10³ molecule equivalent of soluble fluorochrome (MESF) units for the H₂O₂ production (mean ± SD of six independent experiments, donors).

was comparable with and without N₂O. Peak concentrations of calcium were similar in both groups (table 2). Direct activation of PKC was not associated with an increase in cytosolic-free calcium (data not shown).

Reversibility of Functional Changes

The recovery of the oxidative response to FMLP was analyzed further. For each time point, a separate sample

was prepared to avoid sequential stimulations of cells. The impairment of H₂O₂ generation with FMLP stimulation could not be reversed within an observation time of 4 h (table 3). The increased ability of neutrophils to generate H₂O₂ during the 4 h of observation was considered an effect correlating with the spontaneous activation of neutrophils on prolonged storage¹³ because it was visible in N₂O-exposed and control samples. Nevertheless, the extent of suppression after N₂O exposure was similar at all times.

Discussion

Some studies, in contrast to others, have reported an impairment of the neutrophil function, especially of the

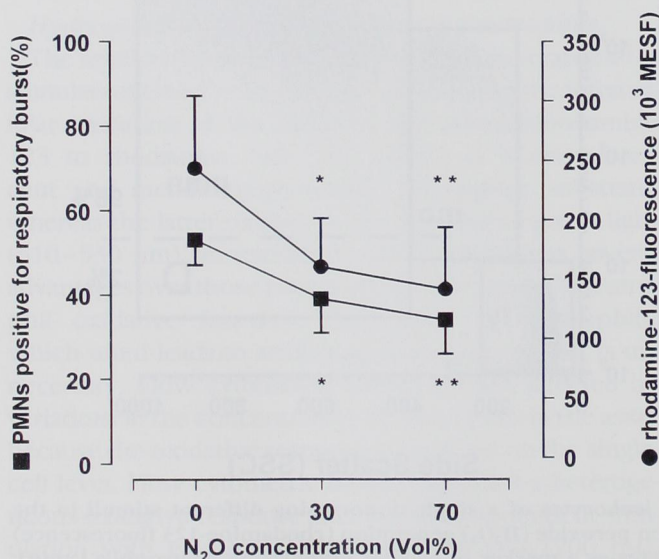


Fig. 3. The mean hydrogen peroxide production (●) and the mean percentage of reacting neutrophils (■) in the presence of nitrous oxide (N₂O) after N-formyl-methionyl-leucyl-phenylalanine stimulation (mean ± SD of six independent experiments (donors)). **P* < 0.05; ***P* < 0.01 for control versus N₂O exposed.

Table 2. Intracellular Ca²⁺ Concentration of Neutrophils following FMLP Stimulation Measured as fluo-3 Fluorescence

Stimulation with FMLP (100 nM)	Control	N ₂ O (70%)	P Value
Time to maximum fluorescence* (s)	33.0 ± 2.7	30.3 ± 3.1	NS
Recovery time† (s)	150.3 ± 10.6	144.9 ± 12.4	NS
Baseline fluorescence (%)	14.1 ± 2.3	14.3 ± 1.5	NS
Maximum fluorescence (%)	101.1 ± 9.9	103.8 ± 9.5	NS

NS = not significant.

The Ca²⁺ dependent fluorescence of fluo-3 loaded cells was standardized relative to the dye loading efficiency based on the ionomycin-induced level of fluorescence (mean ± SD of six independent experiments, donors).

* Time from addition of FMLP to the maximal Ca²⁺ concentration.

† Time until reversal of Ca²⁺ concentration to baseline.

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Table 3. Recovery of H₂O₂ Production following Exposure to N₂O with FMLP as a Stimulus

	During Exposure	1 h	2 h	4 h
Control (10 ³ MESF)	197 ± 98	222 ± 98	337 ± 105	302 ± 108
N ₂ O (70%) (10 ³ MESF)	127 ± 89	127 ± 47	178 ± 89	159 ± 86
N ₂ O/control	0.64*	0.57*	0.53*	0.53*

Values are 10³ molecule equivalent of soluble fluorochrome (MESF) units and as ratio of control and N₂O exposed sample assayed in parallel (mean ± SD of six independent experiments, donors).

* $P < 0.01$ for control versus N₂O exposed.

oxidative function, in the presence of N₂O.²⁻⁵ However, neither the mechanism nor the potential site of this N₂O interaction are known. The purpose of this study was to investigate the influence of N₂O on the oxidative response of neutrophils with respect to immunologically relevant, G-protein-coupled receptors, and the subsequent intracellular signaling.

Signaling Pathway of FMLP and C5a

The bacterial peptide FMLP is a physiologic agonist for a specific receptor on the surface of neutrophils.¹⁴ C5a, a product of the complement cascade, has been shown to act as a major chemoattractant for neutrophils, similarly to FMLP.⁷ Further, phagocytosis of *Escherichia coli* is mainly mediated by FMLP and its receptor on the neutrophil surface.¹⁴ FMLP and C5a trigger multiple neutrophil responses, including exocytosis of enzymes and superoxide generation by the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex.⁷ Binding of FMLP or C5a to their G-protein-coupled receptors¹⁵ induces a transient elevation of inositol-1,4,5-trisphosphate, free cytosolic calcium [Ca²⁺]_i, and DAG^{10,16} (fig. 1). The DAG accumulation is biphasic: The initial phase is catalyzed by phospholipase C and occurs in parallel to the generation of inositol-1,4,5-trisphosphate, whereas the second phase is more sustained and of greater magnitude.¹⁷ The DAG, accumulating in the delayed phase, derives from phosphatylcholine *via* sequential involvement of phospholipase D, which produces phosphatidic acid (PA) and phosphatidate phosphohydrolase.^{18,19} The accumulation of PA and DAG are supposed to mediate sustained cellular responses.^{7,20} As shown in reconstitution assays, PA directly activates NADPH oxidase.²¹ Phosphatidic acid is also transformed by phospholipase A₂ to lysophosphatidic acid, an activator of phospholipase C.²² DAG induces a redistribution of the PKC isoforms β I, β II, and ζ from cytosol to the plasma mem-

brane.¹⁰ Human neutrophils express four PKC isoenzymes (α , β I, β II, ζ). Protein kinase C- β and - ζ are the most abundant isoforms, whereas PKC- α is barely detectable.²³ PMA and DiC8-DAG are synthetic analogs of DAG and induce a similar redistribution and activation of PKC.^{9,10} Therefore both probes allow the effects of DAG to be mimicked. However, activation by PMA is maximal and irreversible, whereas the reaction after DiC8-DAG is weaker, reversible, and more comparable to that after FMLP or C5a stimulation. The membrane-bound form of PKC activates NADPH oxidase by intermediate steps.^{16,24} Through a single electron transfer, this oxidase transforms molecular oxygen into a superoxide anion.¹⁶ The superoxide anion then dismutates to H₂O₂ inside the phagosomes or in the extracellular space.²⁵

Site of the Nitrous Oxide Interaction

Nitrous oxide decreased the oxidative response. This correlated with a decrease in the number of neutrophils that displayed generation of oxidative products with stimulation with FMLP or C5a (table 1). The inhibition was concentration dependent (fig. 3).

A reduced number of receptors might lead to a decreased response,²⁶ as observed. However, no reduction in the number of FMLP receptors was detected, although changes in neutrophil receptor expression have been described after anesthesia.²⁷ The finding of a comparable [Ca²⁺]_i response with and without N₂O may suggest an impairment of the response downstream of phospholipase C. Nevertheless, N₂O may have an effect on the G proteins or phospholipase C because the intracellularly released Ca²⁺ may not be due solely to inositol-1,4,5-trisphosphate-dependent mechanisms. In this context, an activation of phospholipase C by PA and lysophosphatidic acid²² or of guanylate cyclase by N₂O, resulting in increased intracellular cyclic guanosine monophosphate levels, must be consid-

ered potential explanations for the inhibition of the oxidative response. However, the inhibition of the oxidative response, but not of the $[Ca^{2+}]_i$ response, indicates that N_2O exerts a postreceptor modulation of signal transduction.

Neither the DiC8-DAG nor the PMA-induced oxidative response was altered in the presence of N_2O , which indicates an intact signaling downstream of PKC and an unimpaired function of NADPH oxidase in the presence of N_2O . However, neither PMA nor DiC8-DAG require all of the cofactors that DAG requires to activate PKC, and thus a modulation by N_2O at the level of PKC activation is not excluded. Saito *et al.*²⁸ showed an interference of N_2O with PKC. Nevertheless, it should be noted that the tested PKC was isolated from rat neuronal tips, and thus probably belonged to isoforms differing from those found in human neutrophils. Assuming an intact function of PKC, the decreased oxidative response of neutrophils in the presence of N_2O might be due to decreased levels of PA or DAG. The former may directly activate NADPH oxidase and has been shown to mediate through its intracellular level the enhanced oxidative response of neutrophils in the presence of proinflammatory cytokines such as tumor necrosis factor- α .²⁰ The enhanced response in the presence of tumor necrosis factor- α is associated with a changed ratio between nonresponsive and responsive neutrophils, with more of the latter.⁶ Nitrous oxide increases the number of nonresponsive neutrophils, perhaps associated with altered second messenger levels. However, experiments including the direct measurement of the involved second messengers PA, lysophosphatidic acid, DAG, and inositol-1,4,5-trisphosphate (fig. 1) would be needed to substantiate these conclusions. In addition, more defined cellular models such as neutrophils in the absence of other potentially interfering leukocytes, or cells from defined cell lines transfected with the investigated chemotactic receptors are required to characterize the mechanism of interaction at the molecular level.

A scavenging of reactive oxygen derivatives by N_2O , a direct interference with H_2O_2 -generating enzymes, or an inhibition of the oxidation or detection of the dye used are also potential causes for an altered H_2O_2 production or detection. Of importance for such unspecific interactions is the stoichiometric relation among oxygen products, N_2O , and the dye used. Therefore, the concentrations of 100 nM for FMLP, 200 pM for C5a, and 7.5 μM for DiC8-DAG were chosen to achieve the

same level of oxidative response with the different stimuli. These concentrations were also chosen to allow analysis of the heterogeneous response of neutrophils at low degrees of stimulation, and to allow comparison with other authors' data on halogenated volatile anesthetics.^{29,30} However, the finding of an unchanged DiC8-DAG- or PMA-induced oxidative response in the presence of N_2O excludes these possible causes. This is also supported by the observation of an unchanged spontaneous oxidative burst of monocytes in the presence of N_2O (fig. 2C). The regulation of this monocyte reaction involves signaling pathways that differ from those mediating neutrophil responses.

Irreversibility of the Nitrous Oxide Effect

A striking result of our study was the irreversibility of the impairment of neutrophil signaling after N_2O exposure. That this finding is related to our experimental method can be excluded because, as recently published,³⁰ the effects of halogenated, volatile anesthetics were completely reversible under the same conditions. This suggests an effect unique to N_2O with a sustained change in the properties of one or more of the involved signaling components. Levin and Blanck³¹ reported that halothane and isoflurane irreversibly decrease the ability of calmodulin to bind calcium. Nitrous oxide, which was not addressed in their report, might also be able to alter the properties of calmodulin. This would lead to the findings we observed because the proper functioning of calmodulin is necessary for the initialization of the oxidative response.^{32,33} A sustained change in protein properties might also occur in relation to the methionine metabolism. Nitrous oxide affects methionine synthase *in vitro* and *in vivo* by a one-electron oxidation of cobalamin in the enzyme.^{34,35} Further, neutrophils have been shown to have a much higher turnover of methionine than other cell types. As much as 25% of the methionine residues are oxidized in unstimulated neutrophils, whereas oxidized methionine is barely detectable in other cell types.³⁶ Although the oxidation of methionine residues does not always result in complete protein inactivation,³⁶ it seems possible that neutrophils exposed to N_2O might be especially susceptible to oxidative damage by a sustained alteration of their methionine metabolism.

Conclusions

Neutrophils display a decreased oxidative response to FMLP or C5a stimulation in the presence of N_2O . This

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is characterized by a lower percentage of responsive neutrophils and consequently an overall decrease in H_2O_2 production. Nitrous oxide did not affect the number of receptors, nor did it influence the direct induction of the oxidative response by the PKC activators DiC8-DAG and PMA. This suggests an interference of N_2O upstream of NADPH oxidase. Whether the described effects of N_2O are relevant for clinical anesthesia is still controversial. However, neutrophils can serve as a good model to study signaling in human cells because the underlying signaling pathways are ubiquitously present in all cells. The further characterization of the N_2O effects on cellular signal transduction is of interest to help achieve a better understanding of the molecular mechanisms underlying the pharmacologic action of N_2O .

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