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Inhibition of NADPH Oxidase-Mediated Superoxide Radical Formation in PMA-Stimulated Human Neutrophils by 4-Hydroxynonenal—Binding to -SH and -NH_2 Groups[1]

WERNER G. SIEMS[a,*], ELISABETTA CAPUOZZO[b], DANIELA VERGINELLI[b], COSTANTINO SALERNO[c], CARLO CRIFÒ[b], and TILMAN GRUNE[d]

[a]*Herzog-Julius Hospital of Rheumatology and Orthopaedics, Kurhausstraβe 13-17, D-38655 Bad Harzburg, Germany;* [b]*Department of Biochemical Sciences, and* [c]*Laboratory of Clinical Biochemistry, University of Rome, P.le A. Moro, I-00185 Rome, Italy;* [d]*Clinics of Physical Therapy and Rehabilitation, University Hospital Charité, Medical Faculty of the Humboldt University, Schumannstraβe 20-21, D-10098 Berlin, Germany*

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4-Hydroxynonenal (HNE), a major lipid peroxidation product, effectively inhibits the superoxide radical formation by NADPH oxidase of phorbol myristate acetate (PMA)—stimulated human PMNL. The I_{50} value for the inhibition of NADPH oxidase-mediated superoxide radical formation by 4-hydroxynonenal was found to be 19 μM. The HNE inhibition involves the reaction with both -SH and -NH_2 groups. Superoxide formation as final result of the NADPH oxidase cascade was almost completely restored by addition of dithiothreitol. In presence of hydroxylamine only a minor restoration of superoxide radical formation was found. A combination of dithiothreitol and hydroxylamine yielded the greatest recovery. Two other aldehydes with the same chain length as HNE but different binding to lysine, histidine and cysteine residues, trans-2,3-nonenal and nonanal, gave I_{50} values for the inhibition of NADPH oxidase-mediated superoxide formation rate of 110 μM or >300 μM, respectively.

Keywords: 4-hydroxynonenal, NADPH oxidase, human neutrophils, PMNL, PMA-stimulation, superoxide radical formation, lipid peroxidation product

Abbreviations: DTT, dithiothreitol; HA, hydroxylamine; HNE, 4-hydroxy-2,3-trans-nonenal (4-hydroxynonenal); PMA, phorbol myristate acetate; PMNL, polymorphonuclear leukocytes

INTRODUCTION

4-Hydroxyalkenals such as the major lipid peroxidation product 4-hydroxy-2,3-trans-nonenal (4-hydroxynonenal, HNE) can promote and potentiate cell injury induced by oxygen radicals. HNE is a compound with genotoxic, cytotoxic, mutagenic and chemotactic activity and pro-

* Corresponding author. Fax: +49-5322-790200.

[1] The paper is dedicated to Prof. Hermann Esterbauer (1936–1997). The authors are greatly indebted to this leading scientist in the field of free radical research.

duces a multitude of effects such as depletion in glutathione, disturbance of calcium homeostasis, inhibition of DNA, RNA and protein synthesis, initiation of lipid peroxidation, inhibition of mitochondrial respiration, and inhibition or activation of some specific enzymes.[1–4] HNE reacts with lysine, histidine, and cysteine residues.[3,5,6] Therefore, it can seriously affect the function of proteins.

HNE has been found to stimulate the oriented migration of polymorphonuclear leukocytes (PMNL) at concentrations ranging from 10^{-7} to 10^{-5} M.[7] The chemotactic activity of HNE might be mediated by the stimulation of phosphoinositide-specific phospholipase C.[8] Only at concentrations higher than 500 μM HNE inhibited the PMNL motility, whereas a significant inhibition of phospholipase C activity occurred at 100 μM.[8] If the level of HNE is increased in organs or tissues, an increased number of PMNL is moving to those areas.[9]

It was demonstrated that HNE levels are increased in inflammatory regions.[10] In those regions the PMNL superoxide generating NADPH oxidase is active, it responds to a very wide range of foreign stimuli. The different stimulus-dependent transduction mechanisms which can be involved in the activation process of neutrophil superoxide generating oxidase were intensively studied.[11] It was shown previously that HNE and resulting HNE-protein adducts are generated as a result of NADPH oxidase activity in the phagosomes of human neutrophils. It was concluded that these lipid peroxidation products may contribute to microbial killing and/or damage of neutrophil phagolysosomal proteins.[12]

This study was carried out to investigate the influence of HNE on superoxide production by NADPH oxidase in stimulated human PMNL, and therefore on superoxide radical formation as an important part of inflammatory processes. Previously, the influence of HNE on N-formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated PMNL was measured.[13] Here the effect of HNE in phorbol myristate acetate (PMA)-stimulated PMNL is investigated.

MATERIAL AND METHODS

Human leukocyte preparations containing 90–98 % of neutrophils and apparently free of contaminating erythrocytes were obtained by one-step procedure involving centrifugation of heparinized blood, freshly drawn from healthy donors and layered on Ficoll-Hypaque medium (Mono-Poly Resolving medium, ICN Pharm., Costa Mesa, CA).[14] The cells were suspended in isotonic phosphate-buffered saline pH 7.4 with 5 mM glucose and stored on ice. Each preparation produced cells with a viability higher than 90% up to 6 hours after purification. The viability of the cells was measured by trypan blue exclusion test.

NADPH oxidase activity was determined by measuring the rate of superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm by a modification of the method described by Lehmeyer *et al.*[15] The incubation mixture contained 2×10^6 cells/ml, 80 μM cytochrome c, and 0.5 mM calcium chloride. Aldehydes such as HNE, trans-2,3-nonenal or nonanal were added. After 10 min of preincubation at 37°C, the reaction was started by adding 1 μg/ml PMA. The controls contained, in addition, superoxide dismutase 20 μg/ml. In selected experiments, the influence of dithiothreitol (DTT) and/or hydroxylamine (HA) addition was investigated. Those experiments were carried out at 37°C, too. DTT or HA were added to the incubation mixture after measuring the cytochrome c reduction curve for 2 to 3 min. The change of the curve slope of cytochrome c reduction from linear to linear slope again after addition of HA or DTT was always completed within less than 30 s.

The inhibition of the protein kinase C (PKC) activity by HNE was determined from the ^{32}P phosphate transfer from [gamma-^{32}P]ATP (Amersham)[16] after preincubation with HNE at different

concentrations for 5 min in a reconstituted system with membrane and cytosolic fraction. Membrane and cytosolic fractions were obtained by discontinuous sucrose density gradient centrifugation.[17] Protein concentration was determined with the bicinchoninic acid assay (Pierce Chem. Co., Rockford, IL).[18] The incubation was carried out in presence of 0.5 mM EDTA, 0.5 mM EGTA, 10 mM $MgCl_2$, 500μM $CaCl_2$, 1 μM GTP[gamma-S]. The reaction was started by adding [gamma-^{32}P] ATP. After 15 min of incubation at 37°C the reaction was stopped by adding ice-cold 10% (w/v) trichloroacetic acid. The precipitated material was quantitatively transferred to glass fiber filters. After washing with excess trichloroacetic acid, filters were counted. The PKC activity was calculated as percent of control PKC activity measured without HNE.

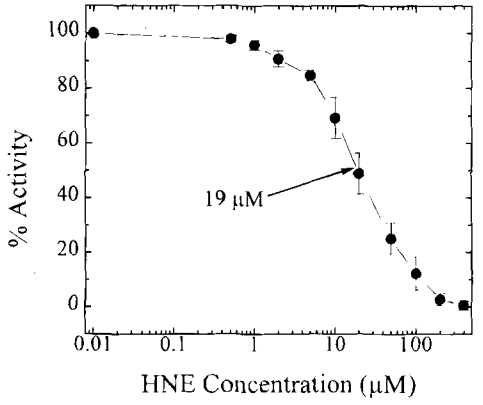

FIGURE 1 Inhibition of NADPH oxidase-mediated superoxide radical formation of phorbol myristate acetate (PMA)-stimulated human neutrophils by HNE. The remaining superoxide radical formation is expressed as percentage of superoxide formation evoked by PMA 1μg/ml in the absence of HNE, which was taken as 100%. 100% was 7.82 nmol/min/10^6 PMNL. The results are means ± S.E.M. of 10 experiments.

RESULTS

HNE rapidly diminishes the superoxide radical formation of NADPH oxidase in PMA-stimulated human neutrophils. The I_{50} value for inhibition of NADPH oxidase-mediated superoxide radical formation by HNE under conditions of PMA-stimulation of neutrophils was measured to be 19 μM (Fig. 1).

For dissoziation of protein-HNE adducts with the aim to restore the superoxide radical formation via NADPH oxidase hydroxylamine (HA) and/or dithiothreitol (DTT) were used. DTT can remove HNE at least partially from sulfhydryl groups. HA is known to reverse the Schiff's base bond, to form an oxime derivative with the aldehyde, which is simultaneously released from the protein lysine residues. The restoration of NADPH oxidase activity by 1 mM HA and/or 1 mM DTT was measured after inhibition of superoxide radical formation by 20 μM HNE. In presence of 1 mM HA only a minor restoration of enzyme activity was found. NADPH oxidase activity was almost completely restored by addition of 1 mM DTT (Table I). The effect of other aldehydes with chain length of nine carbon atoms, trans-2,3-nonenal and nonanal, and additionally the effect of glutaraldehyde, on NADPH oxidase-mediated superoxide formation of PMA-stimulated PMNL is given in Table II. HNE and trans-2,3-nonenal will react with lysine, histidine and cysteine residues, while nonanal does not react with sulfhydryl groups. Nonanal caused a smaller decrease in NADPH oxidase activity than HNE or trans-2,3-nonenal, and the activity could be

TABLE I Restoration of NADPH oxidase-mediated superoxide radical formation in phorbol myristate acetate-stimulated human neutrophils inhibited by 20μM HNE following incubation with dithiothreitol, DTT (1 mM) and hydroxylamine, HA (1 mM)[a]

	nmol/min/10^6 PMNL	% of control
Control	7.58 ± 0.23	100.0 ± 3.1
plus HA	7.56 ± 0.25	99.8 ± 3.2
plus DTT	7.88 ± 0.29	104.0 ± 3.8
HNE	3.47 ± 0.03	45.8 ± 0.3
plus HA	4.80 ± 0.15	63.4 ± 1.9
plus DTT	6.77 ± 0.12	89.3 ± 1.5
plus HA + DTT	7.13 ± 0.03	94.0 ± 0.4

[a] Conditions as described in the text. Values given as nmol superoxide radicals/min/10^6 PMNL and as percent of control. Control values were obtained without addition of aldehyde, DTT, and hydroxylamine. Data are given as means ± S.E.M. (n = 4).

TABLE II Restoration of NADPH oxidase-mediated superoxide radical formation of PMA-stimulated human neutrophils inhibited by different aldehydes (each at 100 μM) following addition of 1mM hydroxylamine, HA and 1 mM dithiothreitol, DTT[a]

Aldehyde	Control	+HA	+HA + DTT
None	100.0	98.9 ± 2.6	103.6 ± 4.2
4-Hydroxynonenal	13.6 ± 1.4	23.1 ± 0.4	39.9 ± 0.4
trans-2,3-Nonenal	53.5 ± 2.9	63.5 ± 1.5	75.7 ± 1.5
Nonanal	70.8 ± 2.5	98.9 ± 2.0	98.8 ± 2.7
Glutaraldehyde	4.5 ± 0.2	65.4 ± 5.4	81.2 ± 5.5

[a] Values given as percent of control (mean ± S.E.M.), expressed as remaining superoxide generation. Control values corresponding to 100% were obtained without addition of aldehyde, hydroxylamine, and dithiothreitol. Each data point represents four experiments.

fully restored using HA (Table II). The I_{50} values for binding of trans-2,3-nonenal and nonanal were about 110 μM and higher than 300 μM, respectively. Glutaraldehyde exerted a strong inhibition of superoxide formation with an I_{50} value of 25 μM, i.e. in the range of the I_{50} value of HNE. For HNE and trans-2,3-nonenal inhibition, the restoring effect of DTT was greater than the restoring effect of HA. For glutaraldehyde inhibition the restoring effect of HA was much more pronounced than that of DTT, and restoring effects of nonanal inhibition are observed only in presence of HA. DTT did not exert additional restoring effects after inhibition of superoxide formation in PMA-stimulated PMNL treated with nonanal (Table II).

The inhibition of the PKC activity by HNE was determined after preincubation with HNE at different concentrations in a reconstituted system with membrane and cytosolic fraction at 37°C. The different HNE concentrations led to the following remaining PKC activity (given as percent of the control activity in absence of HNE which was 0.042 μmol/mg protein per min): 1 μM HNE 90.5 ± 5.1%; 10 μM HNE 74.9 ± 3.1%; 50 μM HNE 54.6 ± 6.2%; 100 μM HNE 6.3 ± 1.1%; 400μM HNE 3.7 ± 0.7% from 4 experiments. From those measurements a 50% inhibition of PKC in the cell free system by about 60 μM HNE was calculated.

DISCUSSION

The results indicate that the inhibition of NADPH oxidase-mediated superoxide radical generation in PMA-stimulated human neutrophils by HNE is due to its reactions both with amino and with sulfhydryl groups (see Tables I and II). HA and DTT were used to restore the NADPH oxidase-mediated superoxide radical formation by removal of HNE from amino or sulfhydryl groups. The reactions with sulfhydryl groups seem to be more important quantitatively for NADPH oxidase inhibition by HNE. Trans-2,3-nonenal and nonanal facilitate the investigation of aldehyde binding to amino or SH groups of the NADPH oxidase itself and/or of other proteins involved in the cascade of neutrophil activation leading to increased superoxide radical formation. HNE and trans-2,3-nonenal will react with both lysine and cysteine residues, while nonanal will react only with lysine. It is clear that the I_{50} value for nonenal which reacts only with lysine residues, is much greater than the I_{50} for HNE and trans-2,3-nonenal, which react with both lysine and cysteine residues.

The I_{50} value for inhibition of NADPH oxidase-mediated superoxide radical formation by HNE is in the low range of values which were measured for different cytosolic, mitochondrial and plasma membrane enzyme activities: adenylate cyclase 2.7μM,[19] ADP ribosyl transferase 4.6μM,[20] glucose-6-phosphatase 70 μM,[21] Na^+-K^+-ATPase 120μM,[22] DNA polymerases alpha and beta 370 and 290 μM,[23] adenine nucleotide translocator > 400 μM,[24] 5′-nucleotidase > 5 mM.[25]

Most inhibitors of PMNL NADPH oxidase which were known are exogeneous compounds, namely drugs, e.g. antiinflammatory and antirheumatic drugs such as diclofenac, ibuprofen, piroxicam or auranofin, antibiotics such as neomycin, flavonoids such as quercetin, or the NADPH analogue trifluoperazine.[26] Under the endogenous compounds which are sensitive inhibitors of the enzyme only adenosine has a lower I_{50} value than HNE, but this is only true for

inhibition of the FMLP stimulated enzyme. For the inhibition of PMA stimulated superoxide radical formation by NADPH oxidase, HNE is the endogenous compound with the strongest inhibitory effect.

The I_{50} of HNE on superoxide production in FMLP-stimulated PMNL was measured to be about 12 μM.[13] The value measured here for the inhibition of PMA-stimulated human PMNL (19 μM) is in the same range. The mechanisms of PMNL stimulation, exerted by FMLP or by PMA are different, involving different proteins within the cascade of stimulation. The accordance of I_{50} values for FMLP and PMA stimulation is an argument for direct HNE binding to the NADPH oxidase as being responsible for the inhibition. In a reconstituted cell free system a 50% inhibition of PKC by 60 μM HNE was estimated. From that result in comparison with the 50% inhibition value of 19μM for the whole cascade of superoxide radical generation one could conclude, that the PKC inhibition is involved in the inhibition of superoxide radical formation in PMA-stimulated human neutrophils by HNE, but that there may be other targets within the NADPH oxidase cascade than PKC which are more sensitive towards HNE.

Our results confirm and extend the conclusion that HNE, at levels higher than 1 μM, inhibits the respiratory burst of human PMNL evoked by different stimuli, such as PMA,[27,28] opsonized zymosan[29] and FMLP.[13] It is hypothesized that HNE can influence the NADPH oxidase activity under conditions of oxidative stress in which HNE concentrations up to the micromolar range were measured, e.g. 6 μM in reperfused small intestine.[30,31] It is assumed that in hydrophobic regions of membranes much higher HNE concentrations occur. For membranes in isolated peroxidized microsomes even a HNE concentration of approximately 4.5 mM was calculated.[32] Those HNE gradients lead to the suggestion that HNE may modulate the extent of superoxide radical generation of human PMNL in inflammatory processes.

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