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THE INFLUENCE OF SUPEROXIDE ON THE PRODUCTION OF HYPOCHLOROUS ACID BY HUMAN NEUTROPHILS

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Human neutrophils stimulated with opsonized zymosan promoted hypochlorous acid (HOCl)-dependent loss of monochlorodimedon. Formation of HOCl was completely inhibited by catalase, and it was also inhibited up to 70% by SOD. There was no inhibition by desferal, DTPA, mannitol or dimethylsulphoxide, which excluded the involvement of ·OH. Our results indicate that generation of O_2^- by neutrophils enables these cells to enhance their production of HOCl. Furthermore, inhibition of neutrophil processes by SOD and catalase does not necessarily implicate ·OH. We propose that O_2^- may potentiate oxidant damage at inflammatory sites by boosting the myeloperoxidase-dependent production of HOCl

KEY WORDS: Myeloperoxidase, superoxide, hypochlorous acid, neutrophils.

INTRODUCTION

Hypochlorous acid (HOCl) is a potent oxidant discharged by stimulated neutrophils. It reacts readily with biological molecules, inactivating α_1-proteinase inhibitor, other neutrophil enzymes and inflammatory molecules, and is cytotoxic to a wide variety of mammalian cells.[1] Since HOCl can account for at least 30% of the O_2^- generated during the respiratory burst,[2,3] it may be responsible for the majority of oxidant damage mediated by neutrophils.

Myeloperoxidase, the most abundant neutrophil protein, catalyses the production of HOCl from H_2O_2 and Cl^-.[4] H_2O_2 is supplied as a secondary product by the respiratory burst through the dismutation of O_2^-.[5] Howerver, O_2^- also reacts directly with myeloperoxidase[6] and modulates the chlorinating activity of the purified enzyme.[7,8] Under conditions where compound II accumulates, O_2^- can treble the production of HOCl by reducing compound II back to active ferric myeloperoxidase.[7] Although this mechanism has been demonstrated with the purified enzyme,[7,8] there is no evidence from previous studies[2,9,10] that it operates in the neutrophil. We have examined in detail the effect of O_2^- on HOCl production by neutrophils[11] and in this presentation we report our major findings.

MATERIALS AND METHODS

Materials

Neutrophils were isolated from the blood of healthy donors by Ficoll-Hypaque

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centrifugation, dextran sedimentation, and hypotonic lysis of contaminating red cells.[12] Myeloperoxidase was purified from human neutrophils to a purity index (A_{430}/A_{280}) of > 0.7, and its concentration was determined using $\epsilon_{430} = 91,000\,M^{-1}cm^{-1}$.[13] Zymosan was opsonized in 30% human plasma with end over end rotation for 30 minutes at 37° C. Superoxide dismutase (SOD), catalase, monochlorodimedon (MCD) and zymosan were purchased from Sigma. All other chemicals were of the highest grade available.

Methods

Reactions were carried out in PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mg ml^{-1} of glucose. When incubations were carried out in the absence of Cl^-, the buffer was 10 mM phosphate, pH 7.5, containing 1.0 mM $MgSO_4$, 47 mM Na_2SO_4 and 1 mg ml^{-1} of glucose. Neutrophils were incubated in a total volume of 1 ml in 1.5 ml sealed microfuge tubes with end over end rotation for 15 minutes at 37° C. At the end of this period tubes were placed in melting ice for 10 minutes and centrifuged to pellet neutrophils and zymosan. The production of HOCl was based on the loss of MCD (ϵ_{290} 19,000 $M^{-1}cm^{-1}$).[14] O_2^- production was measured as SOD-inhibitable cytochrome c reduction ($\epsilon_{550\ (reduced\text{-}oxidized)}$ 21,100 $M^{-1}cm^{-1}$).

RESULTS

Effects of superoxide dismutase and catalase on the loss of MCD

When neutrophils were stimulated with opsonized zymosan they promoted the loss of MCD. Catalase and SOD inhibited the loss of MCD by 93% and 73% respectively (Figure 1). The effects of these proteins were due to their enzymatic activity since the heat-inactivated enzymes and bovine serum albumin were ineffective.

The loss of MCD in the absence of SOD was directly proportional to the production of O_2^- (Figure 2), which was used as a measure of total oxidant ($O_2^- + H_2O_2$) production. In the presence of SOD the loss of MCD plateaued, so that SOD gave greatest inhibition at the highest rates of O_2^- production but did not inhibit at lower

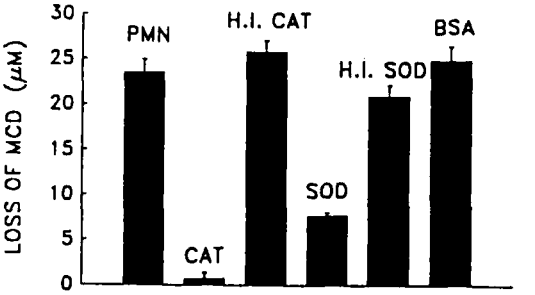

FIGURE 1. The effects of catalase and superoxide dismutase on the loss of MCD mediated by neutrophils stimulated with opsonized zymosan. Neutrophils (4×10^6 ml^{-1}) were incubated with 5 mg ml^{-1} of opsonized zymosan and 70 μM MCD in total volume of 1 ml at 37° C for 15 min. Concentrations of catalase (CAT), SOD and bovine serum albumin (BSA) were 100 μg ml^{-1}, 20 μg ml^{-1} and 100 μg ml^{-1}, respectively. Enzymes were heat inactivated (H.I.) by boiling for 15 min. Superoxide production was 170 μM in 15 min. Values represent meeans and ranges of duplicate experiments.

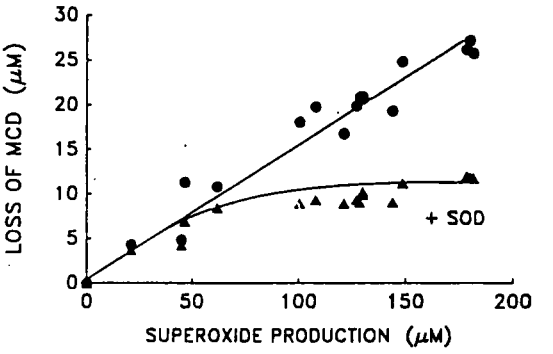

FIGURE 2. The effect of superoxide production on the loss of MCD mediated by neutrophils stimulated with opsonized zymosan. Conditions were as described in Figure 1 except the concentration of opsonized zymosan was varied between 0 and 5 mg ml^{-1} in the presence (▲) or absence (●) of 20 μg ml^{-1} of SOD. Superoxide produced during 15 min of incubation was measured in parallel experiments by the cytochrome c assay. Data are from experiments with several neutrophil preparations.

rates. SOD did not affect the release of myeloperoxidase.[11] Thus, it can be concluded that in the presence of SOD the increased production of H_2O_2 eventually saturated the activity of myeloperoxidase. However, in the absence of SOD, O_2^- was responsible for maintaining the activity of myeloperoxidase so that H_2O_2 was efficiently converted to HOCl.

Effects of oxidant inhibitors and scavengers on the loss of MCD

Inhibition by catalase and SOD could indicate that the loss of MCD was due to ·OH produced by the O_2^--driven Fenton reaction.[15] To distinguish between HOCl and ·OH as the neutrophil oxidant responsible for the loss of MCD, reactions were carried out in the presence of either exogenous myeloperoxidase, or inhibitors of HOCl or ·OH production. Loss of MCD was completely suppressed by the heme

TABLE 1
The effect of myeloperoxidase and oxidant inhibitors on the loss of MCD mediated by neutrophils stimulated with opsonized zymosan. Conditions were as described in Figure 1. Superoxide production was 112 μM/15 min in the presence of Cl^- and 67 μM/15 min in its absence.

Conditions	MCD Loss (μM)	% Change
PMN + MCD + OZ	20.5 ± 1.1 (3)	—
+ 250 nM MPO	35.7 ± 1.9 (4)	+74
+ 100 μM azide	0.4 ± 1.1 (4)	−98
+ 1 mM cyanide	0.1 ± 1.1 (4)	−100
− Cl^-	2.6 ± 0.9 (4)	−80*
+20 mM Me_2SO	22.7 ± 1.2 (2)	+10
+ 40 mM Me_2SO	22.7 ± 1.2 (2)	+11
+ 5 mM mannitol	20.0 ± 2.6 (2)	−2
+ 10 mM mannitol	22.1 ± 1.1 (2)	+8
+ 100 μM desferal	20.4 ± 1.0 (2)	−1
+ 100 μM DTPA	20.2 ± 2.2 (4)	+1
+ 100 μM methionine	2.7 ± 1.1 (4)	−87

*Corrected for decreased O_2^- production. Values represent means and standard deviations of (*n*) experiments.

enzyme inhibitors azide and cyanide, and was enhanced by adding exogenous myeloperoxidase (Table I). It was also suppressed when cells were suspended in Cl^--free buffer to prevent production of HOCl. Under these conditions, the cells produced only approx. 60% of the O_2^- generated in Cl^--buffer. This in itself would result in a corresponding decrease in oxidant production. However, even when this decrease was accounted for, loss of MCD was inhibited by 80%. Cl^--free buffer had no effect on the amount of myeloperoxidase released.[11] Dimethyl-sulfoxide (Me_2SO) and mannitol react rapidly with ·OH and are commonly used as ·OH scavengers. However, they failed to inhibit the loss of MCD (Table I). In addition, there was no inhibition by desferal or diethylenetriaminepentaacetic acid (DTPA), both of which complex iron in a form that is unable to catalyse the O_2^--driven Fenton reaction.[15] Methionine, which reacts rapidly with HOCl,[16] strongly inhibited the reaction.

DISCUSSION

We have demonstrated that human neutrophils stimulated with opsonized zymosan promote the loss of MCD by a process that depends on myeloperoxidase, H_2O_2 and Cl^-. Previous studies with the purified enzyme[4,17] have shown that HOCl is the species responsible for this reaction. Our results are consistent with this and show that extracellularly generated HOCl promotes the loss of MCD by neutrophils stimulated with opsonized zymosan. A novel finding in this investigation is that production of HOCl by neutrophils can be inhibited by SOD. At low rates of oxidant production SOD did not inhibit the loss of MCD. However, with increasing oxidant production, SOD inhibited the loss of MCD by up to 70%. Although inhibition by SOD and catalase could implicate ·OH, this radical was excluded because the loss of MCD was dependent on myeloperoxidase and was unaffected by ·OH scavengers or inhibitors of the Fenton reaction. Our results indicate that neutrophils can utilize O_2^- to enhance their extracellular production of HOCl.

The striking similarity between the effects of O_2^- on HOCl production by purified myeloperoxidase[7] and neutrophils stimulated with opsonized zymosan (Figure 2), suggest that the mechanism established for the purified enzyme (Figure 3) is operative in neutrophils. O_2^- enhanced production of HOCl because it prevents inactive compound II from accumulating. Chlorination occurs in the presence of a flux of O_2^-, even though O_2^- promotes formation of compound III, because this form of mye-

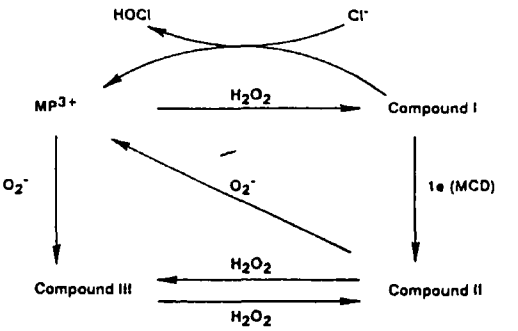

FIGURE 3. The mechanism of myeloperoxidase-dependent chlorination in the presence of a flux of superoxide (7).

loperoxidase reacts with H_2O_2 to give compound II, which is then reduced to active ferric myeloperoxidase by O_2^-.

SOD should inhibit production of HOCl by neutrophils only under conditions where compound II accumulates and its turnover is limiting. Agents other than O_2^-, such as ascorbate[18] and urate[7] can reduce compound II. When they are present, the inhibitory effect of SOD will be masked. However, the high flux of O_2^- produced by neutrophils should ensure that O_2^- is the major reductant *in vivo*. In this investigation the MCD used to detect HOCl also promotes the accumulation of compound II.[19] However, we have recently shown that high concentrations of H_2O_2 inhibit myeloperoxidase by promoting the formation of compound II, as do tryptophan and serum components in the presence of H_2O_2.[19] This inhibition is prevented by a flux of O_2^-.[8]

An important conclusion arising from this investigation is that SOD and catalase can inhibit reactions of neutrophils that are not due to ·OH. This is especially relevant since recent studies have shown that isolated neutrophils do not produce ·OH,[20] and that myeloperoxidase is an effective inhibitor of ·OH production.[21] ·OH has been suggested as the damaging agent in a wide variety of inflammatory conditions, as well as in neutrophil self-destruction,[22] bacterial killing by neutrophils,[23] and *Candida* killing by monocytes[24] because SOD and catalase inhibited these processes. Inhibition of O_2^--driven HOCl production by both enzymes, as observed in the present study, provides an alternative explanation for these results. Hence, these results imply that O_2^- may potentiate inflammatory tissue damage by enhancing the production of HOCl, and that the anti-inflammatory effect of SOD may, in part, be due to the inhibition of this reaction.

Acknowledgement

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References

1. S.J. Weiss and A.F. LoBuglio (1982) Phagocyte-generated oxygen metabolites and cellular injury. *Laboratory Investigation*, **47**, 5–18.
2. S.J. Weiss, R. Klein, A. Slivka and M. Wei (1982) Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. *Journal of Clinical Investigation*, **70**, 598–607.
3. C.S. Foote, T.E. Goyne and R.I. Lehrer (1983) Assessment of chlorination by human neutrophils. *Nature*, **301**, 715–716.
4. J.E. Harrison and J. Schultz (1976) Studies on the chlorinating activity of myeloperoxidase. *Journal of Biological Chemistry*, **251**, 1371–1374.
5. R. Makino, T. Tanaka, T. Iizuka, Y. Ishimura and S. Kanegasaki (1986) Stoichiometric conversion of oxygen to superoxide anion during the respiratory burst in neutrophils. *Journal of Biological Chemistry*, **261**, 11444–11447.
6. Y. Odajima and I. Yamazaki (1972) Myeloperoxidase of the leukocytes of normal blood. III The reaction of ferric myeloperoxidase with superoxide anion. *Biochimica Biophysica Acta*, **284**, 355–359.
7. A.J. Kettle and C.C. Winterbourn (1988) Superoxide modulates the activity of myeloperoxidase and optimizes the production of hypochlorous acid. *Biochemical Journal*, **252**, 529–536.
8. A.J. Kettle and C.C. Winterbourn (1989) The influence of superoxide on the kinetics of myeloperoxidase measured with a H_2O_2 electrode. *Biochemical Journal*, **263**, 823–828.
9. C.C. Winterbourn, R. Garcia and A.W. Segal (1985) Production of the superoxide adduct of myeloperoxidase (compound III) by stimulated neutrophils and its reactivity with hydrogen peroxide and chloride. *Biochemical Journal*, **228**, 583–592.
10. R.A. Clark, P.J. Stone A. El-hag, J.D. Calore and C. Franzblau (1981) Myeloperoxidase-catalysed

inactivation of α_1-protease inhibitor by human neutrophils. *The Journal of Biological Chemistry*, **256**, 3348–3353.
11. A.J. Kettle and C.C. Winterbourn (1990) Superoxide enhances the production of hypochlorous acid by stimulated human neutrophils. *BBA*, In Press.
12. A. Boyum (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation; and of granulocytes by combining centrifugation and sedimentation at 1g. *Scandinavian Journal of Clinical Investigation (Suppl.)* **21**, 77–89.
13. T. Odajima and I. Yamazaki (1970) Myeloperoxidase of the leukocytes of normal blood. I. Reaction of myeloperoxidase with hydrogen peroxide. *Biochimica Biophysica Acta*, **206**, 71–77.
14. L.P. Hager, D.R. Morris, F.S. Brown and H. Eberwein (1966) Chloroperoxidase. II. Utilization of halogen ions. *The Journal of Biological Chemistry*, **241**, 1769–1777.
15. B. Halliwell and J.M.C. Gutteridge (1985) Free radicals in human disease. *Molecular Aspects of Medicine*, **8**, 89–193.
16. M.F. Tsan (1982) Myeloperoxidase-mediated oxidation of methionine and amino acid decarboxylation. *Infection and Immunisation*, **36**, 136–141.
17. C.C. Winterbourn (1985) Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochimica Biophysica Acta*, **840**, 204–210.
18. B.G.J.M. Bolscher, G.R. Zoutberg, R.A. Cuperus and R. Wever (1984) Vitamin C stimulates the chlorinating activity of human myeloperoxidase. *Biochimica Biophysica Acta*, **784**, 189–191.
19. A.J. Kettle and C.C. Winterbourn (1988) The mechanism of myeloperoxidase-dependent chlorination of monochlorodimedon. *Biochimica Biophysica Acta*, **957**, 185–181.
20. M.S. Cohen, B.E. Britigan, D.J. Hassett and G.M. Rosen (1988) Do human neutrophils form hydroxyl radical? Evaluation of an unresolved controversy. *Free Radical Biological Medicine*, **5**, 81–88.
21. C.C. Winterbourn (1986) Myeloperoxidase as an effective inhibitor of hydroxyl radical production. *The Journal of Clinical Investigation*, **78**, 545–550.
22. M.L. Salin and J.M. McCord (1975) Free radicals and inflammation: Protection of phagocytosing leukocytes by superoxide dismutase. *The Journal of Clinical Investigation*, **56**, 1319–1323.
23. R.B. Johnston Jr., B.B. Keele Jr., H. Misra, J.E. Lehmeyer, L.S. Webb, R.L. Haehner and K.V. Rajagopalan (1975) The role of superoxide anion generation in phagocytic bactericidial activity. Studies with normal and chronic granulomatous disease leukocytes. *The Journal of Clinical Investigation*, **55**, 1357–1372.
24. M. Sasada, A. Kubo, T. Nishimura, T. Kakita, T. Moriguchi, K. Yamamoto and H. Uchino (1987) Canadacidal activity of monocyte-derived human macrophages. Relationship between candidal killing and oxygen radical generation by human macrophages. *Journal of Leukocyte Biology*, **41**, 289–294.

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