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Evidence for Singlet Oxygen Involvement in Cell-free Myeloperoxidase/H_2O_2/Chloride System: Exclusion of Hydroxyl Radical Involvement

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=국문요약=

Cell-free Myeloperoxidase/H_2O_2/Chloride System에서 Singlet Oxygen이 관여한다는 실험적 증거 : Hydroxyl Radical의 관여를 배제함

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인체 백혈구에서 추출한 myeloperoxidase(MPO)에 의한 NADH산화 및 methional에서의 ethylene생성을 관찰하여 cell-free MPO/H_2O_2/Cl^- system에서 관여하는 oxygen metabolites가 무엇인지를 규명하기 위하여 본 실험을 수행하였다.

1) NADH는 MPO/H_2O_2/Cl^-에 의하여 산화됨을 확인하였다. 즉 MPO, H_2O_2 및 Cl^-가 존재하는 medium에서 NADH의 산화는 azide와 catalase에 의하여, 그리고 medium에서 Cl^-를 제거하였을 때 완전히 억제되었다.

2) 이와같은 MPO/H_2O_2/Cl^-에 의한 NADH산화는 $^1O_2$제거물질인 1,4-diazabicyclo(2,2,2) octane (DABCO)에 의하여 완전히 억제 되었으나 OH·의 제거물질인 mannitol, benzoate, formate 및 methanol에 의해서는 영향을 받지 아니하였다.

3) 또한 methional을 MPO/H_2O_2/Cl^-으로 처리하였을 때는 ethylene이 전혀 검출되지 아니하였으나 기타 OH·을 생성할 것으로 알려진 산화계인 xanthine/xanthine oxidase 및 $Cu^{++}-H_2O_2$에 의해서는 현저한 ethylene생성을 관찰하였다.

이상의 결과는 cell-free MPO/H_2O_2/Cl^- 산화계에서는 $^1O_2$이 산화반응에 관여하는 주된 산소 대사물이며 OH·은 생성되지 아니함을 알 수 있었다.

INTRODUCTION

Polymorphonuclear leukocytes(PMNs) play an integral role in host defense(Babior, 1978) and are capable of destroying a wide variety of targets, including bacteria(Johnston et al., 1975), fungi(Diamond et al. 1980), red cells (Weiss and LoBuglio, 1980) and tumor cells (Clark and Szot, 1981). In the target cell destruction, it appears that oxygen-dependent mechanisms associated with utilization of O_2 play a major role. Following membrane pertubation, PMNs exhibit a burst in O_2 consumption with generation of superoxide anion ($O_2^-\cdot$), hydrogen peroxide(H_2O_2) and possibly hydroxyl radical(OH·) and singlet oxygen ($^1O_2$), and these oxygen metabolites are suggested to participate in the destructive processes(Klebanoff, 1980).

The oxygen-dependent mechanisms may be grouped into two systems depending upon whether myeloperoxidase(MPO) is involved in the cytotoxicity. Both need H_2O_2 as an essential component.

In MPO-independent mechanism, toxic oxygen metabolite may be OH· generated by direct interaction of O_2^-· and H_2O_2(Clifford and Repine, 1982; Tauber and Babior, 1977). O_2^-· as a primary product is formed by univalent reduction of O_2 and H_2O_2 from its dismutation which occurs readily either spontaneously or catalyzed by superoxide dismutase. A mechanism for the formation of OH· was proposed by Haber and Weiss(1934).

$$O_2^-\cdot + H_2O_2 \longrightarrow OH\cdot + OH^- + O_2$$

Possibly H_2O_2 also reacts with iron(Fe^{++}) from lactoferrin or bacteria to form OH·(Clifford and Repine, 1982).

H_2O_2 can be toxic by the action of MPO in the presence of halides such as Cl^- or I^-. There is substantial evidence supporting the roles for MPO/H_2O_2/halide system in the cytotoxicity exhibited by PMNs(Clark, 1983; Clark et al., 1975). In many cell or cell-free systems showing MPO-mediated toxic effects, OH· has been excluded as a toxic agent. Instead other agents such as HOCl or $^1O_2$ have been suggested (Allen, 1975; Klebanoff et al., 1976).

But recently Weiss et al.(1978) observed production of ethylene from 2-keto-4-thiomethylbutyric acid(KMB), which has been regarded as evidence for OH· production(Diguiseppi and Fridovich, 1980) and the inhibition ethylene production by azide and cyanide which are known to be MPO inhibitors suggesting that the peroxidase is involved in OH· production in the stimulated PMNs. Klebanoff and Rosen(1978) also suggested roles of MPO in OH· production from PMNs.

In view of potential roles of MPO system in host defense and a variety of cytotoxicity by PMNs it is important to know involving oxygen metabolites in this oxidative system. In this study, an attempt to determine what oxygen metabolites are involved in MPO/H_2O_2/Cl^- system was made by observing effects of various quenchers for oxygen species on NADH oxidation and assaying ethylene production in cell-free MPO system.

MATERIALS AND METHODS

Methional(β-methylthiopropionaldehyde), xanthine oxidase, β-nicotinamide adenine dinucleotide, reduced form(NADH) and Chromosorb 102 were obtained from Sigma Chemical Co.; xanthine and sodium azide from Merck; sodium benzoate and sodium formate from Fisher Scientific Co.; mannitol from Hayashi Pure Chem.; hydrogen peroxide from Shinyo Chemical Co.; 1,4-diazabicyclo(2,2,2) octane (DABCO) from Aldrich Chem. Co.. Other chemicals are of analytical reagent grade.

1) Preparation of human leukocytes

Buffy coat was collected by centrifugation of 1 L of blood from different donors(which was mixed with 1/4 volume of solution containing 0.025 M citric acid, 0.05 M sodium citrate and 0.075 M glucose) at 1,000g for 15 min. The buffy coat was mixed with equal volume of 0.15 M KCl and centrifuged at 1,000g for 10 min. The resulting pellet was washed with cold distilled water for 1 min to lyze contaminating erythrocytes and 1.5 M KCl was added to bring the suspenion to isotonic strength. The cells were recentrifuged and the above lysis procedure was followed until no erythrocyte remained. Approximately 6×10^9 cells could be obtained by this procedure.

2) Preparation of leukocyte granules and granule extract

The leukocyte preparation was used to isolate the leukocyte granules from which granule extract was prepared according to the methods described by Baugh and Travis(1976)

3) Partial purification of MPO

MPO-rich fraction was obtained from the granule extract according to the procedures described by Matheson and Travis(1981). The extract was dialyzed at 4°C overnight against 0.05 M Tris-HCl, 0.1 M NaCl and 0.001 M dithiothreitol, pH 8.0. The precipitate was removed by centrifugation at 2,000g for 10 min. The green supernatant which contained MPO was lyophilized and dissolved in 10 ml of the above buffer solution. The concentrated solution after extensive dialysis against 0.05 M sodium phosphate, pH 6.1 was stored at −20°C and used as the enzyme preparaion. One unit of MPO activity was defined as a change in absorbance of 1.0 optical density unit per min at 510 nm.

4) Measurement of NADH oxidation

Oxidation of NADH was carried out in a Unicam SP 1750 spectrophotometer. A typical reaction mixture contained 0.11 mM NADH, 0.0525 unit MPO, 0.15 M KCl, 0.5 mM H_2O_2, 50 mM sodium phosphate, pH 6.1 or other components indicated in the experiments. H_2O_2 was added at zero time. The total volume was 3 ml and temperature was 37°C. The oxidation was followed by recording the changes in absorbance at 340 nm.

5) Determination of ethylene generation

To test whether OH·is generated in MPO/H_2O_2/Cl^- system, ethylene was determined from methional treated with the MPO system according to the gas chromatographic method of Beauchamp and Fridovich(1970). One ml of reaction mixture containing 0.5 mM methional, 0.15 M KCl, 0.5 mM H_2O_2 and 50 mM sodium phosphate, pH 6.1 was incubated in 12.5 ml glass vials sealed with rubber stopper at 37°C in a water bath with vigorous shaking. 0.5 ml portion of the gas phase sampled with a gas-tight syringe was analyzed for ethylene on a GC-V Pye Unicam ionization gas chromatograph equipped with 1/8 inch × 3 meter stainless steel column packed with Chromosorb 102. The temperatures of the column, detector and injector were maintained at 70, 210 and 80°C, respectively. The flow rate was 25 ml/min for carrier gas(N_2), 35 ml/min for hydrogen and 300 ml/min for air. The amount of ethylene produced was calculated from integrated areas of chromatogram of pure ethylene gas.

The assay for ethylene generation from methional was also performed with other oxidation systems, xanthine/xanthine oxidase and Cu^{++} −H_2O_2 to compare their ethylene-producing capability with that of MPO system. 0.5 mM methional was incubated in the reaction medium containing either 10 munits xanthine oxidase, 0.2 mM xanthine and 0.1 mM EDTA or 20 μM Cu^{++} and 0.5 mM H_2O_2 in a final volume of 1.0 ml buffered at pH 7.4 with 50 mM sodium phosphate. All other conditions for ethylene gas analysis were the same as described above. Xanthine oxidase was assayed according to the method described by Greenwald and Moy(1979).

RESULTS

1) Oxidation of NADH by MPO system

The MPO preparation obtained from human leukocyte granule extract catalyzed oxidation

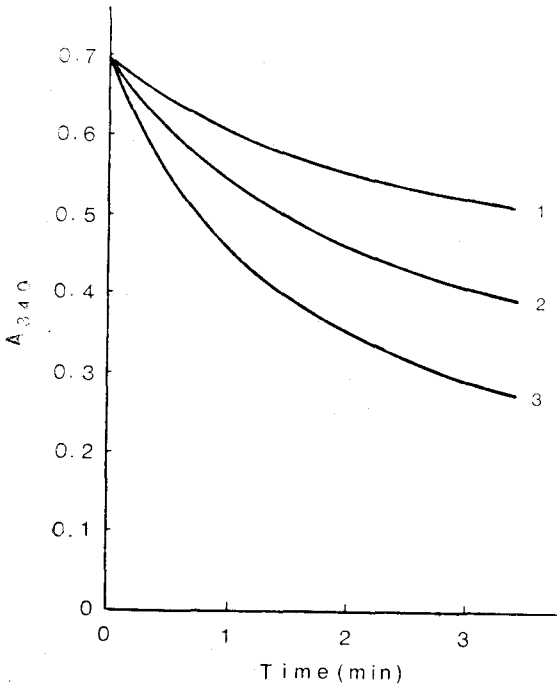

Fig. 1. Oxidation of NADH by myeloperoxidase system. Reaction mixture (3ml) contained 0.11 mM NADH, 0.0525 units MPO, 0.15 M KCl, 50 mM sodium phosphate, pH 6.1 and varying concentrtions of H_2O_2. Curve 1; 0.1 mM, 2; 0.25 mM and 3; 0.5 mM of H_2O_2.

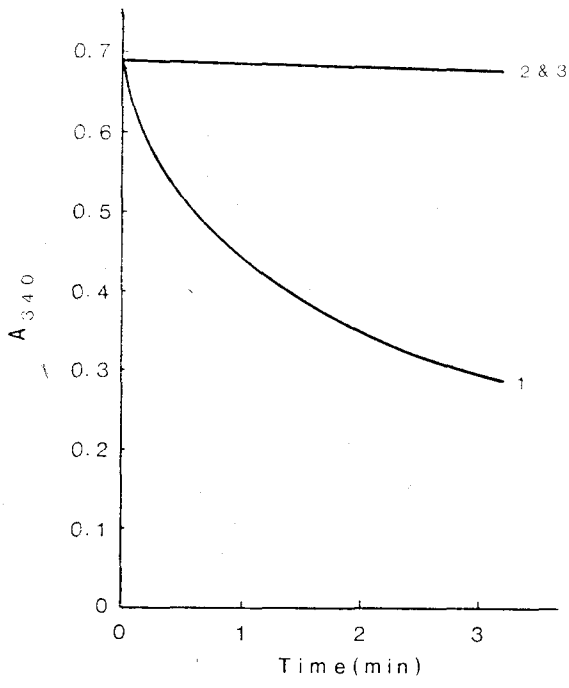

Fig. 2. Effects of soidum azide and catalase on NADH oxidation by myeloperoxidase system. NADH oxidation was measured in the presence of either sodium azide or catalase under the same experimental conditions as in Fig. 1. but with fixed concentration of H_2O_2, 0.5mM. Curve 1; no additions, 2; 1 mM sodium azide and 3; 8μg/ml catalase.

of NADH in the presence of H_2O_2 and Cl^- and the rate of oxidation was dependent on H_2O_2 concentration. As shown in Fig. 1, the rates were 19.3, 33.8 and 54.8 μM/min in the presence of 0.1, 0.25 and 0.5 mM H_2O_2, respectively(they were calculated from the initial slopes of oxidation curves using a molar extinction of 6.2×10^3 M^{-1} Cm^{-1} of NADH at 340 nm). At higher concentrations of H_2O_2 than 0.5 mM, the oxidation rate was decreased (data not shown) possibly because of autoinactivation of MPO by excess H_2O_2(Matheson and Travis, 1981). With fixed amounts of the enzyme preparation(0.0525 units) and NADH (0.11 mM), maximum oxidation was observed with 0.5 mM H_2O_2 in the presence of 0.15 M KCl. Thus, the conditions were used as a standard system in the following experiments where all the characteristics of MPO system were evaluated.

The NADH oxidation observed was further tested to see if it was due to MPO/H_2O_2/halide interaction which constitutes an effective oxidative system to show bactericidal and cytotoxic activities of PMNs(Clark, 1983). In the presence of 1 mM azide which is known to be a MPO inhibitor(Klebanoff, 1968), NADH oxidation was completely inhibited(Fig. 1, Curve 2). Complete inhibition was also observed with 8μg/ml catalase(Fig. 2, Curve 3). Essential requirement of halides was tested in Fig. 3 where Cl^- was chosen as a halide.

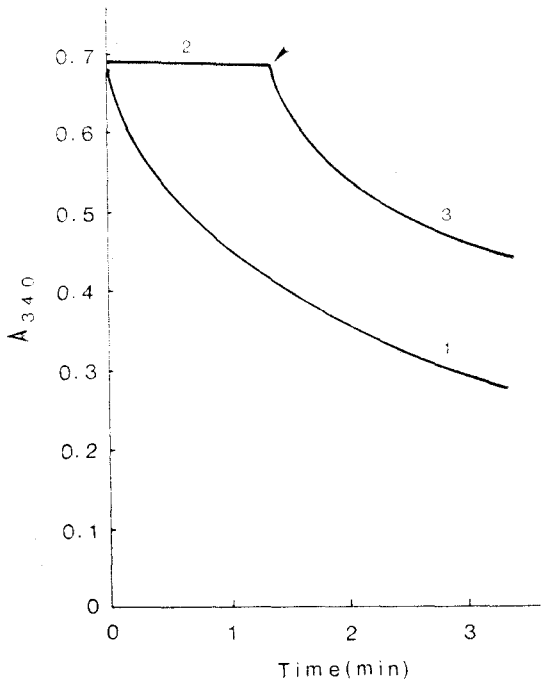

Fig. 3. Effect of chlorides on NADH oxidation. Curve 1 shows NADH oxidation in the complete reaction mixture containing the components as described in Curve 1 of Fig. 2. In Curve 2, reaction was carried out in the mixture omitting KCl. At arrow, 0.3 ml of 1.5 M KCl was added to give a final concentration of 0.15 M. Curve 3 shows resumption of NADH oxidation after the addition of KCl.

When NADH incubated in the medium omitting Cl^-, no oxidation was observed(Fig. 3, Curve 2). But when KCl was added to give a final concentration of 150 mM, the oxidation was started again(Fig. 3, Curve 3) in the same rate to that observed in the presence of KCl in the beginning(Fig 3, Curve 1). These findings indicate that NADH oxidation essentially requires three components, the enzyme, H_2O_2 and Cl^- as a cofactor.

2. Effects of OH· and $^1O_2$ scavengers on NADH oxidation

To test involvement of OH· in the oxidation, effects of its quenchers were studied. Mannitol, sodium benzoate, sodium formate and methanol were employed. None of these quenchers even up to 50 mM did show significant inhibition on the NADH oxidation(Fig. 4. and 5.). In contrast, a $^1O_2$ quencher, DABCO inhibited the oxidation and its inhibitory effect was concentration-dependent; with 50 mM of this quencher the oxidation was completely abolished(Fig. 6.)

Table 1. Comparison of ethylene production from methional by MPO and other oxidation systems

Oxidation systems	Ethylene formed(nmoles) in 5 min	10 min
1. MPO/H_2O_2/Cl^- system	0	0
2. Cu^{++}—H_2O_2 system		
Complete mixture	25.8	37.3
H_2O_2 omitted	0	0
Cu^{++} omitted	0	0
3. X/XO system		
Complete mixture	21.0	30.3
XO omitted	0	0

Medium conditions of each system and determination of ethylene gas were described in Materials and Methods.

3) Comparison of ethylene production from methional by MPO/H_2O_2/Cl^- and other oxidation systems

In the quencher studies, OH· was excluded as a mediator in the MPO-mediated oxidation of NADH. As an alternative to disprove the involvement of this radical, ethylene was measured from methional treated with MPO/H_2O_2/Cl^- system(Table 1). As expected, the MPO system did not produce ethylene. On the other hand, xanthine/xanthine oxidase system which was evidenced to generate OH· through both quencher studies(Kellogg and Fridovich, 1977) and ethylene assay(Beanchamp

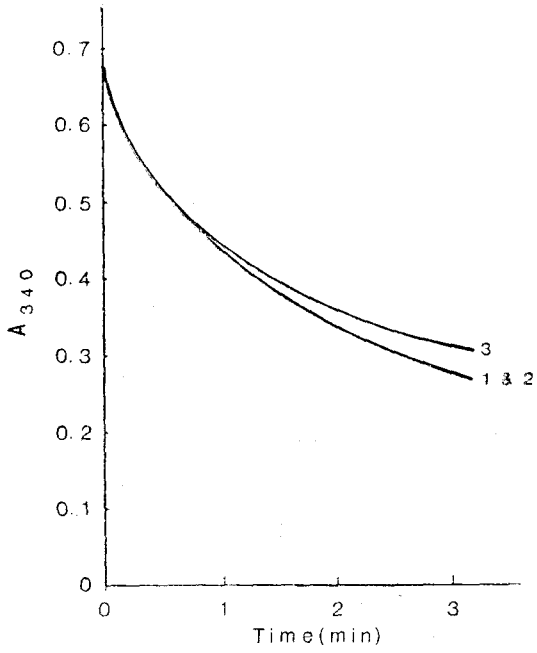

Fig. 4. Effect of mannitol on NADH oxidation by myeloperoxidase system. Reaction was done under the same conditions as in Curve 1 of Fig. 2 in the presence of varying concentrations of mannitol. Curve 1; 0 mM, 2 and 3; 20 and 50 mM mannitol, respectively.

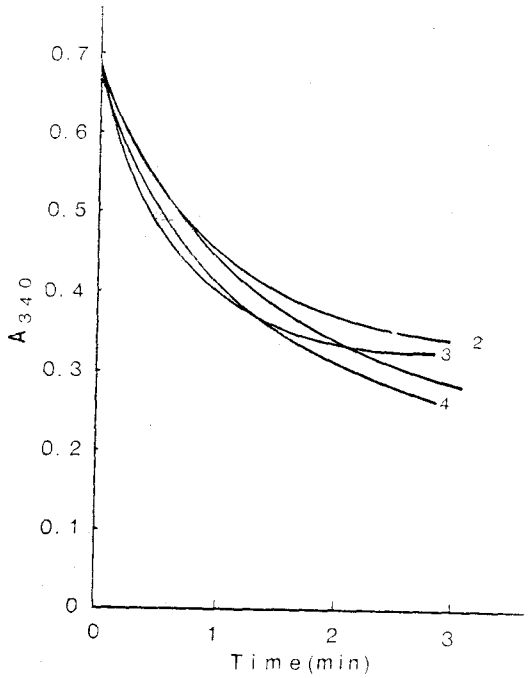

Fig. 5. Effects of other hydroxyl radical quenchers on NADH oxidation. Reaction was performed under the same conditions as in Fig. 4. but in the presence of 50 mM of the quenchers. Curve 1; no quencher, 2; sodium benzoate, 3; sodium formate and 4; methanol.

and Fridovich, 1970) clearly demonstrated the production of ethylene gas(25.8 and 37.3 nmoles at 5 and 10 min of incubation, respectively). When xanthine oxidase was omitted, ethylene was not detected. Further, when 10 μg of superoxide dismutase or 100 μg of catalase was added, the production at 10 min was reduced to 10.1 and 25.0% of the control (data not shown). As shown in other reports (Beauchamp and Fridovich, 1970; Diguiseppi and Fridovich, 1980) this indicates that in the xanthine/xanthine oxidase system OH· is produced from the interaction of O_2^{-}· and H_2O_2 and the resulting radical attacks methional to produce ethylene. Lack of ethylene production in the MPO system was further confirmed by observing the production of the gas in another peroxidative system, $Cu^{++}-H_2O_2$· The latter system was shown to oxidize a number of organic compounds including NADH(Chan and Kesner, 1980; Deasy et al., 1974; Ishimitsu et al, 1979). In this experiment, its oxidative action to NADH was also confirmed. As shown in Fig. 7, the oxidation was inhibited completely by either catalase or EDTA. In contrast to MPO system, $Cu^{++}-H_2O_2$ when react with methional produced ethylene; with 20μM Cu^{++} and 0.5 mM H_2O_2, 21.0 and 30.3 nmoles of ethylene were produced at 5 and 10 min of incubation, respectively. The ethylene production also required both Cu^{++} and H_2O_2.

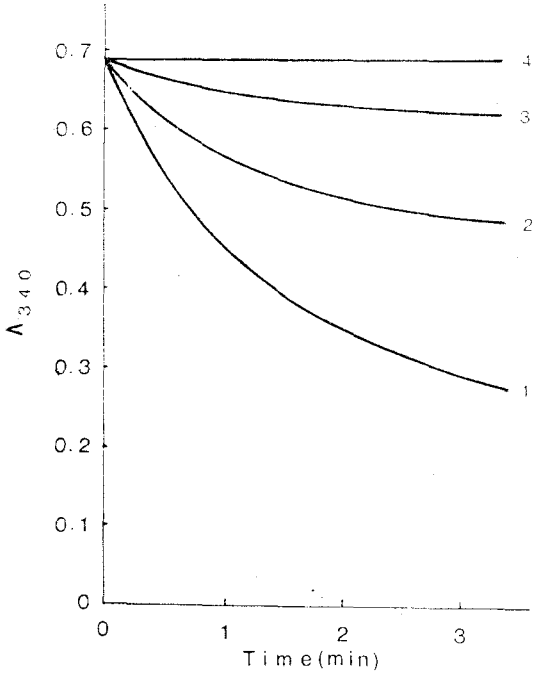

Fig. 6. Effect of 1,4-diazabicyclo(2, 2, 2)octane (DABCO) on NADH oxidation. Reactions were done under the same conditions as in Fig. 5 in the presence of varying concentrations of DABCO. Curve 1, 2, 3 and 4; 0, 10, 20 and 50 mM of DABCO, respectively.

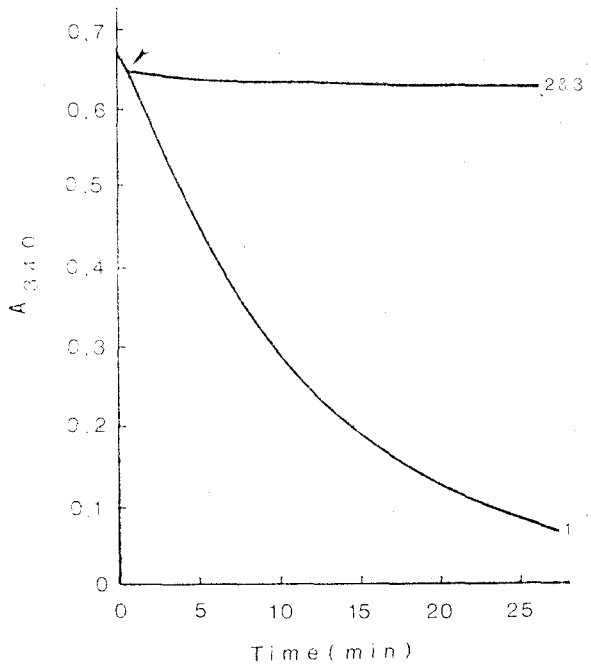

Fig. 7. Effect of $Cu^{++}-H_2O_2$ on NADH oxidation. 3 ml of reaction medium contained 0.11 mM NADH, 20 μM $CuSO_4$, 0.5 mM H_2O_2 and 50 mM sodium phosphate, pH 7.4. Reaction was started by addition of H_2O_2. Curve 1; control, 2 and 3; at arrow 0.2 ml of 5 mM EDTA and 0.01 ml of 5 mg/ml of catalase were added, respectively.

DISCUSSION

The oxidative action of enzyme preparation used in this study was characterized with NADH as a substrate. The rate of the NADH oxidation was dependent on H_2O_2 concentration (Fig. 1) and the oxidation was only possible in the presence of Cl^- (Fig. 3). In the complete reaction medium containing the enzyme, H_2O_2 and the halide, the substrate oxidation was completely inhibited by either catalase to decompose H_2O_2 or azide which is known to be a inhibitor of MPO. These findings indicate that the oxidation of NADH was due to MPO/H_2O_2/halide system. Thus, it is established that the enzyme preparation has properties of MPO, and NADH oxidation with the enzyme preparation can serve as a system where mechanism of MPO-mediated oxidation could be evaluated.

In the present study, the observed oxidation of NADH was not inhibited by all the quenchers of OH· tested, mannitol, formate, benzoate and methanol(Fig. 4 and 5) while a $^1O_2$ quencher, DABCO showed complete inhibition in this respect(Fig. 6). The results obtained from the quencher studies support that NADH oxidation was mediated by $^1O_2$,, and OH· was excluded. So it seems that at least in cell-free MPO system, $^1O_2$ is involved as a principal mediator.

In contrast to the results demonstrating the involvement of $^1O_2$ in the present quencher

studies as well as other reports(Allen, 1975; Klebanoff et al., 1976; Tsan and Chen, 1980), MPO was suggested to be linked to generation of OH·. Diamond et al.(1980) observed that in cell-free MPO system, damage to hyphae of Candida albicans was inhibited by $^1O_2$ scavengers including DABCO but also by a scavenger of OH·, dimethyl sulfoxide. The role of MPO in OH· generation was more implicated in in situ cell system. Klebanoff and Rosen(1978) reported that ethylene production from KMB was decreased in MPO-deficient PMNs as well as normal PMNs in the presence of azide and cyanide. Weiss et al.(1978) also observed that ethylene production from KMB by normal PMNs was inhibited by the MPO inhibitors. In their studies, conversion of KMB to ethylene(Diguiseppi and Fridovich, 1980) was used as evidence of OH· production.

Therefore, as an alternative to test OH· involvement, ethylene production from methional was also determined in the present MPO/H_2O_2/Cl^- system(Table 1). The MPO system, however, did not produce ethylene at all. It was consistant with the findings obtained from the quencher studies showing all the OH· scavengers used demonstrated no effect on NADH oxidation(Fig. 4 and 5). On the other hand, ethylene was produced by xanthine/xanthine oxidase system which was proved to generate OH· possibly through Haber-Weiss reaction.(Beauchamp and Fridovich, 1970; Diguiseppi and Fridovich, 1980). Ethylene was also generated by Cu^{++}—H_2O_2 system which showed the activity to oxidize NADH (Fig. 7). The production of ethylene by the latter system was in accord with the report of Ishimitsu et al.(1979) showing that phenylalanine oxidation to tyrosine by Cu^{++}—H_2O_2 was inhibited by OH· quenchers, although other studies(Chan and Kesner, 1980 and Chung et al., 1984) excluded OH· or even $^1O_2$ in this Cu^{++} -catalyzed peroxidation and suggested still undetermined oxygen species as a mediator. With clear demonstration of ethylene production by other H_2O_2- medieted oxidation systems to produce OH·, the observed lack of ability to produce ethylene from methional can also be evidence that the cell-free MPO system does not generate OH·.

Several possibilities can be considered as a cause to give different results in many studies on oxygen metabolites involved in MPO/H_2O_2/halide system. One possibility is that the situation of MPO system of PMN in situ may be different from that of simple cell-free system. PMN itself is a complex system which has a variety of compounds which may interact with MPO system to produce OH· or other organic radicals. In addition to OH·, organic radicals also produce ethylene from methional indicating that the ethylene assay can not be a specific test for OH·(Pryor and Tang, 1978) This possibility may be supported by the reports showing that peroxidase produced organic radicals as well as OH· depending upon the substrates present, such as thyroxine (Takayama and Nakano, 1977), p-coumaric acid(Halliwell and Ahluwalia, 1976), hydroquinone, ascorbic acid and dihydroxyfumaric acid(Yamazaki et al., 1960). Further, Yang (1969) observed ethylene production from methional or KMB by peroxidase in the presence of sulfite. Even in the cell free system, if the target to be attacked by MPO system is cell such as hyphae, situation may be similar. Moreover, under these circumstances possibly OH· may be generated from the interaction of H_2O_2 and metal ions contaminated in the cells such as Fe^{++}(Clifford and Repine, 1982).

Second possibility is that decrease in ethylene

production by azide or cyanide may be due to their OH· quenching effect rather than inhibition of MPO, since the compounds are suggested to have an ability to scavenge OH· (Singh, 1982). Particularly, this possibility seems to be more probable if ethylene production from methional by stimulated leukocytes may be due to OH· resulting from the interation of O_2^-· and H_2O_2(Tauber and Babior, 1977; Weiss et al., 1977).

The cytotoxicity expressed by PMNs seems to be largely dependent on MPO/H_2O_2/halide system of the oxygen-dependent mechanisms (Clark, 1983). The mechanism of action of the MPO mediated cytotoxic system is not simple and it is probable that MPO, H_2O_2 and the halides interact to produce a toxic agent or agents which affect the target cells. They may be different depending on halides employed as well as substrates to be attacked. But chloride is fully active at concentrations well within the physiologic range and thus be the most relevent cofactor under in situ conditions. In the cell-free MPO/H_2O_2/Cl^- system, $^1O_2$ not OH· was suggested as a mediator possibly through hypochlorous formation which reacts with H_2O_2 to from $^1O_2$ (Allen, 1975; Klebanoff et al., 1976).

SUMMARY

The present study was performed to determine oxygen metabolites involved in cell-free MPO/H_2O_2/Cl^- system by observing the effects of their scavengers on NADH oxidation and ethylene production from methional by the action of MPO prepared from human leukocytes.

It was clearly demonstrated that NADH was oxidized by the cell-free MPO/H_2O_2/Cl^- system as evidenced by complete inhibition of the oxidation of the substrate in the presence of either azide or catalase, or by omitting Cl^-.

The MPO-mediated oxidation of NADH was completely abolished by a $^1O_2$ quencher, DABCO but not by OH· scavengers, mannitol, benzoate, formate and methanol.

In ethylene assay, no ethylene was detected from methional in the MPO/H_2O_2/Cl^- system with evident production of the gas by xanthine oxidase and Cu^{++}—H_2O_2 systems which are suggested to generate OH·.

From the results obtained, it is concluded that $^1O_2$ is a major mediator with exclusion of OH· involvemnt in the cell-free MPO-mediated oxidation.

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