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# EFFECT OF OZONE ON ERYTHROCYTE MEMBRANE ADENOSINE TRIPHOSPHATASE *

ROBERT J. KINDYA and PHILLIP C. CHAN

*Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, N.Y. 11203 (U.S.A.)*

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## Summary

Human erythrocyte membrane fragments were exposed to $O_3$ over varying lengths of time. Ozone was found to have a deleterious effect on the ouabain-sensitive ATPase (EC 3.6.1.3) in the membrane fragments. After 1 min of exposure to $O_3$, which was generated at a rate of 4.0 μmol/min, ouabain-sensitive ATPase activity decreased to 26% of the control. Ouabain-insensitive ATPase was found to be unaffected by $O_3$ exposure under the test conditions. Additions of ascorbic acid or cysteine, prior to $O_3$ exposure, partially protected the enzyme from inactivation. However, the inactivating effect of $O_3$ could not be reversed by addition of either ascorbic acid or cysteine after exposure. Superoxide dismutase or catalase did not afford significant protection. The enzyme could not be protected by Ellman's reagent. The inactivating effect of $O_3$ on the ouabain-sensitive ATPase was also demonstrated in exposure of intact erythrocytes. No detectable change was observed in glycolytic activity in the hemolysate prepared from $O_3$-treated erythrocytes, however. It was postulated that inactivation of the membrane ATPase by $O_3$ may be responsible for the destructive effect of $O_3$ on the red cell.

## Introduction

In 1958 Brinkman and Lamberts [1] reported that inhalation of $O_3$ inhibited the desaturation of oxyhemoglobin in the capillaries of the skin of an occluded digit. They followed up this work by demonstrating that low concentrations of $O_3$, inhaled for a short period of time, potentiated the sphering of erythrocytes in mice, rats, rabbits, and man [2].

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Abbreviation: $Nbs_2$ for Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid).

In 1967 Goldstein and Balchum [3] conducted an investigation based of the well-known ability of $O_3$ to react with the double bonds of unsaturated fatty acids. Employing a system using the enzyme acetylcholinesterase, which is located solely on the red cell membrane, as an indicator of membrane damage, they showed that the red cell membrane was readily affected by $O_3$. Studies on the kinetics of the reaction between $O_3$ and acetylcholinesterase and between $O_3$ and intracellular sulfhydryl levels also revealed that depression of the enzyme activity preceded sulfhydryl oxidation and the acetylcholinesterase effect was independent of experimental manipulation of intracellular sulfhydryl concentration. Additional evidence of membrane damage included the finding of increased osmotic fragility of red cells exposed to $O_3$ [3].

To date there has not been any study on direct relationships between the disruption of specific enzyme systems of the red cell by $O_3$ and the toxic effects that have been observed. Goldstein used acetylcholinesterase only as an indicator of $O_3$ damage to the membrane. He did not relate inactivation of the enzyme to eventual cellular destruction.

Numerous investigators [4—6] have shown that there is a close correlation between the activity of $(Na^+ + K^+)$-activated ATPase in erythrocyte membrane and active transport of cations across the membrane via the sodium pump. Earlier observations by Nakao et al. [7] indicated that maintenance of the shape of the red cell was dependent on the concentration of intracellular ATP. Sphering of the cell was observed when its intracellular ATP concentration fell to 10% of the control. These results appear to link maintenance of the shape of the red cell to the ATPase-related sodium pump. Therefore, it may be postulated that the potentiation of spherocytosis of erythrocytes observed by Brinkman and Lamberts [1] may possibly be related to inactivation of the $(Na^+ + K^+)$-activated ATPase in the red cell membrane.

This study was conducted to investigate the effect of $O_3$ on the activity of the $(Na^+ + K^+)$-activated ATPase in the red cell membrane.

## Materials and Methods

All chemicals used were of the highest purity available. Ouabain (strophantin-G) was obtained from Sigma Chemical Co. Catalase (Catalog no. 15674) was from Boehringer Mannheim Corp. Superoxide dismutase was isolated according to the procedure of McCord and Fridovich [8].

*Ozone generation.* Ozone was generated by passing oxygen through a Supelco micro-ozonizer (Catalog no. 06-0615). The oxygen stream was passed through at a flow rate of 25 ml/min. The control unit on the ozonizer was set at the minimum. Under these conditions $O_3$ was generated at a rate of 4.0 $\mu$mol/min. The rate of $O_3$ production was monitored by bubbling the gas stream into 1% (w/v) KI in 0.1 M phosphate (pH 7.0) as described by Saltzman [9].

*Preparation of the erythrocyte membrane fragments.* Whole blood was obtained from healthy volunteers and used within 3 h. All preparations of erythrocyte membrane fragments were made using fresh blood. Erythrocyte "ghosts", free of hemoglobin, were prepared by hemolysis according to the method of Dodge et al. [10], with minor modification [11]. The protein concentration of the membrane preparation was determined using the method of Lowry et al. [12].

*Treatment of the erythrocyte membrane preparation with ozone.* Ozone was bubbled directly into a 1.0 ml suspension of the erythrocyte membrane fragments to which 5 μl caprylic alcohol had been added as an antifoaming agent. In a control experiment, caprylic alcohol was found to have no effect on any of the assays or other procedures followed.

*ATPase assay.* ATPase activity was measured according to the procedure described by Walz and Chan [11]. The standard reaction mixture, in a final volume of 2.0 ml, contained 100 mM NaCl, 25 mM KCl, 5 mM $MgCl_2$, 100 mM imidazole · HCl (pH 7.6), 2.5 mM ATP (as Tris salt), membrane fragments equal to a final concentration of 0.30—0.35 mg protein/ml, and other additions as indicated. The reaction was incubated at 37° C, with continuous shaking, for 1 h. The reaction was terminated by addition of 1.0 ml 15% (w/v) trichloroacetic acid. The resulting precipitate was removed by centrifugation. The clear supernatant fluid was assayed for inorganic phosphate activity according to the method of Horwitt [13]. Specific activity of ATPase is expressed as nmol inorganic phosphate released/mg membrane protein per h.

The results presented in the tables and the figure are from representative experiments. Although the absolute values of membrane ATPase specific activity varied somewhat from membrane preparation to membrane preparation this variation did not have significant influence on the observations made in this study. Each of the effects of $O_3$ and other treatments were tested in at least three different membrane preparations and the results were consistant in all replicate experiments.

*Exposure of intact red cells to ozone.* Before exposure, the red cells were washed three times with 0.17 M NaCl. 10 ml packed cells were then diluted with an equal volume of 0.17 M NaCl. $O_3$ was bubbled directly into the red cell suspension which contained 20 μl caprylic alcohol as an antifoaming agent. The suspension was stirred continuously during exposure. The 20-ml suspension was exposed to 12 μmol $O_3$/min. After exposure the cells were washed twice with the 0.17 M NaCl and erythrocyte membrane fragments were then prepared as before.

*Assay for glycolytic activity.* This assay was used to assess the effect of $O_3$ exposure on the glycolytic pathway in erythrocytes. Intact red cells were exposed to $O_3$ in a manner similar to that described above. After exposure, the red cells were washed twice with 0.17 M NaCl. They were then quickly hemolyzed in two volumes of ice-cold water. The reaction mixture, in a final volume of 2.0 ml, contained 100 mM KCl, 25 mM NaCl, 5 mM $MgCl_2$, 10 mM glucose, 1 mM ADP (as Tris salt), 2.5 mM ATP (as Tris salt), 1.25 mM NAD, 12.5 mM phosphate (pH 7.5), 50 mM Tris · HCl (pH 7.5), and 1.0 ml of hemolysate. The reaction was incubated at 37°C, with continuous shaking, for 1 h and then terminated by addition of 1.0 ml 30% (w/v) $HClO_4$. The resulting precipitate was removed by centrifugation. The clear supernatant fluid was assayed for lactate with lactate dehydrogenase according to the procedure described by Hohorst [14].

## Results and Discussion

Erythrocyte membrane fragments were exposed to $O_3$ for varying lengths of time and then assayed for ATPase activity. The results are depicted in Fig. 1. It

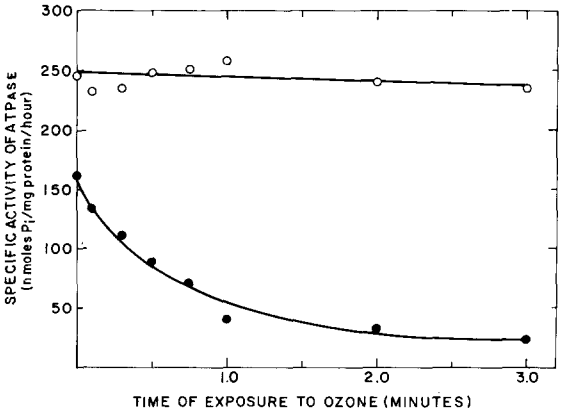


Fig. 1. Effect of length of exposure to $O_3$ on ATPase activity in erythrocyte membrane fragments. Ozone was generated at a rate of 4.0 μmol/min and bubbled directly into a 1.0-ml sample of membrane fragments. The membrane fragments were then assayed for ATPase activity as described in Materials and Methods. ○, activity of ouabain-insensitive ATPase; ●, activity of ouabain-sensitive ATPase.

is apparent that $O_3$ had a deleterious effect on the activity of the $(Na^+ + K^+)$-activated ATPase (ouabain-sensitive ATPase). The enzyme activity decreased with increasing length of exposure. After 1 min of $O_3$ exposure, the activity of the ouabain-sensitive ATPase was only 26% of the control. On the other hand, the activity of the ouabain-insensitive ATPase (which is often referred to as the $Mg^{2+}$-ATPase) did not change significantly throughout the exposure period. For each of the remaining experiments the activity of ouabain-insensitive ATPase was tested and it was found to be unaffected by $O_3$ exposure. For this reason, data regarding the effect of $O_3$ on the ouabain-insensitive ATPase are omitted in the following discussion of the remaining experiments.

Ample evidence implicating free radicals as a basic biochemical mechanism of $O_3$-induced cell damage has been indicated by many experiments showing the similarity of the action of $O_3$ to that of ionizing radiation [1,15,16]. Ozone has been recognized as a radiomimetic substance [1,2]. In this regard it has been shown by Fairchild et al. [17] that substances which protected against the effects of radiation have also afforded protection against the toxic effects of $O_3$. The protective effects of two of these substances were tested with regard to the inactivation of red cell membrane ATPase by $O_3$.

Cysteine and ascorbic acid in two different concentrations (2 and 20 mM) were added to the membrane fragments prior to exposure to $O_3$. ATPase activity was assayed in the usual manner following exposure and the results are presented in Table I. As may be seen by comparing the activity of the enzyme in Tubes 3—6 with control Tube 1, both ascorbic acid and cysteine afforded protection to the enzyme against $O_3$. As expected, the higher concentration gave greater protection. Tubes 7 and 8 were prepared to check whether the inactivating effect of $O_3$ could be reversed by addition of cysteine or ascorbic acid after $O_3$ exposure. To this end these substances were added, in the higher concentration, to the membrane preparation after it had been exposed. As indicated

TABLE I

PROTECTION OF OUABAIN-SENSITIVE ATPase IN ERYTHROCYTE MEMBRANE FRAGMENTS BY ASCORBIC ACID AND CYSTEINE AGAINST INACTIVATION BY OZONE

Preparation of membrane fragments, method of exposure, reaction mixture, and ATPase assay are described in Materials and Methods. Ascorbic acid and cysteine were neutralized prior to addition. Ozone generation was at a rate of 4.0 μmol/min. Activity of the ouabain-sensitive ATPase is expressed as nmol inorganic phosphate released/mg membrane protein per h.

| Tube No. | Additions prior to $O_3$ exposure | $O_3$ exposure (min) | Additions after $O_3$ exposure | ATPase activity | Inhibition (%) |
|---|---|---|---|---|---|
| 1 | — | 0 | — | 157 | 0 |
| 2 | — | 2 | — | 33 | 79 |
| 3 | 2 mM Ascorbic acid | 2 | — | 87 | 45 |
| 4 | 20 mM ascorbic acid | 2 | — | 126 | 20 |
| 5 | 2 mM cysteine | 2 | — | 91 | 42 |
| 6 | 20 mM cysteine | 2 | — | 121 | 23 |
| 7 | — | 2 | 20 mM ascorbic acid | 39 | 77 |
| 8 | — | 2 | 20 mM cysteine | 33 | 79 |

by Tubes 7 and 8 in Table I the inhibitory effect of $O_3$ could not be reversed by either cysteine or ascorbic acid.

As mentioned earlier, Goldstein and Balchum [3] have demonstrated the ability of $O_3$ to react with cellular membranes and, more specifically, with the unsaturated fatty acids which constitute an essential part of the membrane structure. They have shown that $O_3$ breaks down the unsaturated fatty acids through the mechanism of lipid peroxidation [3]. The breakdown of the unsaturated fatty acids might be initiated by either direct ozonolysis or interaction with free radicals derived from $O_3$.

A series of reactions of $O_3$ in aqueous solution has been postulated [18,19]. One such reaction leads to the formation of superoxide radicals ($O_2^-$ and $HO_2$):

$$O_3 + OH^- \rightarrow O_2^- + HO_2$$

Superoxide radicals have been shown to be deleterious to biological systems [20]. They have been implicated in the phenomenon of lipid peroxidation and particularly in the peroxidative hemolysis of red blood cells [21]. They have also been shown to generate $H_2O_2$ [20]. In order to determine whether superoxide radicals and/or $H_2O_2$ might be responsible for the inactivating effect of $O_3$ on ouabain-sensitive ATPase, superoxide dismutase or catalase was added to the erythrocyte membrane preparation prior to exposure to $O_3$. Superoxide dismutase destroys the superoxide radical through disproportionation [20].

The experimental results indicated that superoxide dismutase offered only slight protection to the enzyme while addition of catalase had no detectable effect. As a further check, $H_2O_2$ was substituted for $O_3$ exposure. Results indicated that even when the $H_2O_2$ concentration was as high as 16 mM (four times as much as would be expected to derive from $O_3$ in the control tube), $H_2O_2$ alone inhibited the activity of the ouabain-sensitive ATPase by only 16%. It was concluded from these data that at most only a small fraction of the inactivation of ATPase by $O_3$ can be attributed to either superoxide or $H_2O_2$.

Menzel [22] has shown that $O_3$ is highly reactive towards enzyme sulfhydryl

groups. Chan and Rosenblum [23] have demonstrated that certain sulfhydryl groups are essential in the normal functioning of ATPase in erythrocyte membrane. They have shown that treatment of the erythrocyte membrane fragments with Ellman's reagent ($Nbs_2$), (5,5'-dithio-bis-(2-nitrobenzoic acid)), completely abolished the activity of the ouabain-sensitive ATPase, presumably by converting essential sulfhydryl groups to mixed disulfides. Subsequent regeneration of the sulfhydryl groups by dithiothreitol restored about 80% of the enzyme activity.

This procedure was used to test whether $Nbs_2$ treatment on the membrane fragments may protect the ATPase from attack by $O_3$. The results of this experiment are presented in Table II.

Membranes in Tubes 1—3 were not exposed to $O_3$. The results were similar to those reported earlier [23]. In Tube 2, 94% of the enzyme activity was abolished by treatment with $Nbs_2$. Subsequent treatment with dithiothreitol regenerated 86% of the control activity. Membrane fragments in Tubes 4 and 5 were treated with $Nbs_2$ and then exposed to $O_3$. As expected, the ouabain-sensitive ATPase activity was completely abolished by this treatment (Tube 4). Moreover, as shown in Tube 5, dithiothreitol could not regenerate any ATPase activity in the $Nbs_2$- and $O_3$-treated membrane fragments. It was concluded from these data that $Nbs_2$ was not able to protect the enzyme system from inactivation by $O_3$. Ozone may have oxidized the essential sulfhydryl groups to a higher oxidative state that could not be reversed by dithiothreitol. Similar postulations have been made by others regarding the deleterious effect of $O_3$ on other enzyme sulfhydryl groups [22]. It is also possible that the effect of $O_3$ was directed towards some essential components of the enzyme system other than the sulfhydryl groups.

It has been demonstrated thus far that $O_3$ has a deleterious effect on the ouabain-sensitive ATPase in erythrocyte membrane fragments. It is important to determine whether this effect could be demonstrated on intact cells. During

TABLE II

LACK OF PROTECTION OF OUABAIN-SENSITIVE ATPase BY $Nbs_2$-TREATMENT

Exposure to $O_3$ was as described in Materials and Methods. Ozone was generated at a rate of 4.0 μmol/min. Ouabain-sensitive ATPase activity is expressed as nmol inorganic phosphate released/mg membrane protein per h.

| Tube No. | $Nbs_2$ treatment * | $O_3$ exposure (min) | Dithiothreitol treatment ** | ATPase activity |
|---|---|---|---|---|
| 1 | — | 0 | — | 124 |
| 2 | + | 0 | — | 7 |
| 3 | + | 0 | + | 107 |
| 4 | + | 2 | — | 0 |
| 5 | + | 2 | + | 0 |

* Membrane fragments were incubated with 10 mM $Nbs_2$ at 37°C for 30 min. The fragments were then washed twice with 12 mM Tris . HCl (pH 7.5).

** Regeneration was accomplished by incubating the fragments with 10 mM dithiothreitol at 37°C for 30 min. After incubation, excess dithiothreitol was removed by washing the fragments twice with 12 mM Tris · HCl (pH 7.5). They were then assayed for ATPase activity as described in Materials and Methods.

TABLE III

EFFECT OF EXPOSURE OF INTACT RED CELL TO OZONE ON INACTIVATION OF MEMBRANE OUABAIN-SENSITIVE ATPase

Treatment of intact cells, method of exposure, and assay procedure are described in Materials and Methods. Activity of the ouabain-sensitive ATPase is expressed as nmol inorganic phosphate released/mg membrane protein per h.

| Tube No. | Conditions of exposure | ATPase activity |
|---|---|---|
| 1 | Control, no exposure | 206 |
| 2 | Exposed to $O_2$, 8 min * | 205 |
| 3 | Exposed to $O_3$, 8 min ** | 96 |

* To ascertain the effect of the bubbling procedure, $O_2$ was bubbled into the intact erythrocyte sample at a flow rate of 50 ml/min.

** The intact cells were exposed to a total $O_3$ concentration of 4.8 μmol/ml.

the membrane preparation procedure the red cell membrane undergoes various fragmentations and inversions. Therefore, it is essential to establish whether the treatment of the erythrocyte membrane during preparation of the fragments had any effect on the lability of the membrane ATPase towards $O_3$. Accordingly, intact erythrocytes were exposed to $O_3$ directly and membrane fragments were prepared from these $O_3$-treated cells and assayed for ATPase activity. The results in Table III indicate that the inactivating effect of $O_3$ on the ouabain-sensitive ATPase was also observed on the membrane of intact cells.

The extent of exposure to $O_3$ of these intact cells differed slightly from that of the membrane fragments. Due to the larger sample volume required, the intact cell suspension was exposed to $O_3$ for a longer time than were the membrane fragments. The total concentration of $O_3$ per ml to which the intact cells were exposed was still less than that of the fragments, however. The intact cell suspension was exposed to a total $O_3$ concentration of 4.8 μmol/ml while the membrane fragments were exposed to 8.0 μmol/ml. This may account for the smaller inhibition of the ouabain-sensitive ATPase activity observed in the membrane preparation made from these $O_3$-treated intact red cells than in the membrane fragments which were exposed directly to $O_3$. Exposure of the cells to higher concentrations of $O_3$ was not attempted because bubbling of the $O_3$ through the red cell suspension caused extensive hemolysis. This increase in hemolysis, however, had no effect on the subsequent determinations of enzyme activity because hemolyzed cells were washed away after $O_3$ exposure. Only the remaining intact cells were used to prepare the membrane fragments for ATPase assay.

Since $O_3$ exhibited a deleterious effect on the membrane of intact red cells, the effect of $O_3$ exposure on glycolysis, an intracellular system of erythrocytes, was also investigated.

Intact red cells were exposed to $O_3$, washed three times with 0.17 M NaCl, hemolyzed, and assayed for glycolytic activity. The exposure of the intact erythrocytes was similar to that described in Table III. The red cells were exposed to 4.8 μmol $O_3$/ml.

Experimental results indicated that there was no significant effect on glycolytic activity in the hemolysate of $O_3$-treated red cells under the test condi-

tions. This finding concurs with Goldstein's study comparing the effect of $O_3$ on acetylcholinesterase of the red cell membrane with the oxidation of intracellular sulfhydryl groups.

It has been shown that the red cell membrane ($Na^+$+$K^+$)-activated ATPase is a primary target of $O_3$ attack on an intact red cell. Since the ATPase is essential for maintenance of the shape and integrity of the red cell, the earlier observations on the $O_3$-potentiated sphering of erythrocytes and osmotic fragility may be secondary effects following the inactivation of the ($Na^+$+$K^+$)-activated ATPase in the red cell membrane.

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