Effects of Oxidant Stress on the Hexose Monophosphate Shunt Pathway of Platelets

By Alex Koufos and Arthur L. Sagone

Platelet function may be altered by the oxygen metabolites generated by phagocytic cells. This raised the question whether oxidant injury to platelets is associated with a depletion of reduced glutathione, since this compound is known to be important in the protection of other blood cells against oxidant damage. We studied the effects of the oxygen metabolites generated by the enzyme system xanthine-xanthine oxidase on the glutathione (GSH) stability and the metabolism of the hexose monophosphate pathway (HMPS) of platelets. Platelet suspensions incubated with the enzyme system had marked stimulation of the HMPS pathway and did not have GSH instability. In spite of this, the platelet suspensions had impaired aggregation to adenosine diphosphate (ADP). By using specific scavengers, we found that this altered metabolism and function was related to H2O2 and not H2O2. Since this compound is known to be sensitive to oxidant injury to platelets, we suggest that platelets produce O2 and that this oxygen radical may be degraded by the HMPS pathway via glutathione peroxidase. These data indicate that oxidant injury to platelets occurs in spite of active degradation of H2O2 and suggest that these cells may not be able to protect some sensitive cellular sites during exposure of exogenous H2O2. Our studies also suggest that platelets produce O2 and that this oxygen radical cannot react directly with HMPS of platelets.

Recent studies by several laboratories have suggested that platelet function may be altered as a result of exposure to hydrogen peroxide.1-9 Levine et al. demonstrated that the amount of H2O2 generated by granulocytes is sufficient to impair platelet aggregation.1 This observation suggests that platelet function may be altered in vivo as a result of the H2O2 generated by phagocytic cells. A number of morphological and biochemical changes have been reported in platelets as a result of this oxidant stress and may be related to their altered function.2-9 In addition to H2O2, it has now been established that granulocytes generate reactive oxygen species in addition to H2O2. These include superoxide, hydroxyl radical, and possibly singlet oxygen.10 Recent studies indicate that superoxide may induce aggregation.7 Therefore, these species as well as H2O2 may also modify platelet function in vivo.

Several enzymes are known to be important in the protection of mammalian cells against oxidant injury.11-17 These enzymes include superoxide dismutase, glutathione peroxidase, and catalase. Glutathione peroxidase appears to be the preferential pathway for the degradation of low concentrations of H2O2 in the intact cell.13-17 The degradation of hydrogen peroxide by glutathione peroxidase is dependent on the pentose phosphate pathway. Glutathione peroxidase is known to require reduced glutathione as a cofactor. GSH, which is oxidized during the degradation of H2O2, is regenerated by glutathione reductase, an enzyme dependent on NADPH as a cofactor. The NADPH required for the reduction of GSSG is provided by the hexose monophosphate shunt pathway of the cell.12-17 Presumably, the degradation of superoxide by the intact cell requires its conversion to H2O2.11 The H2O2 would then be degraded by glutathione peroxidase. However, the possibility that O2 may be degraded by other pathways has not been extensively studied. In this regard, there is evidence that under some circumstances, stimulation of the HMPS may reflect direct reaction of NADPH with oxidants.12,16-19 This raised the question whether O2 might be degraded directly by NADPH in the intact platelet. One aim of our experiments was to test this possibility.

Cells that are deficient in enzymes related to the HMPS are known to be sensitive to oxidant injury. A classic example is RBC deficient in glucose-6-phosphate dehydrogenase.12-17 Incubation of these cells with H2O2 is associated with a depletion of reduced glutathione, a biochemical abnormality that appears to be associated with irreversible damage to the cell.17 This raised the question whether exposure of platelets to H2O2 in concentrations that impair platelet function is associated with the oxidation of reduced glutathione in this cell.

For these reasons, we have investigated the effects of several oxygen metabolites on the HMPS pathway and GSH stability of human platelets. We have studied the effect of the enzyme system xanthine-xanthine oxidase, which is known to generate continuous low

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concentrations of several of the oxygen metabolites produced by phagocytic cells. These include superoxide, hydrogen peroxide, and hydroxyl radicals. Thus, this enzyme system should approximate the type of exposure to these agents which platelets might encounter in vivo as a result of contact with stimulated polymorphonuclear leukocytes (PMN). The effect of this enzyme system on the metabolism of the platelets was correlated with its effects on platelet function as determined by aggregation.

**MATERIALS AND METHODS**

**Preparation of Platelets**

Siliconized glassware and plastic tubes and syringes were used throughout. Venous blood (approximately 50 cc) was drawn from normal human volunteers in one-sixth volume ACD. These persons had not taken medications for at least 1 wk prior to the study. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 g for 10 min. Contaminating red and white cells were removed by centrifugation of the PRP at 1000 g for 45 sec. Platelets were then concentrated by centrifugation of the PRP at 1000 g for 10 min. Most of the platelet-poor plasma was then removed. The remaining platelet button in approximately 0.2 cc of plasma was resuspended in Earle's balanced salt solution for metabolic studies or 5 cc Tyrode's buffer for aggregation studies. Platelets were counted on an FN coulter counter. Microscopic examination of the platelet suspensions revealed less than 0.1% contamination by other cells. These were primarily red cells and lymphocytes.

**Platelet Aggregation Studies**

Platelet aggregation was studied in fresh platelet suspensions in Tyrode's buffer at 37°C by the turbidimetric method. We also studied platelet aggregation morphologically. The platelet suspensions (2.50 x 10⁹ in 1 cc of buffer) were preincubated with the enzyme systems xanthine-xanthine oxidase for 15 min before aggregation studies were done with ADP (2 x 10⁻⁴ M). In some experiments, the protective effect of scavengers were also studied. The final concentrations of the reagents used were 0.1 mM xanthine (Sigma, St. Louis, Mo.), 0.0075 U/ml xanthine oxidase (Sigma, Grade 1, 0.75 U/mg protein), 40 μg/ml superoxide dismutase (Truett Laboratories, 3000 U + /mg), 1000 U/ml catalase (Sigma, from beef liver 36,000 U/mg), 40 mM mannitol (Merek, Sharp, and Dohme), ADP 2 x 10⁻⁴ M (Sigma), and allopurinol 10⁻⁴ M. Allopurinol was a gift of Burroughs Wellcome Co. For some experiments, the enzymes were heat-denatured by autoclaving them for 30 min.

**Continuous Measurement of the Hexose Monophosphate Shunt Pathway Activity**

The platelet suspensions (10⁹) were incubated at 37°C in 25-ml flasks with 3 air-tight ports in a media consisting of Earle's balanced salt solution (4 ml) and 5μCi of [14C]-1-glucose or [14C]-formate. The inlet arm of the flask was connected to a gas cylinder containing compressed air with 5% CO₂. The outlet arm of the flask was connected to a 275-ml Cary-Tolbert ionization chamber and Cary Model 401 vibrating reed electrometer. The third arm of the flask was covered with a rubber stopper. The use of the ionization chamber-electrometer apparatus for continuous monitoring of CO₂ produced by platelet suspensions has been described elsewhere. A duplicate system was used so that [14C]CO₂ derived from the substrate could be measured simultaneously from both control and experimental flasks. The incubation flasks were maintained at 37°C throughout these experiments and were stirred continuously.

During the experiments reagents were added through the inlet arm of the flask. The final concentrations of these reagents were similar to those used in the aggregation study. CO₂ production was calculated from the continuous millivolt reading as previously described and expressed as nanomoles of CO₂ produced per hour per 10⁹ cells. The peak oxidation of [14C]-1-glucose occurring with xanthine-xanthine oxidase in the presence and absence of scavengers was used to compare the control and experimental values (see Fig. 2).

**Effect of Oxidant Injury on the GSH Stability of Platelets**

Approximately 2.50 x 10⁹ platelets were suspended in 1 ml of Earle's balanced salt solution and incubated with the enzyme system xanthine-xanthine oxidase for 1 hr. Control suspensions were also prepared and treated similarly. After the incubation, EDTA was added to the suspension so that the final concentration was 5 mM and the specimen was rapidly frozen in dry-ice acetone. Following this, the concentration of reduced sulphydrals was determined by a modification of the methods of Vogt et al. and Beutler et al. Cell suspensions were frozen and thawed three times to lyse the cells. Samples were then boiled in a heated water bath for 10 min to produce maximum deproteinization. Solutions were cleared of particulate matter by centrifugation in a refrigerated Sorvall Model RC2-B centrifuge for 20 min at 25,000 g. Aliquots of 0.75-1.0 ml of the supernatant were removed and added to 2.0 ml of 0.15 M Na and K phosphate buffer adjusted to pH 7.34. Colorimetric determinations were done with a Beckman Model DB spectrophotometer at 412 nm within 30 sec following the addition of 0.25 ml of 40 mg/dl 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and vigorous mixing. The optical density in experimental samples was corrected by subtraction of identically processed blanks containing buffer. These findings contributed insignificantly to final results. Values were expressed as nanomoles of reduced SH/10⁹ cells.

**Statistical Analysis**

Data were analyzed according to the t test for independent samples.

**RESULTS**

**Effect of Oxidant Stress on Platelet Aggregation**

Figure 1 shows the effect of the enzyme system, xanthine-xanthine oxidase, on platelet aggregation to ADP. As seen, platelet suspensions preincubated with the enzyme system for 15 min failed to aggregate to ADP. No impairment in platelet aggregation was noted in suspensions preincubated with the enzyme system and allopurinol (data not shown). These latter experiments demonstrated that xanthine oxidase was required for the impaired platelet function observed in our experiments. Platelet suspensions were also examined morphologically for aggregation. Suspensions preincubated with the enzyme system did not have microaggregates before or after the addition of ADP.

In order to establish that the adverse effects of the enzyme system on platelet aggregation were related to oxidant injury, we studied the protective effect of several agents that are known scavengers of reactive
HMPS ACTIVITY OF H2O2 STIMULATED PLATELETS

was not protective, indicating that active catalase enzyme system. In fact, platelet aggregation against the effects of catalase were completely protected by oxygen compounds. Suspensions supplemented with catalase were completely protected against the effects of the enzyme system. In fact, platelet aggregation might have been mildly enhanced. Heat-denatured catalase was not protective, indicating that active enzyme was required. In contrast, suspensions supplemented with superoxide dismutase, a scavenger of \( \cdot \text{O}_2 \), were not protected.

The generation of singlet oxygen and hydroxyl radical by the xanthine oxidase enzyme system appears to require the interaction of \( \cdot \text{O}_2 \) and \( \cdot \text{H}_2\text{O}_2 \). Therefore, the fact that superoxide dismutase did not protect the platelets against the effect of the xanthine oxidase enzyme while catalase did implies that \( \cdot \text{O}_2 \) and any hydroxyl radicals or single oxygen derived from them were probably not involved in the injury caused by this system.

In order to further exclude a role of hydroxyl radicals, the protective effect of mannitol, an agent known to be a hydroxyl scavenger, was studied. This agent, in concentrations as high as 40 mM, was not protective.

The addition of catalase, SOD, or mannitol alone to platelet suspensions did not impair platelet aggregation to ADP.

**Effect of the Enzyme System on the HMPS Pathway of Platelets**

We next studied the metabolism of the HMPS pathway during incubation with the xanthine-xanthine oxidase enzyme system utilizing the oxidation of \(^{14}\text{C}-1\)-glucose. As indicated in Fig. 2, platelets had a gradual increase in the oxidation of \(^{14}\text{C}-1\)-glucose during the first hour of incubation. At this point, the addition of the enzyme system resulted in a prompt increase in the oxidation of \(^{14}\text{C}-1\)-glucose, which peaked within 45 min. As we have previously reported, the oxidation of \(^{14}\text{C}-1\)-glucose, during the first hour under our conditions reflects primarily HMPS activity rather Krebs cycle activity. The minimal oxidation of \(^{14}\text{C}-6\)-glucose by platelet suspensions at 1 hr was not increased during incubation with the enzyme system, indicating that the increased \(^{14}\text{C}-1\)-glucose oxidation was related to a stimulation of the HMPS pathway. Allopurinol inhibited the stimulation of \(^{14}\text{C}-1\)-glucose by the enzyme system, indicating that xanthine oxidase was required for the reaction.

![Fig. 1. Platelet aggregation to ADP following exposure to the enzyme system xanthine (X)-xanthine oxidase (XO). The y axis indicates the percentage change in light transmission by the platelet suspensions. The experimental suspensions indicated were preincubated with X + XO, X + XO + catalase, or X + XO + SOD. Control suspensions were also preincubated for 15 min and included platelet suspensions without additives, and those with xanthine, xanthine oxidase, SOD, and catalase alone. The experiment is typical of 3 performed.](image1)

![Fig. 2. Effect of xanthine and xanthine oxidase on the oxidation of \(^{14}\text{C}-1\)-glucose by platelet suspensions. The curves represent a continuous measurement of \(^{14}\text{CO}_2\) production by platelets in buffer using the ionization chamber electrometer system (see Materials and Methods). The datum points were drawn from the means of 3 experiments. The bars indicate SD. The y axis indicates the rate of \(^{14}\text{CO}_2\) production from the suspensions. The upper curve indicates the \(^{14}\text{CO}_2\) generated by suspensions to which xanthine and xanthine oxidase were added at 1 hr while the lower curve indicates suppression supplemented with catalase (1000 U/ml) prior to the addition of X + XO. As seen, the addition of X + XO resulted in a prompt increase in \(^{14}\text{C}-1\)-glucose oxidation, which peaked at 45 min. In contrast, suspensions supplemented with catalase failed to show this stimulation. Similar experiments were done in the presence of SOD and mannitol. The peak rates achieved with these agents were compared to controls and are tabulated in Table 1.](image2)
these agents on the peak stimulation of $^{14}$C-1-glucose on platelet suspensions after the addition of the enzyme system are summarized in Table 1. As indicated, catalase virtually ablated the enhanced oxidation of $^{14}$C-1-glucose by the enzyme system. The kinetics of the reaction are indicated in Fig. 2. In contrast, mannitol, a scavenger of OH, had no effect and SOD increased the stimulation twofold. As seen in Fig. 3, only a small portion of the augmentation by SOD could be attributed to its effects on the HMPS in the absence of the enzyme system. It is of interest that a similar augmentation of $^{14}$C-1-glucose oxidation by SOD could be demonstrated if the enzyme was added to the suspensions at the time of the peak stimulation by the xanthine-xanthine oxidase system (Fig. 4).

Table 1. Effect of Scavengers on the Stimulation of $^{14}$C-1-Glucose Oxidation by Xanthine-Xanthine Oxidase

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Catalase</th>
<th>SOD</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>593</td>
<td>57</td>
<td>1141</td>
<td>542</td>
</tr>
<tr>
<td>SD</td>
<td>± 77</td>
<td>± 10</td>
<td>± 171</td>
<td>± 20</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>p Value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Values are given in n mole $^{14}$CO$_2$ produced/hr/10$^8$ platelets and were derived from the peak oxidation occurring in the suspension after the addition of xanthine-xanthine oxidase. No difference in the kinetics were noted (see Fig. 2). Heat-denatured catalase and SOD did not alter the stimulation of $^{14}$C-1-glucose, indicating that active enzyme was required.

The Effect of the Enzyme System on the Reduced Soluble Sulfhydryl (RSS) Concentration of Platelets

For eight experiments, the mean concentration of the RSS of platelet suspensions incubated with the enzyme system for 60 min was 15.00 ± 4.9 n mole/2.5 x 10$^8$ platelets compared to 16.00 ± 5.1 for the controls (NS). These values were similar to fresh platelet suspensions and indicate that there was no evidence of GSH instability during the time of maximal HMPS activity.

The Effect of the Enzyme System on the Oxidation of $^{14}$C-Formate by Platelet Suspensions

Since formate is oxidized to CO$_2$ in the presence of catalase and H$_2$O$_2$, we used this system to determine if H$_2$O$_2$ might be reduced by cellular catalase. Platelet suspensions did not oxidize detectable amounts of formate (Fig. 4) before or after the addition of xanthine and xanthine oxidase. As expected, when platelet suspensions were supplemented with catalase in a concentration that ablated the stimulation $^{14}$C-1-glucose by the enzyme system, there was a marked increase in $^{14}$C-formate oxidation. These observations indicate that supplemental catalase effectively scavenged the H$_2$O$_2$ generated by the enzyme system.

DISCUSSION

Our results indicate that the functional capacity of normal human platelets may be impaired by exposure to H$_2$O$_2$. In our studies, we used the enzyme system,
xanthine-xanthine oxidase, which is known to generate low continuous concentrations of several highly reactive oxygen species. These include superoxide, hydrogen peroxide, hydroxyl radicals, and possibly singlet oxygen.\textsuperscript{2} The protective effect of catalase compared to the other scavengers indicates that the injury caused by this enzyme system is related primarily to H$_2$O$_2$. Our results, therefore, confirm the observation of others, which indicates that low concentrations of H$_2$O$_2$ may impair platelet aggregation.\textsuperscript{14}

In contrast to the effects of H$_2$O$_2$ on platelet function, other reactive oxygen species may produce opposite effects. Recently, Handin et al. have shown that platelets exposed to a high flux of O$_2$ may aggregate.\textsuperscript{7} They also used the enzyme system xanthine-xanthine oxidase to generate reactive oxygen species for their experiments. However, the concentration of the enzyme system required to induce aggregation was much higher than the one used in our study. Similar to our findings, they reported that a concentration of the enzyme system comparable to the one used for our studies did not induce aggregation. They observed that platelets had an increased sensitivity to aggregation with thrombin following a 5-min exposure to this lower flux of O$_2$. They did not study the effect of longer incubations similar to those used in our experiments or the subsequent response of these platelets to other aggregating agents. Therefore, the antagonistic effects of two reactive oxygen species may be important in their overall effect on platelet function and requires further study.

We found that incubation of platelets with the xanthine-xanthine oxidase system was associated with marked stimulation of the hexose monophosphate shunt pathway. The enhanced oxidation of $^{14}$C-1-glucose by the enzyme system could be virtually ablated by concentrations of catalase that were shown to be protective in our aggregation studies. Therefore, the stimulation appears primarily related to H$_2$O$_2$ generated by the enzyme system. Most likely, the enhanced HMPS activity reflects the degradation of H$_2$O$_2$ by glutathione peroxidase. The reduced soluble sulfhydryl concentrations of the platelets remained stable during incubation with the enzyme system. Thus, exposure of platelets to H$_2$O$_2$ in concentrations that impaired aggregation was not associated with depletion of cellular GSH. The possibility that membrane sulfhydryls are impaired by exposure to H$_2$O$_2$, however, has not been excluded and requires further study.

Experiments in which the effects of SOD were studied proved to be of interest. The addition of this enzyme increased the HMPS activity of unstimulated platelets. This is best explained by an increased dismutation of O$_2$ to H$_2$O$_2$ as a result of this enzyme. and is evidence that resting platelets generate O$_2$, as suggested by others.\textsuperscript{26} SOD also enhanced the stimulation of the HMPS pathway in platelet suspensions incubated with the enzyme system. One explanation for this observation is that some of the O$_2$ generated by the enzyme system reacts with xanthine or xanthine oxidase rather than dismutating to H$_2$O$_2$. Thus, when SOD is added to the system, the conversion of O$_2$ to H$_2$O$_2$ would be facilitated and more H$_2$O$_2$ would be available for reaction with glutathione peroxidase. However, this possibility seems unlikely, since McCord and Fridovich have shown that SOD does not increase total H$_2$O$_2$ production by the enzyme system, only the rate of dismutation.\textsuperscript{27}

Thus, it seems more likely that some of the O$_2$ generated by the enzyme system is reacting with the platelets directly, rather than dismutating to H$_2$O$_2$. However, the biochemical nature of this reaction is not clear. Our results also indicate that O$_2$ does not react directly with GSH or NADPH in intact platelets. This may reflect the preferential reaction of O$_2$ with other pathways or an inability of O$_2$ to react rapidly with GSH or NADPH in the concentration found in this cell.

We also attempted to study the importance of catalase in the protection of platelets against injury by H$_2$O$_2$. We were unable to demonstrate significant oxidation of $^{14}$C-formate by platelets as a result of the oxygen metabolites generated by the enzyme system. Although this observation does not exclude some H$_2$O$_2$ degradation by catalase, it does suggest that the majority of H$_2$O$_2$ is degraded by the glutathione pathway of the platelets under our experimental conditions. This observation is not unexpected. In red blood cells, low concentrations of H$_2$O$_2$ appear to be preferentially degraded by the glutathione peroxidase pathway, and degradation does not occur as a result of catalase unless there is a depletion of GSH or an uncoupling of the link between glutathione peroxidase and the HMPS enzymes.\textsuperscript{12-19} Our data therefore suggest that platelets are adequately degrading the H$_2$O$_2$ diffusing into the cell. However, significant amounts of H$_2$O$_2$ may be reacting with the cell membrane or cellular organelles, which may lack adequate concentrations of these protective enzymes. Our results therefore raise the possibility that the protective mechanisms of platelets for the degradation of intracellular H$_2$O$_2$ may not be adequate for protection against H$_2$O$_2$ generated in extracellular environment. This suggests that compartmentalization of the enzymes known to be important in protection against oxidant injury may be present in some tissue. This may explain why the biochemical changes noted by others,
such as depletion of ATP and adenylate energy charge, occur as a result of H₂O₂.

In summary, our observations indicate that platelet aggregation to ADP is impaired following exposure to H₂O₂. This oxidant injury appears to occur inspite of the fact that the H₂O₂ is rapidly reduced by the HMPS pathway and that these cells maintain cellular GSH. Our observations suggest that H₂O₂ may cause perturbations in the membrane or cellular organelles of this cell that are important in its function inspite of active degradation of H₂O₂ by pathways considered important in affording protection against oxidant injury.

REFERENCES

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