Hydrogen Peroxide and Platelet Function

By Rosa T. Canoso, Robert Rodvien, Kristine Scoon, and Peter H. Levine

Hydrogen peroxide, at concentrations in the micromolar range, can influence platelet function. Although \( \text{H}_2\text{O}_2 \) causes no detectable platelet aggregation at these concentrations, it can aggregate platelets which have been previously exposed to any of several aggregating agents. \( \text{H}_2\text{O}_2 \) can enhance both aggregation and the subsequent disaggregation of platelets, as initiated by ADP. Preexposure of platelets to \( \text{H}_2\text{O}_2 \) blunts the subsequent response to ADP. Washed platelets are susceptible to \( \text{H}_2\text{O}_2 \). Catalase-treated \( \text{H}_2\text{O}_2 \) has no effect on platelet function. \( \text{H}_2\text{O}_2 \) does not appear to act by altering either the aggregating agent or plasma thrombin generation. These data suggest that minute amounts of \( \text{H}_2\text{O}_2 \), which could theoretically be generated in vivo at sites of platelet plug formation, could play a regulatory role in the dynamics of growth and/or dissipation of the hemostatic plug. The blood granulocyte could participate in hemostasis and thrombosis via \( \text{H}_2\text{O}_2 \) generation.

During the evolution of thrombi, platelets come into intimate contact with polymorphonuclear leukocytes.\(^1\) The latter generate hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) during the course of their metabolic functions.\(^2,3\) Conceivably, the \( \text{H}_2\text{O}_2 \) produced by polymorphonuclear leukocytes alters platelet function in a way that influences the formation of thrombi. Enough \( \text{H}_2\text{O}_2 \) is produced by phagocytosing polymorphonuclear leukocytes to damage neighboring susceptible erythrocytes.\(^4\) Because the effects of \( \text{H}_2\text{O}_2 \) on platelet function are unknown, the present studies were instituted.

MATERIALS AND METHODS

Platelet rich plasma (PRP) was obtained from healthy individuals who had taken no medications for at least 7 days. Venous blood, drawn through a 19-gauge needle, was collected in plastic syringes and immediately mixed (9:1) with acid-citrate anticoagulant (6 parts 0.1 M \( \text{Na} \) citrate to 4 parts 0.1 M citric acid pH 4.80). For some studies 3.8%, \( \text{Na} \) citrate only was used; the choice of anticoagulant did not alter the quality of the results obtained. The blood was centrifuged at 200 g for 6 min at room temperature and the PRP removed. The remaining blood was then centrifuged at 850 g for 15 min to yield platelet poor plasma (PPP).

The platelet count of the PRP was adjusted to 400-600 \( \times 10^3 \) cu mm by dilution with PPP.

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Supported by a grant from the American Medical Association Education and Research Foundation.

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When studied, the leukocyte count of the PRP never exceeded 1500/cu mm. The PRP was stored at room temperature until used. All glassware was siliconized. To ensure normal function of the resultant platelets, a standard battery of platelet aggregation studies with ADP, collagen, and epinephrine were performed on an aliquot of each sample, and compared to a population of 60 normal individuals. Any samples exhibiting abnormal behavior were excluded from further study.

**Adenosine Diphosphate (ADP)**

Disodium ADP (Sigma Chemicals) was dissolved in veronal buffer (VB, 0.025 M, pH 7.35) at a concentration of 10 mg/100 ml, adjusted to pH 6.8, and frozen in aliquots at −20°C. In the standard experiment 30 μl (3 μg) of this stock solution were added to 0.4 ml PRP aliquots (final concentration, 6.1 μg/ml, or 1.3 × 10⁻³ M). In other experiments, various dilutions of the stock ADP were made in order to obtain primary aggregation responses only.

**Collagen**

Dessicated bovine Achilles tendon (Sigma Chemicals) was used to make a collagen suspension by the method of Hovig.⁵ Aliquots of 5 mg collagen per ml of 0.9% NaCl were kept frozen at −20°C. Addition of 30 μl of this suspension to the PRP during aggregation studies yielded a final concentration of 0.34 mg collagen per ml PRP.

**Epinephrine**

Epinephrine HCl (Parke Davis, Detroit), 1 mg/ml, was stored in the dark until used; 60 μl were added to the PRP during aggregation studies to give a final concentration of 0.13 mg epinephrine per ml PRP (7.1 × 10⁻⁴ M).

**Thrombin**

Bovine thrombin (Parke Davis) was diluted in Tris buffer at pH 7.6. A stock solution of 8 U/ml was stored at −20°C. Thirty microliters of this dilution were used in 0.4 ml of PRP, yielding a final concentration of 0.6 U/ml.

**Hydrogen Peroxide**

Thirty microliters of 30%, H₂O₂ (J. T. Baker Chemical Co., Phillipsburg, N.J.) was diluted in 100 ml of VB. 30 μl of this solution was added to 0.4 ml of PRP to obtain a final concentration of 200 μM. A 600-μM H₂O₂ solution was also used in some of the studies.

**Catalase**

Thymol free catalase from bovine liver (Sigma Chemical Co., St. Louis, Mo.) was diluted with VB to 13,000 U/ml.

**Washed Platelets**

Platelets were washed according to the method of Mustard et al.⁶ and were resuspended in Tyrode's solution, containing 0.35%, bovine albumin (Pentex, Kankakee, Ill.). Aggregation studies were performed on PRP within 3 hr of its collection, using the turbidometric method of Born⁷ as modified by Mustard et al.⁸ Four-tenths of a milliliter of PRP were placed in the cuvette of a Chrono-Log aggregometer (Chrono-Log Corp., Broomall, Pa.) and stirred at 1200 rpm, using a Teflon-coated stir bar. The light transmittance was recorded on a moving strip chart recorder. Two minutes incubation at 37°C were allowed before adding the aggregating agent. In this system, light transmittance increases as a function of the number and size of platelet aggregates. The degree of aggregation was expressed as a per cent change in light transmittance. The light transmittance of a sample of PPP is regarded as 100% transmission and the light transmittance of the PRP as zero.

A standard ADP concentration produced less aggregation at the end of each 90-min set of experiments than at the beginning. To offset this effect of time-related change in response to ADP, the sequence of the experimental maneuvers performed upon each individual's PRP was randomized.
In some experiments, a concentration of ADP just sufficient to produce a primary aggregation wave without secondary aggregation was determined by trial and error. The dose thus arrived at was kept standard for that set of experiments.

The following maneuvers, in random sequence, were performed on 0.4-mI aliquots of each PRP sample:
(1) Addition of H₂O₂ alone, as compared to the addition of veronal buffer alone.
(2) Simultaneous addition of H₂O₂ and ADP, as compared to the simultaneous addition of buffer and ADP.
(3) Addition of ADP, followed 3 min later by the addition of H₂O₂ or buffer.
(4) Addition of H₂O₂ or buffer, followed 3 min later by the addition of ADP.

The above sets of experiments were performed on PRP obtained from 16 normal individuals. Significance of differences within each pair was assessed by the Student's t test.

Platelet Factor 3 Availability
PF3a was determined by three different methods. Using a modification of the method of Zucker and Peterson,⁹ 0.2 ml of diluted Russell's viper venom (Stypven, Burroughs Wellcome) plus calcium chloride, 0.125 M, was added to 0.1 ml PRP prewarmed at 37°C, and clot formation timed (Stypven time). Measurements of such clot formation were made on aliquots of PRP taken from the aggregometer cuvette immediately before and at 7 min after the addition of the aggregating agent. Results were expressed as the number of seconds by which the Stypven time was shortened following aggregation.

The second method for PF3a measurement was that of Rabiner and Hrodek,¹⁰ in which a standard number of platelets in plasma was incubated with celite, following which recalcification times were determined.

The third method for measuring PF3a was a two-stage assay, a modification of the method of Karpatkin and Siskind.¹¹ To 1.0 ml of PRP was added 1.0 ml of "contact product"¹² and 0.1 ml of ADP. This mixture was stirred and incubated at 37°C in the platelet aggregometer. From this cuvette, 0.1-ml samples were removed at standard intervals and pipetted into plastic tubes, followed by the addition of 0.1 ml of 0.025 M CaCl₂, the fibrin end point was observed and recorded in seconds.

In order to eliminate aging of the platelets as an experimental variable, and to assess possible changes in the generation of PF3a, two samples from the same PRP were incubated simultaneously in two identical aggregometers, with and without peroxide. Aliquots were removed every 30 sec for PF3a determination. Curves of PF3 generation could thus be drawn, and the effect of H₂O₂ on the generation rate better assessed.

Serotonin Release
5-Hydroxytryptamine-3,¹³ 57 mCi/mmol (Radiochemical Centre, Amersham, U.K.), was dissolved in 0.9%, saline at 10 μCi/ml and kept at -20°C.

Measurement of ³⁵S-serotonin release was based on the method described by Jerushalmy and Zucker.¹⁴ PRP for this study was prepared from blood mixed 9:1 in 3.8% sodium citrate, and was either used immediately, or was stored in melting ice. After 5 min of warming at 37°C, the PRP was incubated for 30 min at the same temperature with ³⁵S-serotonin (specific activity 50 mCi/mM, final concentration 0.05 μCi/ml). Four-tenths-milliliter aliquots of this labeled PRP were placed in plastic tubes, aggregating agents were added, and the tubes incubated at 37°C for 15 min. The tubes were then shaken for 5 min on a Micromixer (Cooke Engineering Co., Alexandria, Va.).

Samples of PRP (0.1 ml) were removed from the control tubes for measurement of total radioactivity. All tubes were then centrifuged; 0.1 ml of the supernatant plasma was delivered to scintillation vials, and then 0.4 ml NCS tissue solubilizer (Amersham/Searle) was added. Thirty minutes later, 10 ml of Bray's solution were added. Radioactivity was measured in a Nuclear Chicago liquid scintillation counter, 720 series. All samples were run in duplicate, and the mean result was calculated.

Miscellaneous Studies
Kaolin-activated thromboplastin time, thrombin time, recalcification time, one-stage prothrombin time, and prothrombin consumption test were performed using standard methods.¹⁵
Platelet counts were performed by phase-contrast microscopy. Erythrocyte and leukocyte counts were done in a Coulter Counter, Model F (Coulter Electronics Corp., Hialeah, Fla.).

RESULTS

ADP-Induced Aggregation

A number of experimental protocols were completed on 16 PRP samples concerning the effect of H_2O_2 on platelet aggregation. Four main bodies of results emerged and are presented below.

(1) The addition of H_2O_2 (final concentration, 200 μM) to the PRP resulted in no evidence of aggregation in any study. Eighteen per cent of the samples exhibited a slight decrease in light transmission. At 600 μM H_2O_2, 22% of the samples exhibited a biphasic curve of slight increase in light transmission, followed by a proportional decrease. These data are summarized in Fig. 1A.

Light and scanning electron microscopy were used to confirm that micromolar doses of H_2O_2 cause no platelet aggregation.

At high concentrations of H_2O_2, in the millimolar range, minute bubbles appeared in the PRP which decreased the light transmittance for a period of 2–3 min, with subsequent return of light transmission to the initial base line. The same phenomenon was observed in platelet poor plasma, at the millimolar concentrations.

(2) When ADP, in a concentration sufficient to produce primary aggregation only, was added simultaneously with 200 μM H_2O_2, there was a potentiation of primary aggregation (p < 0.01) with a concomitant enhancement of disaggregation (p < 0.001), as compared to the effects of ADP plus buffer. In the majority of the cases, there was complete disaggregation in the presence of
peroxide; i.e., light transmission decreased to base-line level. A typical experiment is shown in Fig. 1B. This disaggregation was only temporary; in all samples low-grade aggregation recurred gradually over several minutes. The results of 16 sets of paired experiments are shown in Fig. 2.

(3) The addition of 200 μM H₂O₂ to platelets which had previously undergone primary aggregation due to ADP invariably produced an aggregation wave. This was followed by disaggregation, the light transmittance falling to below the base line, with eventual return of light transmittance to pre-H₂O₂ levels (Fig. 1C). Peroxide produced these post-ADP responses in all of the samples tested. The addition of 200 μM H₂O₂ to platelets already maximally aggregated by exposure to large doses of ADP produced no change in light transmittance in any of six samples tested.

These post-ADP peroxide-induced phenomena could not be elicited by a second exposure to H₂O₂ in the same PRP sample (Fig. 3C). If the PRP was repeatedly exposed to ADP, a primary aggregation wave was always obtained, with gradual extinction of the phenomenon (Fig. 3A). If each exposure to H₂O₂ was preceded by ADP exposure, the peroxide-induced aggregation response could then be repeated (Fig. 3B).

(4) PRP was incubated for 3 min with 200 μM H₂O₂, and then exposed to ADP (Fig. 4A). There was a decrease in primary aggregation (p < 0.01), as well as a decrease in disaggregation (p < 0.01), when compared with PRP pre-incubated for the same length of time with VB. The degree of inhibition was proportional to the length of incubation, and was greater at the highest H₂O₂ concentration.

At ADP concentrations just large enough to produce secondary aggregation, prior incubation with H₂O₂ decreased the primary aggregation wave and delayed the appearance of the secondary aggregation wave (Fig. 4B). The du-
Fig. 3. Sequential stimulation of platelets with various agents.

Fig. 4. Effect of preexposure of platelets to $H_2O_2$ on their subsequent response to ADP. $H_2O_2$ or buffer is added at the first arrow. (A) $1.3 \times 10^{-6}$ M ADP. (B) $2.6 \times 10^{-6}$ M ADP. (C) $1.3 \times 10^{-5}$ M ADP.
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Fig. 5. Effect of H₂O₂ on thrombin-induced aggregation. (A) H₂O₂ following thrombin. (B) H₂O₂ preceding thrombin.

ration of delay in secondary aggregation was increased by the higher H₂O₂ concentration, and by lengthening the time of incubation. If larger quantities of ADP were used to produce secondary aggregation, incubation with H₂O₂ caused only slight decrease in the secondary aggregation wave (Fig. 4C).

Effect of H₂O₂ on the Aggregating Agent

Platelet aggregation induced by ADP, which was preincubated with micromolar H₂O₂ for 5 min, was not significantly different from the platelet aggregation induced by the simultaneous addition of these agents to PRP, in eight sets of studies.

Other Aggregating Agents

Epinephrine or thrombin was used in place of ADP in several sets of experiments. Both of these agents, given before or after H₂O₂, yielded results qualitatively identical to those described for ADP. A typical set of experiments using thrombin as the aggregating agent is shown in Fig. 5.

Abolition of H₂O₂ Effect by Catalase

Catalase (13,000 U/ml) was mixed with an equal volume of freshly prepared 3.0%, H₂O₂. After incubation at 37°C for 5 min, this catalase-treated stock solution, appropriately diluted, was compared to a similar dilution of untreated H₂O₂ (Fig. 6A). In four sets of experiments, the catalase-treated H₂O₂ no longer had any effect on platelet aggregation.

KCN as an Inhibitor of Endogenous Plasma Catalase

Ten millimolar KCN was adjusted to pH 7.4; 50 µl were incubated with 0.4 ml PRP for 5 min at 37°C. This PRP responded to peroxide in a manner essentially indistinguishable from PRP incubated with VB as a control (Fig. 6B). In half of the samples tested, KCN enhanced the peroxide effect.

Washed Platelets

Washed platelets were resuspended in Tyrode's solution with albumin and used in place of PRP. H₂O₂ was added concomitant with or following ADP,
according to the protocols described above. With respect to the effects of H₂O₂, washed platelets behaved in a manner similar to platelets in plasma (Fig. 7), with one exception. Only minimum disaggregation of washed platelets was observed when ADP alone was added. Marked disaggregation of washed platelets occurred only when H₂O₂ was present (Fig. 7A).

**PF3a Measurements**

The effect of H₂O₂ on PF3a was assessed in several ways. Preincubation of 200 and 600 μM H₂O₂ with PRP for 60 sec was followed by measurement of PF3a by the three methods described above. The H₂O₂ incubation caused no changes which were significantly different from incubation with VB as a control.
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in ten sets of experiments. Following the addition of H₂O₂ to platelets previously exposed to various doses of ADP, a series of parallel aggregation and PF3a studies were performed. Although the H₂O₂ influenced the aggregation responses in a manner already described, no significant alteration of PF3a could be detected. In another set of eight experiments, the presence of H₂O₂ did not alter the rate at which PF3a could be generated, following exposure to various stimuli (Fig. 8).

**1⁴C-Serotonin Release**

¹⁴C-serotonin release was measured on platelet samples at the same time that nephelometric measurements were made. Addition of 200 or 600 μM H₂O₂ alone produced no release. Using ADP as the aggregating agent, we observed no change in serotonin release under conditions of aggregation, as depicted in Fig. 1B, in which only a primary wave was induced. At larger doses of ADP (see Fig. 4C), changes in the secondary wave were induced by prior exposure to peroxide. Variable changes in serotonin release were observed in ten sets of experiments. No statistical significance could be assigned to these results.

**Coagulation Studies**

The one-stage prothrombin time, Kaolin-activated partial thromboplastin time, thrombin time, prothrombin consumption test, and recalcification time of ten normal plasma samples were studied with and without the addition of 200 μM H₂O₂. The time taken for fibrin clot formation was not modified (Table 1). Similar studies on plasmas deficient in various clotting factors revealed no change in clot formation due to H₂O₂ addition.

<table>
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<th>Buffer Only</th>
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<td>Thrombin time</td>
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<td>Prothrombin consumption</td>
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<td>45.6</td>
</tr>
<tr>
<td>Recalcification time</td>
<td>104</td>
<td>107</td>
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Times are expressed in seconds.
DISCUSSION

The results of these studies demonstrate that platelet function can be modified by the presence of micromolar quantities of peroxide. Two hundred or six hundred micromolar H$_2$O$_2$ can induce a nephelometric change suggestive of loss of disk shape, but aggregation is not observed at these doses. However, these quantities of H$_2$O$_2$ do influence the pattern of aggregation caused by ADP and other aggregating agents. In the presence of ADP, peroxide enhances both aggregation and subsequent disaggregation. This dual effect occurs when peroxide is added concomitant with or following exposure to the aggregating agent. Preincubation of platelets with H$_2$O$_2$ for several minutes decreases the subsequent platelet response to ADP.

The major effect of H$_2$O$_2$ is to alter the primary wave of platelet aggregation. As expected, no change in serotonin release or PF3a was associated with these phenomena. H$_2$O$_2$ has minor effects on the secondary wave of platelet aggregation. Our inability to demonstrate changes in serotonin release or PF3a in this setting may reflect the relative insensitivity of these techniques to minor changes in secondary aggregation.

The effects of added peroxide to PRP can be abolished by pretreatment of the peroxide solution with catalase. Inhibition of any plasma catalase by KCN only partially alters peroxide's effects on PRP. These findings are consistent with the high Km of catalase and the minute concentrations of peroxide necessary to cause these effects.

The target for the effects of H$_2$O$_2$ may be the platelet itself, the plasma environment, or the aggregating agent. Separation of the platelets from the plasma by washing does not alter the quality of the peroxide effects. No effect of micromolar H$_2$O$_2$ on the studies of the plasma coagulation system could be observed in vitro. In addition, the effects of peroxide on the platelet were observed independent of the choice of the aggregating agent. Finally, there is evidence that the aggregating agent itself is not modified by the H$_2$O$_2$. All of these results are most consistent with a direct effect of H$_2$O$_2$ on the platelet. Of the possible platelet sites where significant biological oxidation can occur, the cell membrane is the most obvious. Whether the H$_2$O$_2$ effects are due to lipid peroxide formation, or are mediated by changes in sulfhydryl groups, membrane receptor sites, etc., are matters for future study.

Only one other study of H$_2$O$_2$ and platelets is known to the authors. In this work, H$_2$O$_2$ was noted to be able to cause platelets to aggregate. The doses used, however, were in the millimolar rather than the micromolar range. The significance of aggregation at such high H$_2$O$_2$ levels is not clear.

The amount of peroxide which can be produced in vivo, or the levels attainable at or near the platelet, are difficult to quantitate. There is general agreement that the methods of measurement underestimate peroxide production. We arbitrarily chose a dose of peroxide which conceivably could be attainable by extrapolation from the available data on in vitro studies of various tissues.

We believe that the interactions of platelets and peroxide demonstrated above may have biologic significance. The neutrophil is not the only cellular source of H$_2$O$_2$ production. Moreover, H$_2$O$_2$ may also be generated in vivo.
in the presence of various therapeutic agents, such as primaquine and sulfonamide. The generation of peroxide at a site of thrombus formation may alter the development of the thrombus. This alteration could occur via the changes in either aggregation or in disaggregation of platelets shown in this paper. In this manner, drugs which produce or inhibit peroxidation might also alter thrombus formation.

It is hoped that further study of the effects of H$_2$O$_2$ on the platelet will contribute to a better understanding of platelet-platelet and/or platelet-granulocyte interactions.

ACKNOWLEDGMENT

The authors wish to thank Dr. Robert Schwartz for helpful advice, Miriam Uyeminami, Jayne Mazurkowitz, and Andrew McCauley for technical assistance, and Eleanor Jonassen and Eileen O’Brien for aid in preparation of the manuscript.

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