Concurrent Studies of Oxygen Consumption and Aggregation in Stimulated Human Platelets

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A new approach to the study of the "burst" of oxygen consumption in stimulated human platelets is described. Following addition of collagen, thrombin, or arachidonic acid to washed platelets, O2 consumption and aggregation were measured concurrently. In contrast to thrombin and arachidonic acid, collagen induced a calcium-dependent O2 burst after a lag phase of 25–35 sec. Elevation of platelet cAMP levels completely inhibited the oxygen burst and aggregation response. Blockade of energy metabolism also prevented the oxygen burst; however, the aggregation response was not completely suppressed. Eicosatetraynoic acid and indomethacin—inhibitors of arachidonic acid oxygenation—also abolished the collagen-induced O2 burst but not aggregation. These findings indicate that the platelet O2 burst is utilized for formation of prostaglandin endoperoxides, which are apparently not essential for collagen-induced aggregation. Comparison of the oxygen consumption and aggregation curves elicited by arachidonic acid in the presence of increasing amounts of ADP scavengers demonstrated the obligatory participation of released ADP in aggregation induced by this fatty acid. Stored platelets did not exhibit an oxygen burst when challenged by collagen or thrombin, although the aggregation response was relatively intact. In contrast, addition of arachidonic acid to stored platelets elicited both an O2 burst and aggregation, suggesting that phospholipase A2 activity, or steps leading to its activation, are more labile to storage than the platelet cyclooxygenase. It is concluded that concurrent comparison of oxygen consumption and aggregation responses permits a direct evaluation of the role of endoperoxide formation in human platelet aggregation.

PLATELET STIMULATION and/or aggregation can be induced by a wide variety of chemical and pharmacologic agents. Following addition of thrombin or arachidonic acid to washed human platelets there is an abrupt, transient increase in oxygen consumption.2,6 This "oxygen burst" has been linked to the formation of

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oxygenated products of free arachidonic acid. The currently accepted sequence of
events involves activation of a putative platelet phospholipase by thrombin,
collagen, or ionophore A23187, which then hydrolyzes endogenous arachidonic
acid from platelet phospholipids, rendering free arachidonic acid available for
oxygenation. We recently reported that collagen stimulation of washed platelets
induces an oxygen burst, as did Feinstein et al.

In the present paper we describe conditions under which the O2 burst occurs and
how it can be modified in washed platelets as well as in platelets stored for 24 hr at
4°C. Furthermore, we compared the effect of agents known to influence the
thrombin-induced oxygen burst with their effect on the burst associated with
collagen stimulation. Among the agents studied were (1) 5,8,11,14-eicosatetray-
noic acid (ETYA)* and indomethacin, which are thought to inhibit arachidonic
acid oxygenation; (2) prostaglandin D2 (PGD2), and prostacyclin (PGI2) and
dibutyryl cAMP (dBcAMP), which increase intracellular cAMP; (3) antimycin
and 2-deoxy-D-glucose (DOG), which inhibit energy metabolism. In addition,
collagen-induced platelet aggregation was measured concurrently with oxygen
consumption. In this way the relative contributions of the two known pathways of
collagen-induced aggregation could be assessed.

Following exposure to phagocytic stimuli, leukocytes exhibit an oxygen burst
linked to the production of superoxide radicals (O2-) and hydroxyl radicals
(OH). However, production of O2- by human platelets is not enhanced by an
aggregation stimulus. In order to further confirm this observation in platelets,
experiments were also carried out to explore the influence of superoxide dismutase
(SOD) and catalase on both the oxygen burst and aggregation.

MATERIALS AND METHODS

Platelet collection and processing. For each experiment one unit of whole blood from donors at the
New York Blood Center was used. Each volunteer denied having taken medications during the
preceding 2 wk. Blood was drawn into a plastic pack system (Fenwal, 4R1709 [L-465], Deerfield, Ill.).
Processing at 4°C was initiated within 50 mm of venesection.

Initial centrifugation was carried out in a Sorvall RC-3 centrifuge at 2000 rpm (1000 g) for 10 min.
Rotor speed was brought up slowly (~ 60 sec), thereby obviating formation of creases in the bag with
consequent trapping of red cells. Platelet-rich plasma (PRP) was expressed into the first satellite bag,
and the bag containing residual erythrocytes and leukocytes was discarded. Prior to all subsequent
centrifugation steps, the satellite bag was blown up with air until taut. This facilitated separation of the
liquid phase from the pellet. The PRP was spun at 900 rpm (225 g) for 10 min to eliminate remaining
erythrocytes and leukocytes. PRP was then expressed into the second satellite bag, and the first satellite
bag was discarded. For every 10 g of PRP contained in the bag, 1 ml of "citrate solution" (38 mM citric
acid, 75 mM sodium citrate) was added. The air-filled PRP-citrate bag was spun at 2500 rpm (1600 g)
for 10 min and the platelet-poor plasma discarded. The sedimented platelets were suspended in 3 ml
Tris-citrate (75 mM Tris, 100 mM KCl, 12 mM citric acid, pH 6.4) by gentle massage of the bag. An
additional 50 ml Tris-citrate was added when suspension was complete. Following centrifugation at
2500 rpm (1600 g) for 10 min, the washing procedure was repeated. The final sediment was resuspended
in 3 ml 0.15 M KCl. The platelet count was then adjusted to an average of 12 X 10^7/ml utilizing the
microhematocrit technique. When examined by phase-contrast microscopy the platelet suspensions
were essentially devoid (when 50 fields were scanned, one granulocyte was seen) of polymorphonuclear

*Abbreviations used in this paper: ETYA, 5,8,11,14-eicosatetraynoic acid; PGD2, prostaglandin D2;
PGI2, prostaglandin I2, prostacyclin; dBcAMP, dibutyryl cyclic adenosine 3',5'-monophosphate; DOG,
2-deoxy-D-glucose; SOD, superoxide dismutase; PRP, platelet-rich plasma; EGTA, ethylene glycol
bis-(β-aminoethyl ether)-N,N'-tetraacetic acid; CP/CPK, creatine phosphate/creatine phosphokinase;
TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate HCl.
cells and monocytes. A rare erythrocyte or lymphocyte was occasionally observed. Stock suspensions from which experimental samples were removed were maintained at 4°C. Processing time from blood collection to initiation of experiments was less than 4 hr.

**Oxygen consumption studies.** Polarographic measurements were made with a Model 53 oxygen monitor equipped with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The electrode was fitted to a Gilson water-jacketed cell (OX-15253, Gilson Medical Electronics, Middleton, Wisc.) connected to a Lauda circulator (K-2/RD) maintained at 37°C ± 0.02°C. Measurements were recorded with a Riken Denshi two-channel instrument (SP-H5V, Payton Associates, Buffalo, N.Y.) with full-scale deflection adjusted to correspond to a 500 nat oxygen/mL. Thus a change in oxygen concentration of 10% of full scale per minute for an incubation mixture of 1.5 ml containing 3 × 10^9 platelets indicates an oxygen consumption rate of 75 nat oxygen per 3 × 10^9 platelets per minute. The data in this paper are referred to as “net burst rates,” i.e., the observed maximal rate of oxygen consumption during the burst corrected for the platelets’ basal respiratory activities as measured just prior to the addition of the stimulus. In some instances the amount of oxygen consumed during the burst in excess of the basal consumption was measured as the “extent of burst.”

**Platelet aggregation** was monitored with a Payton aggregation module. The aggregometer output was connected to the second channel of the recorder, thus providing concurrent tracings of oxygen consumption and platelet aggregation.

**Experimental design.** Reaction mixtures for oxygen consumption studies (final volume 1.5 ml) were stirred at 1100 rpm (37°C). Then 3 × 10^9 platelets (0.25 ml of stock suspension) were added to a solution containing 0.15 M KCl, 0.3% defatted bovine serum albumin, and 7 mM potassium phosphate buffer (pH 7.4). Where indicated, ethylene glycol bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) 5 mM was also included. The washing procedure and suspending medium used in our studies had been successfully employed in previous investigations of platelet oxygen consumption by Pickett and colleagues. Immediately following platelet addition a diffusion stopper was placed in the cell to prevent entry of atmospheric oxygen into the reaction chamber. Baseline oxygen consumption was determined at the conclusion of a 4-min incubation period that allowed for temperature equilibration and preincubation with inhibitors. ETYA, indomethacin, apyrase, creatine phosphate/creatine phosphokinase (CP/CPK), antimycin, DOG, SOD, and catalase were added 3 min prior to introduction of collagen; PGI2, PGD2, and dBcAMP were added 2 min before collagen; 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate HCl (TMB-8) was added 1 min prior to collagen. Stimuli were introduced through a capillary port in the stopper using a Hamilton syringe (1710LT). All aspects of the aggregation studies were identical to the oxygen consumption experiments except that the final volume of the reaction mixtures was 0.5 ml. Thus the concentrations of platelets used for aggregation and O2 measurements were the same. This was done in order to compare aggregation and O2 consumption under identical conditions. The concentration of platelets used for aggregometry was higher than is usually employed.

**Chemicals.** Reagents were obtained as follows: collagen, Hormon-Chemie, München, West Germany; epinephrine, Parke-Davis, Detroit, Mich.; sodium arachidonate, Nu Chek-Prep, Elysian, Minn.; fibrinogen, grade L, Kabi, Stockholm, Sweden; SOD, Miles Laboratories, Elkhart, Ind.; vasopressin, adenosine diphosphate (ADP), catalase, DOG, antimycin, indomethacin, dBcAMP, CP, and CPK, Sigma Chemical, St. Louis, Mo. PGD2 was kindly provided by Dr. David Mills; TMB-8 was a gift from Dr. Richard Feinman; ETYA was provided by Dr. W. E. Scott, Hoffmann-La Roche, Nutley, N.J.; human thrombin was supplied by Dr. John Fenton; PGI2 was a gift from Dr. Babette Weksler. Apyrase was prepared by a modification of the method of Molnar and Lorand as utilized in the laboratory of Dr. J. F. Mustard, McMaster University, Hamilton, Ont. The apyrase preparation was assayed for its inhibitory effect in ADP-induced aggregation in PRP and used as indicated in the text, assuming that 1 unit inactivates 2 nmol ADP/min.

Antimycin, indomethacin, PGD2, PGI2, and ETYA were all added as freshly prepared ethanol solutions. Appropriate solvent controls for each addition were always included.

**RESULTS**

**Collagen-Induced Oxygen Burst**

Addition of collagen to a platelet suspension in the absence of EGTA resulted in a burst of oxygen consumption after a lag phase of 25–35 sec. The maximal rate of oxygen consumption occurred at the start of the burst, concomitant with the onset
A precipitous fall in oxygen concentration was recorded early in the burst. This was attributed to platelet aggregates, which were observed adhering to the membrane of the electrode. The data obtained under these readily apparent circumstances were not included in the study.

Both net burst rate and extent of the "oxygen burst" were directly proportional to the quantity of collagen added (data not shown). The postburst rate of oxygen consumption returned to the same level as that observed prior to stimulation (Fig. 1A). This indicated that aggregation did not mechanically interfere with the functional capacity of the electrode.* After completion of the initial oxygen burst, a second addition of either collagen (up to 100 μg/ml) or thrombin (up to 6 U/ml) was not followed by a second burst. However, addition of sodium arachidonate (48 μM) after the first collagen stimulus did indeed elicit a second burst. Further additions of arachidonate were followed by O₂ bursts of lesser intensity until the oxygen in the chamber was totally consumed.

The increase in oxygen consumption associated with collagen stimulation was calcium dependent, since no oxygen burst occurred when EGTA was included in

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*In 14 of 137 experiments a precipitous fall in oxygen concentration was recorded early in the burst. This was attributed to platelet aggregates, which were observed adhering to the membrane of the electrode. The data obtained under these readily apparent circumstances were not included in the study.
the platelet suspension. This is in sharp contrast to thrombin and arachidonate, both of which induce an oxygen burst in the presence or absence of EGTA.

**Perturbation of the Collagen-Induced Oxygen Burst and Aggregation**

The collagen-induced oxygen burst was further investigated by incubating the reaction mixture with a variety of compounds prior to collagen addition. Again oxygen consumption and the aggregation response were measured concurrently.

**Effects of inhibitors of aggregation due to ADP release and arachidonic acid oxygenation.** Apyrase, an inhibitor of aggregation due to ADP, had virtually no effect on the magnitude of the burst rate but did reduce the aggregation response (Fig. 1B). In addition, the lag phase of the O₂ burst was prolonged. A two- to threefold increase in apyrase concentration did not affect the magnitude of the burst rate, although it further reduced but did not eliminate the aggregation response. Removal of ADP by another, unrelated mechanism, CP/CPK at concentrations of 13.2 units CPK/ml and 1.32 mM CP, gave results identical to the higher apyrase concentrations. In contrast, ETYA, an inhibitor of both the cyclooxygenase and lipoxygenase pathways of arachidonic acid oxygenation, blocked the collagen-induced oxygen burst but did not completely abolish the aggregation response (Fig. 1C).

In an effort to block both arachidonic acid oxygenation and the effects of ADP release, ETYA and apyrase were preincubated in combination. This resulted in complete inhibition of both the collagen-induced oxygen burst and aggregation (Fig. 1D)—an effect not observed with ETYA or apyrase alone. Identical results were obtained when indomethacin (8 μM) was substituted for ETYA in the experiments depicted in Figs. 1C and 1D. The latter result was somewhat unexpected, since indomethacin theoretically shunts arachidonic acid oxygenation toward the lipoxygenase pathway. Furthermore, when the platelet count in the reaction chamber was doubled in order to increase the sensitivity of the system, there was still no measurable collagen-induced oxygen burst in the presence of indomethacin.

**Effects of agents that raise platelet cAMP levels.** Preincubation of platelets with PGD₂ or PGI₂ resulted in complete inhibition of both the oxygen burst and aggregation upon stimulation with collagen. Both compounds have been reported to increase cAMP levels in platelets. When the platelets were pretreated with 1 mM dBcAMP, an oxygen burst was not detected but slight aggregation was noted. However, increasing the concentration of dBcAMP to 2 mM abolished the aggregation response. The above results are summarized in Table 1.

**Effects of inhibitors of energy metabolism.** Antimycin, an inhibitor of oxidative phosphorylation, strongly reduced the basal rate of oxygen consumption but only partially blocked the oxygen burst and had no effect on aggregation. Preincubation with DOG had no effect on the basal rate, burst rate, or aggregation. On the other hand, antimycin and DOG in combination totally abolished the oxygen burst although slight aggregation was observed. Since preincubation with antimycin and DOG did not completely inhibit collagen-induced aggregation, further experiments were carried out in which apyrase was added to a preincubation mixture containing antimycin and DOG. Under these conditions the oxygen burst, as well as the aggregation response, was completely inhibited (Table 1).
Control experiments were also carried out with preincubation mixtures containing antimycin plus apyrase or DOG plus apyrase. The results indicated that all three agents were required to block both the oxygen burst and aggregation.

**Studies with SOD and catalase.** As can be seen in Table 1, neither SOD nor catalase added to the platelet suspension, alone or in combination, had any measurable effect on the net burst rate, basal rate, or aggregation.

**Effects of TMB-8.** TMB-8, an antagonist of intracellular calcium translocation, has been reported to block platelet secretion. When this compound was added to the reaction mixture, the basal rate of oxygen consumption rapidly decreased to 40% of its initial value. Upon addition of collagen there was no oxygen burst, and aggregation was completely inhibited (Table 1). In the presence of 300 μM TMB-8 there was no oxygen burst, but a slight aggregation response was elicited by collagen.

**Effects of other aggregating agents on the oxygen burst.** The following compounds did not induce an oxygen burst when added to washed platelet suspensions in the presence or absence of EGTA (5 mM): ADP (with and without fibrinogen), epinephrine, vasopressin, and sodium fluoride.

**Studies of the Oxygen Burst in Stored Platelets**

Platelet suspensions that had been continuously maintained at 4°C for 24 hr showed the same basal rate of oxygen consumption as when they were freshly prepared. However, upon the addition of collagen (40 or 66 μg/ml) no O₂ burst was measurable, although the aggregation response was present albeit slightly reduced (Fig. 2). Similar results were obtained with thrombin (2 U/ml).

The failure of thrombin to induce an oxygen burst in stored platelets was further investigated by stimulating with as much as 10 U/ml in the presence of EGTA.

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**Table 1. Perturbation of Oxygen Burst and Aggregation Induced by Collagen (40 μg/ml)**

<table>
<thead>
<tr>
<th>Addition</th>
<th>O₂ Burst (μmol/min/10⁶ Platelets)</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>52</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Group I: Agents that increase platelet cAMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGI₂ (60 μM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PGD₂ (10 μM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dBcAMP (1 mM)</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>dBcAMP (2 mM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group II: Inhibitors of energy metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimycin (100 ng/ml)</td>
<td>44*</td>
<td>+ + + +</td>
</tr>
<tr>
<td>DOG (10 mM)</td>
<td>52</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Antimycin, DOG</td>
<td>0*</td>
<td>+ +</td>
</tr>
<tr>
<td>Antimycin, DOG, apyrase (16.7 U/ml)</td>
<td>0*</td>
<td>0</td>
</tr>
<tr>
<td>Group III: Enzymes related to superoxide metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD (10 μg/ml)</td>
<td>52</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Catalase (10 μg/ml)</td>
<td>52</td>
<td>+ + + +</td>
</tr>
<tr>
<td>SOD, catalase</td>
<td>52</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Group IV: Inhibitor of Ca²⁺ translocation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMB-8 (300 μM)</td>
<td>0†</td>
<td>+ +</td>
</tr>
<tr>
<td>TMB-8 (600 μM)</td>
<td>0†</td>
<td>0</td>
</tr>
</tbody>
</table>

*In addition, the basal rate of O₂ consumption was reduced to 10% of normal levels.
†Basal O₂ consumption was reduced to 60% of normal levels.
However, even under these conditions an oxygen burst was not elicited. Following the above dose of thrombin, sodium arachidonate (48 μM) was added to the same reaction mixture; a definite oxygen burst was induced (Fig. 3). The burst rate in this stored platelet suspension was approximately half that observed when the suspension was freshly prepared. The same result was obtained when arachidonate alone was added to the stored suspension. These results indicate that in stored platelets thrombin and collagen do not elicit an oxygen burst, even though platelet...
cyclooxygenase activity is still relatively unimpaired, as evidenced by the response to arachidonic acid. They also indicate that availability of endogenous platelet arachidonic acid for oxygenation may be impaired in stored platelets.

**Role of Released ADP on Aggregation and Oxygen Consumption Induced by Thrombin and Arachidonate**

It was previously noted that when platelets were stimulated by collagen in the presence of apyrase the aggregation response was delayed and diminished, although the net burst rate was normal. However, the shape of the oxygen consumption curve was qualitatively different from the control curve (compare Fig. 1B with Fig. 1A) in that the lag phase was prolonged and the onset of the burst slower without any effect on the maximum rate. To further investigate this effect of apyrase, experiments were carried out with thrombin and arachidonate.

Apyrase (17 U/ml) had no effect on the oxygen burst or on aggregation induced by thrombin (2 U/ml). However, when apyrase (17 U/ml) was incubated with the platelet suspension and arachidonate (48 μM) added, an immediate oxygen burst occurred, but there was a 2-min delay in the aggregation response, which was slightly diminished. Doubling the apyrase concentration to 34 U/ml was also followed by an immediate oxygen burst, but aggregation was completely inhibited. The results of these experiments are depicted in Fig. 4. Similarly, when apyrase was

![Fig. 4. Oxygen consumption and aggregation induced by thrombin or arachidonic acid in the presence of apyrase. (A) Thrombin, 2 U/ml, induced a normal aggregation response and O₂ burst in the presence of 17 U/ml apyrase. (B) Arachidonate (48 μM) elicited a normal O₂ burst, although aggregation was delayed when 17 U/ml apyrase were used. (C) When the apyrase concentration was doubled to 34 U/ml, the O₂ burst was unaffected but there was no aggregation.](image-url)
replaced by CP/CPK (1.32 mM CP and 13.2 units CPK/ml), arachidonate (48 μM) induced an oxygen burst but no aggregation occurred. However, when lower concentrations of CP and CPK were used, aggregation was only partially inhibited. Since the oxygen burst induced by arachidonic acid was not accompanied by aggregation in the presence of apyrase or CP/CPK, it may be concluded that arachidonic acid–induced aggregation is mediated by released ADP. This is in contrast to thrombin, which is capable of inducing a normal oxygen burst and a normal aggregation response in the presence of apyrase.

DISCUSSION

The experiments described here represent a novel approach to the study of the burst of oxygen consumption associated with human platelet stimulation. The O₂ burst has been directly correlated with the aggregation response, and the data suggest that the extra O₂ consumed during the burst represents oxygenation of arachidonate to prostaglandin endoperoxides. Furthermore, the role of endoperoxide formation in aggregation can be directly evaluated by comparing oxygen consumption and aggregation curves in the presence or absence of perturbing agents.

Since collagen is thought to be one of the initial in vivo platelet stimuli, it was of interest to discern whether or not the collagen–platelet interaction was associated with a burst of oxygen consumption. Collagen induced a calcium-dependent burst of oxygen consumption that occurred concurrently with the onset of aggregation and was characterized by a lag phase of 25–35 sec (Fig. 1A).

On the other hand, thrombin and arachidonate induced an immediate oxygen burst in the presence or absence of calcium, in agreement with reports from other laboratories.²⁻⁶ The lag phase that distinguished the collagen-induced oxygen burst and aggregation response from those of thrombin and arachidonate may represent the time interval required for collagen to interact with the platelet surface prior to initiation of the appropriate response.

Although indomethacin and ETYA, known inhibitors of arachidonic acid oxygenation, abolished the collagen-induced oxygen burst, they did not completely block the aggregation response. It was therefore concluded, on one hand, that the oxygen burst induced by collagen is due to oxygenation of endogenously liberated arachidonic acid and, on the other hand, that collagen-induced aggregation is not due solely to arachidonic acid transformation. Moreover, whereas ETYA inhibits arachidonate oxygenation via both the lipoxygenase and cyclooxygenase pathways, indomethacin does not affect lipoxygenase activity.²⁵ Since both ETYA and indomethacin completely abolished the O₂ burst (Fig. 1C), it is suggested that (1) the platelet oxygen burst represents oxygenation of arachidonate to prostaglandin endoperoxides and (2) the lipoxygenase pathway does not play a direct role in the burst per se. These conclusions are supported by data on the time course of formation of cyclooxygenase products in contrast to that of lipoxygenase products.²⁵⁻³¹

Apyrase or CP/CPK was utilized for removal of extracellular ADP. Only when these agents were combined with either ETYA or indomethacin was collagen-induced aggregation completely blocked. These results indicate that collagen can induce platelet aggregation by more than one mechanism, in agreement with the findings of other investigators.¹²,¹³
Both the oxygen burst and aggregation response induced by collagen were inhibited by agents that increased intracellular cAMP levels (Table 1). This observation suggests that decreases in cAMP, which are ordinarily associated with aggregation, occur prior to the initiation of phospholipase A₂ activity.

In agreement with results previously reported by Mürer,² who used thrombin as the platelet stimulus, we observed that only the combination of antimycin and DOG totally abolished the oxygen burst initiated by collagen (Table 1). However, Mürer’s thrombin experiments were carried out in the presence of EDTA; therefore no aggregation occurred. We noted a slight but definite degree of aggregation in the presence of antimycin and DOG that was prevented by the addition of apyrase to the preincubation mixture. It thus appears that the oxygen burst and aggregation response require a certain amount of metabolic energy regardless of whether it is derived from mitochondrial or glycolytic sources.²,³²

Results of experiments utilizing SOD and catalase (Table 1) indicated that endogenously generated superoxide radicals had no effect on the collagen-initiated oxygen burst or aggregation, confirming previous observations.¹⁸ Since the oxygen burst in leukocytes is associated almost exclusively with the generation of superoxide radicals and hydrogen peroxide,¹⁴,¹⁵ it is obvious that the leukocyte and platelet oxygen bursts are associated with different cellular functions.

Studies of the burst of oxygen consumption in stored platelets were of interest in that they indicated the relative stability of platelet cyclooxygenase activity, since an oxygen burst was elicited with arachidonic acid but not with collagen or thrombin (Figs. 2 and 3). The experiments further demonstrated that phospholipase A₂ activity, or steps leading to its activation, appear to be more storage labile than is cyclooxygenase. Moreover, in fresh platelets it was possible to elicit repeated O₂ bursts with arachidonate as opposed to collagen and thrombin (see also ref. 6). This suggests that the cyclooxygenase in intact platelets is capable of oxygenating arachidonic acid as this fatty acid is made available.

Malmsten et al.³³ and Smith et al.³⁴ have proposed that the endoperoxides PGG₂/PGH₂ and/or thromboxane A₂ induce platelet aggregation by way of ADP release. Experiments with apyrase and CP/CPK, reported herein (see Fig. 4), tend to support this contention, at least when arachidonic acid is used as the stimulus. In the presence of these agents arachidonic acid induced a normal oxygen burst that was not accompanied by an aggregation response. Preliminary studies indicate that these results can also be obtained in PRP. In the presence of CP/CPK³⁵ (5 mM CP and 40 U/ml CPK) arachidonate induces an oxygen burst but aggregation is completely inhibited.

In conclusion, the experimental results suggest that the platelet O₂ burst represents formation of prostaglandin endoperoxides occurring at the onset of aggregation. These phenomena, when elicited by collagen, require a minimal amount of ionic calcium and are inhibited by raising intracellular cAMP levels or by blocking energy metabolism. No role is apparent for superoxide radicals, another expression of basic differences between leukocytes and platelets, which can also be used as a measure of potentially troublesome cross-contamination. In addition it is suggested that the pathogenesis of one of the “storage lesions” of platelets is their lack of response in terms of an oxygen burst upon stimulation by collagen or thrombin. Furthermore, evidence has been provided that arachidonic
acid-induced aggregation is mediated via released ADP, as supported by the inhibitory action of apyrase or CP/CPK.

REFERENCES

25. Hamberg M, Samuelsson B: Prostaglandin endoperoxides. Novel transformations of arachi-
1. Proc Natl Acad Sci USA 71:3400–3404, 1974
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NM Bressler, MJ Broekman and AJ Marcus