Superoxide Production and Reducing Activity in Human Platelets

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ABSTRACT Human platelets contain the cuprozinc (cytoplasmic) and manganese (mitochondrial) forms of superoxide dismutase. Nevertheless, superoxide radicals were detectable in the surrounding medium of metabolically viable platelet suspensions by using two assay systems: cytochrome *c* and nitroblue tetrazolium. The quantity of superoxide generated by platelets (5×10^5 superoxide radicals/platelet per 10 min) was constant and did not increase after aggregation by agents such as collagen and thrombin. The superoxide-generating system was present in the supernate of both aggregated and resting platelets and therefore was not platelet-bound. Platelet superoxide production was unaffected by prior ingestion of aspirin, indicating that the prostaglandin and thromboxane pathways were not involved. Both resting and aggregated platelets exhibited a reductive capacity toward cytochrome *c* and nitroblue tetrazolium which was unrelated to superoxide production. Furthermore, the aggregation process always resulted in a marked increase in this reduction. The nonsuperoxide reduction associated with aggregation was found to be membrane bound and to decrease with an apparent first order reaction rate ($k_1 = 0.067$ min^{-1}). In addition, accumulative, time-dependent nonsuperoxide-related cytochrome *c* reduction was also detected. Since there is no superoxide dismutase in plasma, the presence of superoxide radicals in the surrounding medium of platelets may have in vitro significance for platelet and leukocyte concentration and storage and in vivo significance for hemostasis, coagulation, and thrombosis. The nonsuperoxide-related reducing activities may represent a biochemical basis for platelet-blood vessel interactions, with particular reference to blood vessel integrity.

INTRODUCTION

Aerobic cells contain the enzyme superoxide dismutase (SOD),[1] which serves as protection from destructive effects of superoxide radicals (1, 2). These free radicals form as toxic intermediates in oxidative metabolic processes because the spin state of oxygen favors univalent pathways of reduction (1–3). SOD catalyzes the reaction: $O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$. Human polymorphonuclear leukocytes utilize superoxide radicals for bacterial killing (4–6), while in the disorder chronic granulomatous disease, generation of superoxide radicals is defective (6, 7). Leukocytes also generate O_2^- in response to cell surface perturbation in the absence of phagocytosis (8, 9). Superoxide production in lung tissue has been implicated in the pathogenesis of poisoning by the herbicide, paraquat (10), and in the pulmonary fibrosis resulting from long-term ingestion of nitrofurantoin (11). Thus, clinical consequences of exposure of cells and tissues to superoxide radicals are becoming apparent.

In the course of an unrelated study involving a lipid-protein fraction from human platelets (12) SOD was identified and an investigation of the role of this enzyme in platelet function was undertaken. Since superoxide radicals are capable of damaging cell membranes and macromolecules (1–3), and since there is essentially no SOD in plasma (13), the presence of superoxide radicals in the external milieu of stimulated or unstimulated platelets may have significant implications for hemostasis, coagulation, and occlusive vascular disease. Furthermore, the generation of O_2^- could be an important determinant of platelet viability during processing, concentration, and storage for clinical transfusion purposes.

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[1] *Abbreviations used in this paper*: NBT, nitroblue tetrazolium; SOD, superoxide dismutase.

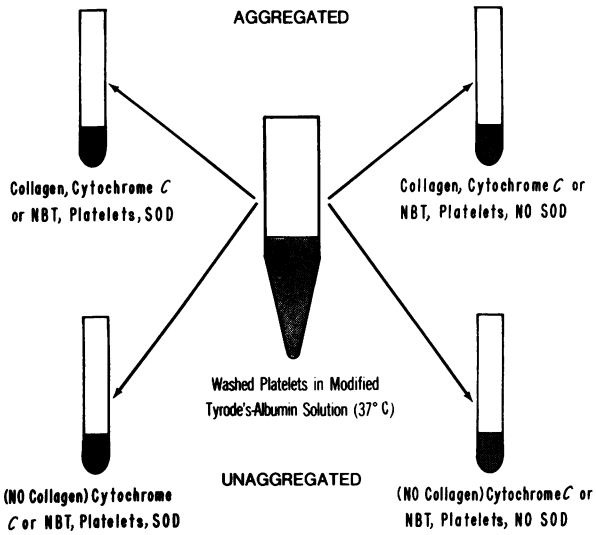

FIGURE 1 Diagram of procedure utilized for platelet aggregation and simultaneous assay of cytochrome *c* or NBT reduction in the presence or absence of SOD. Platelets were transferred from stock suspension to four assay tubes (containing appropriate reagents) in a sequential manner at 15-s intervals. Successive sets of experiments were spaced approximately 30 min apart. In the cytochrome *c* assay it was necessary to note the exact time of platelet addition since values increased with the age of platelets in the stock suspension. This was due to cytochrome *c* reduction unrelated to superoxide generation. Thus the cytochrome *c* data were plotted on a time scale with the venipuncture taken as zero time (Figs. 4–7).

This report describes the qualitative and quantitative distribution of SOD in human platelets. The appearance of O_2^- in the surrounding medium of washed platelets incubated alone and in the presence of aggregating agents will be documented. Finally, data on the reductive capacity of 'resting' and aggregated platelets (unrelated to O_2^- production) will be presented.

METHODS

Platelet collection and processing. For each experiment 1 U of whole blood was drawn into a plastic bag containing citrate dextrose solution (U.S.P. formula A) as anticoagulant. (No. 4RO112, Fenwal Laboratories, Inc., Morton Grove, Ill.) We obtained an explicit history concerning medications and dietary status from each donor. Blood packs were transported in insulated containers, and processing was initiated within 40 min.

Initial centrifugation was carried out in a Sorvall RC-3 centrifuge (Du Pont Instruments, Newtown, Conn.) (HG-4L rotor) set for 7 min and 1,000 *g* (22°C). An additional 3 min was required for braking time (14). Platelet-rich plasma was expressed from the bag into 50-ml conical polypropylene tubes and centrifuged at 1,100 *g* for 15 min (25–30°C). Washing and suspending media were those devised by Mustard et al. (15), and deionized water was used throughout. This buffer system contains albumin in a concentration of 3.5 g/liter. Each platelet pellet was gently suspended (polyethylene dropper) in 5 ml of washing fluid (37°C) and the suspensions were pooled into two tubes, each diluted to a total volume of 35 ml. Two washes were carried out with centrifugation at 37°C for 15 min (1,100 *g*). Before each centrifugation the platelets were allowed to incubate for 15 min (37°C). The platelets were finally pooled and counts adjusted to an average of 2.8×10^9/ml (4.78 mg protein/ml) utilizing the microhematocrit technique of Tollefsen and associates (16). Contaminating erythrocytes and leukocytes were removed by 'trapping' in the cone of the centrifuge tubes. Examination of stained smears prepared from final platelet suspensions revealed that total leukocyte contamination was less than 0.1%, of which 99.8% were lymphocytes.

All experiments reported were carried out with samples removed from the 'stock' suspension, which was maintained at 37°C. The processing time from blood collection to final suspension was 3–4 h. Radioactive serotonin uptake was used as an indicator of metabolic viability of the platelets (17). In addition, oxygen consumption, fluctuations in pH, and alterations in aggregation responses to collagen were monitored.

Assays for SOD. The method of McCord and Fridovich (18) was employed for quantification of platelet SOD. Washed, frozen, and thawed platelets, sonified in a cup-horn instrument (model L Sonifier Converter, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) (four 15-s intervals, output setting at '8,' 15°C), as well as supernatant fractions from ultracentrifuged platelet homogenates, (19) were used for assay. To inhibit cytochrome *c* oxidase (19), a potential source of error (20), KCN (10 μm) was included in each assay. Manganese SOD was measured by inhibiting the copper-zinc form with 1 mM cyanide (21, 22). Triton X-100 (0.033%, final concentration) was included in the assays for solubilization of any SOD that may have been particle-bound (23).[2] Measurements were made in a Cary 16 spectrophotometer with full scale expanded to 0.2 absorbance units. A calibration curve was constructed with beef erythrocyte SOD. (Truett Laboratories, Dallas, Tex. Preparations used in aggregation studies were dialyzed and lyophilized before use). To avoid reduction or oxidation of cytochrome *c* by endogenous platelet substances and at the same time remain in the linear portion of the standard curve, it was necessary to limit the range of platelet protein assayed to 45–200 μg. Sample blanks (no xanthine-xanthine oxidase) served as controls.

Platelet SOD was also studied with the use of polyacrylamide gel electrophoresis as described by Beauchamp and Fridovich (20). However, the resolving gels were chemically polymerized (25) rather than photopolymerized. Samples (100 μg protein) were placed on the gels in Tris-glycine buffer containing 20% glycerol (25). Gels were stained for SOD activity by the procedure of Salin and McCord (22).

Assays for O_2^- generation

Ferricytochrome *c* (18) and nitroblue tetrazolium (NBT) (6, 26–28) were used as indicating scavengers for O_2^- in two separate assay procedures. Both involved an indirect method of intercepting superoxide radicals by exogenously provided SOD.

Cytochrome c assay. The test system (Fig. 1) consisted of four polypropylene tubes (no. 2059 [17 × 100 mm], Falcon

[2] Commercially obtained xanthine oxidase (Sigma Chemical Co., St. Louis, Mo.) was further purified by the method of Rajagopalan and Handler (24), with the addition of sodium salicylate (2 mM) to all buffers. Personal communication. Dr. G. Nathans, Pritzker School of Medicine, Chicago, Ill.

Plastics, Division of BioQuest, Oxnard, Calif.) which were incubated at 37°C in a Dubnoff shaker for indicated periods of time. Two tubes contained cytochrome *c* (no. C-2506 horse heart, type III, Sigma Chemical Co., St Louis, Mo.) (final concentration 182 μM) and the aggregating agent under study. Collagen (Hormon-Chemie, 8000 München 45, Postfach 101, West Germany) (devoid of SOD activity) was used in quantities of 10 or 20 μg and thrombin[3] at 1 U per tube. Two control tubes contained cytochrome *c* but no aggregating agent. SOD (Truett Laboratories, Miles Laboratories Inc., Elkhart, Ind.) was added to one tube of each pair in a final concentration of 45 μg/ml. Reagents were warmed to 37°C for 30 s and reactions initiated by addition of 0.4 ml platelet suspension, resulting in a total volume of 0.55 ml per incubation mixture. After incubation, reactions were stopped by placement of the tubes in ice and centrifugation (microfuge B, Beckman Instruments, Inc., Palo Alto, Calif.) in the cold room for 3 min (15,000 rpm). Supernates were transferred to conical glass tubes and stored at −60°C overnight. Supernates were thawed, promptly diluted fivefold with potassium phosphate buffer (0.1 M, pH 7.4), and absorbance spectra measured *vs.* buffer in a Cary 16 recording spectrophotometer (545–565 nm). The most reproducible duplicate readings were obtained when absorbance at the isosbestic point (555 nm) was subtracted from that at the maximum (547 nm). The quantity of reduced cytochrome *c* in each sample was calculated from the decrease in this value after oxidation by a few crystals of ferricyanide with an absorbance coefficient of 21.1 mM^{-1} cm^{-1} (reduced minus oxidized) (29, 30, 8). Occasionally the total cytochrome *c* concentration was checked by subsequent reduction with dithionite. Blank values (complete reaction mixtures without platelets) were subtracted from assay values. Results were expressed as nanomoles reduced cytochrome *c*, adjusted to 1×10^9 platelets, and plotted against a time scale with the venipuncture taken as zero. Superoxide production was calculated as the difference between points obtained in the absence and presence of SOD. Inactivated SOD was used in control experiments. 10 mg of enzyme (Miles Laboratories, Inc.) were dissolved in 10 ml Tyrode salt solution and autoclaved for 20 min at 120°C. The autoclaved material was then dialyzed *vs.* Tyrode salt solution (pH 7.35) for 24 h at 4°C. When the preparation was reassayed at the end of the procedure, only faint traces of SOD activity were detected. The above procedures were carried out in the absence of fluorescent light (31).

NBT assay. The incubation system was identical to that of the cytochrome *c* assay (Fig. 1). A modification of the procedure of Dejesus et al. (28) for measuring NBT reduction by platelets was used. NBT stock solution (0.92 mg/ml in Tyrode salts) was freshly prepared, and after dissolution at 37°C was passed through Whatman no. 1 filter paper. The pH was readjusted to 7.35 with 5% CO_2 gas. Final concentrations in the incubation mixture were: NBT, 0.4 mg/ml, and SOD, 8.2 μg/ml. Unless otherwise indicated, 40 μg of collagen was added to induce aggregation. Assays were initiated by addition of 0.5 ml platelet suspension to the other reactants, which were not prewarmed. Total volume of incubation mixtures was 1.15 ml. Incubations (20 min in most experiments) were terminated by addition of 5 ml 0.5 N HCl, vortex mixing, and placement in ice. The tubes were centrifuged at 3,500 *g* for 45 min (4°C), supernates discarded, and sediments overlayed with 1 ml pyridine. After storage overnight (4°C), the sediments were broken up with glass rods and the tubes capped and placed in boiling water for 10 min. Samples were recentrifuged for 45 min (3500 *g*, 25°C) and the optical density of the pyridine extracts read at 515 nm *vs.* pyridine. 'Zero time' blanks were prepared by addition of HCl before

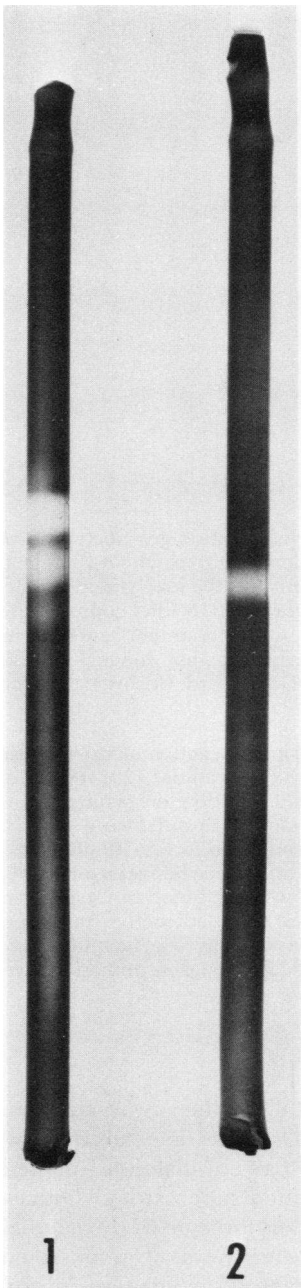

FIGURE 2 Polyacrylamide gel electrophoretic analysis of 100 μg of soluble fraction protein derived from a platelet homogenate that was ultracentrifuged on a sucrose gradient (19). The achromatic zone represents the location of platelet SOD that inhibited NBT reduction (20). Gel 1: beef erythrocyte SOD standard (Truett Laboratories. Preparations used in aggregation studies were dialyzed and lyophilized before use.); gel 2: platelet fraction.

[3] Kindly provided by Dr. John Fenton, New York State Department of Health, Albany, N. Y.

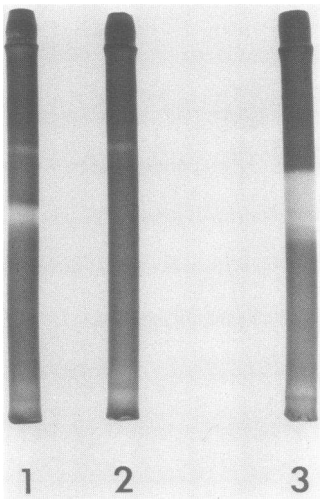

FIGURE 3 Polyacrylamide gel electrophoretic pattern of 100 μg of protein derived from whole platelets (33). Gel 1: the upper band represents the manganese (mitochondrial) form of SOD. The lower band is the cuprozinc (cytoplasmic) enzyme. Gel 2: after treatment with 2 mM cyanide the cuprozinc enzyme is no longer visible. Gel 3: beef erythrocyte SOD standard. (Miles Laboratories, Inc.)

platelet suspensions. Reaction mixtures without platelets did not reduce NBT. Cyanide (20 μM) in the incubation mixtures (28) had no effect on results and was not included in later experiments. Results were expressed in terms of optical density, adjusted to 1×10^9 platelets or to 1 mg platelet protein. The difference between total and SOD-inhibitable NBT reduction was attributed to O_2^- generation.

Other procedures. Radioactive serotonin uptake and release were measured by the technique of Valdorf-Hansen and Zucker (32). Protein determinations were as previously described (19).

RESULTS

Platelet SOD. The cuprozinc and manganese forms of SOD were detectable in whole platelets. Frozen and thawed platelets, pooled from several donors, contained superoxide dismutase equivalent to 1fg of bovine erythrocyte SOD/platelet. About 13% of the enzyme activity was cyanide-insensitive, indicating the presence of manganese (mitochondrial) SOD (21, 33). Assays carried out on the soluble fraction derived from an ultracentrifuged platelet homogenate (19) indicated that cuprozinc SOD was essentially a cytoplasmic enzyme.

Fig. 2 depicts a polyacrylamide gel electrophoretic separation of the soluble portion obtained from a subcellular platelet fractionation after staining for SOD activity (22). A single band, which represents the zone in which platelet SOD inhibited reduction of NBT, can be seen. When frozen and thawed platelets were treated with Triton X-100 and extracted with ethanol and chloroform (33), the mangani and cuprozinc forms of SOD were present on the gels. Treatment of the gels with cyanide (2 mM) inactivated the cuprozinc enzyme. Thus only the mangani form of SOD was visible. The results are depicted in Fig. 3.

Properties of platelet suspensions. Experiments could not be conducted in platelet-rich plasma since there was interference by plasma components in both the cytochrome *c* and NBT assays. Furthermore, the number of platelets in platelet-rich plasma was below the sensitivity range of the detection systems. Thus it was necessary to carry out the studies in a washed platelet system (15). The pH was stable over the course of the experiments (average change, 0.2 U) and [^{14}C]serotonin uptake averaged 90% during that time, indicating that the platelets were metabolically viable (17). Oxygen consumption was intact, as monitored by a Clark electrode (model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio). Aggregation responses to collagen and thrombin were maximal throughout the test period and were preceded by a shape change.

Superoxide detection by cytochrome c assay. O_2^- radicals were detected in the surrounding medium of unstimulated and collagen-aggregated platelets in each of 15 experiments. The quantity of O_2^- detected did not change with the age of the platelet suspension, nor was there a significant difference between aggregated and nonaggregated platelets (Table I). A prominent feature of all experiments was non-superoxide-related cytochrome *c* reduction. This reduction, which was not inhibitable by SOD, was detected in nonaggregated as well as aggregated platelet incubates and increased with the age of the platelet suspension. Furthermore, when the platelets were removed from the system at successive time intervals by centrifugation, the increase in non-O_2^- reducing capacity was detectable in the supernates. That portion of the non-O_2^- reducing capacity which is present in the supernate is responsible for the slope of the curves shown in Figs. 4 and 5. The substance (s) responsible for this time-dependent cytochrome *c* reduction was not identified. Reduced glutathione was not detected (34) in the supernate

TABLE I
O_2^- Production by 'Resting' and Collagen-Aggregated Platelets

Conditions	O_2^--dependent cytochrome *c* reduction
	nmol/l × 10^9 platelets/10 min
'Resting'	0.76±0.35 SD
Aggregated	0.69±0.23 SD

The above figures were derived from a total of 84 experimental points. The statistical difference between the two values is not significant (Student *t* test).

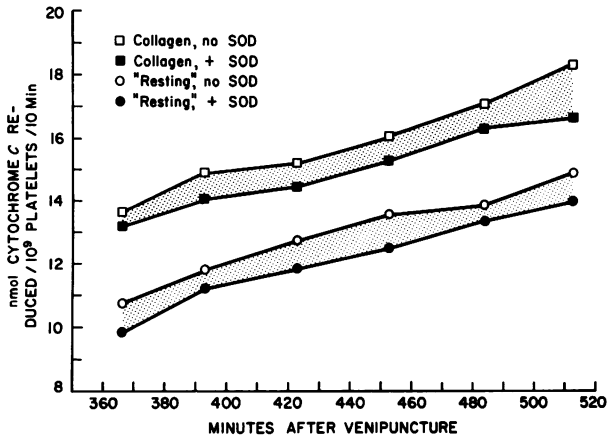

FIGURE 4 Cytochrome *c* reduction by collagen-aggregated and 'resting' platelet suspensions in the presence or absence of SOD (45 μg/ml). At the indicated time points platelets were removed from the stock suspension and aggregated or shaken in the presence of cytochrome *c* for 10 min. The shaded areas represent superoxide generation by aggregated (upper) and 'resting' (lower) platelet suspensions. The quantity of O_2^- produced by aggregated and nonaggregated platelets was similar. The slope of the curves as well as the elevated position of the two upper lines (representing aggregated platelets) are due to non-O_2^--dependent cytochrome *c* reduction.

and addition of a quantity of serotonin which would be expected to be released from 1×10^9 platelets did not reduce cytochrome *c*.

In addition to the time-dependent non-O_2^- cytochrome *c* reduction described above, a marked increase in non-O_2^- reduction always occurred in association with the aggregation process. The elevated position of the two curves representing aggregated platelets (Fig. 4) is due to this increase in reduction. The association of the increase in nonsuperoxide-related reduction with aggregation was further explored. The cytochrome *c* assay was carried out in an aggregometer and the reaction terminated at the upswing of the recorded collagen-induced aggregation curve (31 s). Under this circumstance the increase in non-O_2^--related reduction was absent. In contrast, when the reaction was terminated in 115 s (irreversible aggregation) the increase in non-O_2^--related reduction was present. This phenomenon was demonstrable clinically when platelets from a donor who had ingested medication containing glyceryl guaiacolate (35) showed a negligible aggregation response to collagen.[4] In the absence of macroscopic aggregation, the increase in non-O_2^--related reduction did not occur. Nevertheless, superoxide production itself was detected in collagen-treated and -untreated platelet suspensions. It can be seen in Fig. 5 that the points obtained with collagen-treated and 'resting' platelets were virtually superimposable.

Since thrombin stimulates platelets by a mechanism different from that of collagen (36), experiments on thrombin-induced aggregation were performed. The results obtained were identical to those observed with collagen.

As agitation is required for platelet aggregation, the effect of shaking in the absence of an aggregating agent was evaluated. Results of these experiments indicated that platelet motion was not the determinant of O_2^- generation or total cytochrome *c* reduction.

When autoclaved SOD was used in place of the active enzyme there was no difference in cytochrome *c* reduction between reaction mixtures containing the autoclaved preparation and those containing no enzyme. This eliminates the possibility of a nonspecific inhibitory effect related to the presence of protein in the assay.

Superoxide detection by NBT assay. This system was used for confirmation and extension of results obtained with cytochrome *c* assays. A major difference between the NBT and cytochrome *c* results was the absence of interference by time-dependent non-O_2^- reduction. This enabled us to carry out experiments at convenient time intervals without correlating results with the age of the platelet suspension. Superoxide radicals were detected in aggregated and 'resting' platelets in 15 separate experiments. Non-O_2^--related NBT reduction was present in unstimulated platelets and, as in the cytochrome system, aggregation was accompanied by a significant increase in such reduction (Fig. 6).

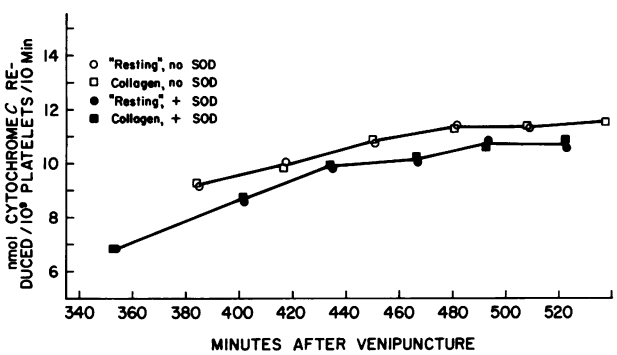

FIGURE 5 After ingestion of medication containing glyceryl guaiacolate, the subject's washed platelets failed to aggregate in response to collagen. The increase in cytochrome *c* reduction unrelated to superoxide generation which accompanies aggregation was absent (compare with upper curves of Fig. 4). However, O_2^- production was detected in all of the platelet suspensions, indicating that it was not inhibited by this drug.

[4] Scattered microscopic aggregates were observed by phase contrast.

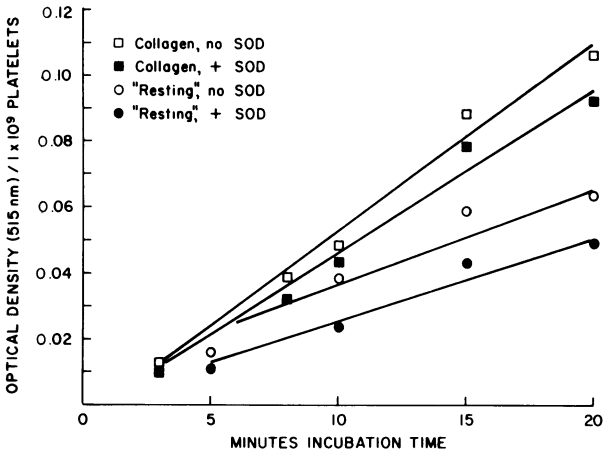

FIGURE 6 NBT reduction by collagen-aggregated and 'resting' platelet suspensions in the presence or absence of SOD. The abscissa indicates the time of incubation after stimulation. For most experiments a 20-min incubation time was employed. The ordinate depicts optical density of the blue formazan (extracted into pyridine) in terms of platelet protein. Results were comparable when related to 1×10^9 platelets. On a statistical basis the quantity of superoxide generated by aggregated and 'resting' platelets was similar. Lines on the graph were constructed by the method of least squares.

NBT reduction after aspirin ingestion. Superoxide- and nonsuperoxide-related NBT reduction was studied with the platelets of a donor who had ingested four 300-mg tablets of acetylsalicylic acid 24 h previously. Aggregation responses to varying concentrations of collagen and their effect on NBT reduction were evaluated. As shown in Fig. 7, aspirin ingestion had no effect on superoxide production in 'resting' or stimulated platelets. When low concentrations of collagen were used, aggregation and [^{14}C]serotonin release did not take place and the increase in non-O_2^--related reduction was also absent. At higher collagen concentrations the inhibitory effect of aspirin was overcome, aggregation and release occurred, and the increase in non-O_2^- NBT reduction was apparent. Thus it can be concluded that superoxide production by platelets does not appear to be dependent upon the prostaglandin and thromboxane pathways (37) since it was not inhibited after aspirin ingestion.

Presence of leukocytes in platelet suspensions. Granulocytes and monocytes were essentially absent from the final platelet suspensions. The only contaminating cells seen on stained smears were lymphocytes, which do not generate superoxide radicals (38, 39). An additional experiment was carried out in which leukocyte contamination was reduced further. Only the upper 60% of the platelet-rich plasma layer in the blood bag was processed. This plasma was respun at 100 *g* and the supernatant platelets processed further. There were virtually no contaminating leukocytes in this preparation. Nevertheless superoxide radicals were detected in the same quantities as in previous experiments. Furthermore, phorbol myristate acetate, a known stimulus of O_2^- production in leukocytes (8), did not stimulate additional superoxide in the platelet suspensions. We have thus concluded that the O_2^- measured was in fact generated by the platelets.

Additional studies of superoxide- and nonsuperoxide-related reduction. It was of interest to discern whether platelet stimulation was accompanied by a 'burst' of superoxide production comparable to the 'oxygen burst' reported to occur in platelets (40–42). Thus NBT was added 5 or 12 min after collagen stimulation ('Delayed NBT Experiments,' Table II), followed by an additional 20-min incubation period. Controls were also carried out in which NBT was present at the beginning of stimulation. As shown in Table II, superoxide was not produced in a 'burst.' It can be seen that the O_2^- detected in the 5- and 12-min samples was quantitatively the same as in the controls wherein NBT was present at the beginning of stimulation. On the other hand, results concerning nonsuperoxide NBT reduction associated with aggregation indicated that this reducing activity could have become available in a 'burst.' When NBT was added after a 5- or 12-min delay, reduction was progressively smaller than that measured when NBT was initially present ('0 time') (Table II). This was in sharp contrast to the observations made on superoxide

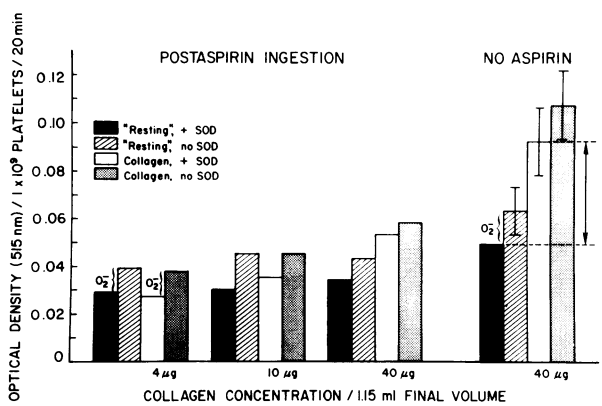

FIGURE 7 NBT reduction by platelets from a donor who had ingested aspirin. At a concentration of 4 μg collagen there was no aggregation or [^{14}C]serotonin release; nevertheless, superoxide generation was detected in both 'stimulated' and unstimulated platelets. The increase in nonsuperoxide-related NBT reduction usually associated with aggregation (indicated by arrow) was absent. This is also apparent with platelet suspensions stimulated by 10 μg of collagen in which aggregation did not occur. When 40 μg collagen was used, aggregation and [^{14}C]serotonin release took place and the increase in nonsuperoxide-related NBT reduction was apparent, although of lesser magnitude than control values. The bars shown at the right represent mean values and standard deviations from six experiments (12 points).

production, which was constant and equal in aggregated and nonaggregated platelets.

Experiments were carried out to determine whether the systems responsible for superoxide- as well as nonsuperoxide-related reduction associated with aggregation were platelet-bound or free in the supernate. Collagen-stimulated platelets were incubated in buffer (12 min) in the absence of NBT, followed by placement in ice (10 min) and centrifugation at 3,000 rpm for 20 min (4°C). The supernates were stored in ice (1–3 h) until all test samples and controls were prepared. Aliquots derived from 1×10^9 platelets were then added to the standard NBT assay. As shown in Table III, the non-O_2^- NBT reduction was platelet-bound, but the superoxide-generating mechanism was present in the supernate. These results will be analyzed in detail in the Discussion.

The relationship of physical platelet aggregation to the non-O_2^- reduction associated with the aggregation process was studied by inhibiting gross and microscopic thrombin-induced aggregation with EDTA. NBT reduction and [^{14}C]serotonin release were measured in the usual manner. The results are shown in Table IV, where it can be seen that stimulated but unaggregated platelets were still capable of eliciting 54% of the increase in non-O_2^- reduction achieved in the control samples. Concomitantly, release in the presence of EDTA was 62% of that in the aggregated control. Table IV also shows that the presence of EDTA had no effect on the production of superoxide radicals. It is therefore apparent that the events leading to full platelet aggregation, including release, are required for the non-O_2^- reducing activity associated with the aggregation process.

DISCUSSION

Human platelets contain 1 fg SOD/platelet,[5] which is approximately one-fifth of that present in the leukocyte (22) and one-ninth of that in the erythrocyte (13). Approximately 13% of platelet SOD is the manganese-dependent form located in mitochondria (2). The latter finding favors previous contentions that mitochondrial metabolism plays an important role in platelet function (36, 40).

Fridovich has proposed that the univalent reduction of oxygen accounts for a constant fraction of the total oxygen reducing capacity of all cells (2). The SOD in each cell should dismutate all of the O_2^- formed. Therefore, no O_2^- should be expected to appear extracellularly. However, in the case of stimulated leukocytes, superoxide radicals are detectable in the external environment of the cells (4, 22). Goldstein and associates (8) have proposed that extracellular O_2^- may have been generated at the cell surface, where it was not accessible to SOD. Alternatively, the quantity of O_2^- produced in the stimulated cell might be in excess of the ability of intrinsic SOD to dismutate it. There is now substantial evidence that superoxide radicals cannot be tolerated by biological systems (3, 43). Since plasma and extracellular fluids contain little or no SOD (44, 13), protection of other cells and constituents of plasma from deleterious effects of O_2^- depends entirely upon spontaneous dismutation. Unfortunately, the life-span of O_2^- is extended by six orders of magnitude if spontaneous dismutation is the only mechanism of disposal (13). It was therefore pertinent to determine whether superoxide radicals were present in the extracellular medium of human platelets.

The results of cytochrome *c* and NBT assays demonstrated that superoxide production was detectable in aggregated and unaggregated platelets. Analysis of the data indicated that the quantities of O_2^- produced (5×10^5 O_2^- radicals/platelet per 10 min) in aggregated

TABLE II
*'Delayed NBT Experiments'**

Conditions‡	O_2^--dependent NBT reduction§		Non-O_2^- NBT reduction associated with aggregation	
	Aggregated	Unaggregated		
			OD§	%‖
NBT present in reaction mixture ('0 time')	0.011	0.012	0.036	100
NBT added after 5 min	0.010	0.012	0.025	69
NBT present in reaction mixture ('0 time')	0.024	0.019	0.062	100
NBT added after 12 min	—	0.019	0.028	45

* Described in text.
‡ Samples were incubated for 20 min after NBT addition.
§ Expressed as OD 515 nm.
‖ Per cent of '0 time' value for each experiment.

TABLE III
Superoxide and Nonsuperoxide NBT Reduction in Platelet Supernates

Component assayed	O_2^--dependent NBT reduction		Non-O_2^- NBT reduction associated with aggregation process
	Aggregated	Unaggregated	
Uncentrifuged platelet suspension (control)*	0.024‡	0.019	0.062
Supernate§ (12-min preincubation)	0.005	0.006	0.002

* NBT present initially. Total incubation 20 min.
‡ Results expressed as OD 515 nm/10^9 platelets.
§ After platelets were incubated with or without collagen for 12 min, the supernate was removed and assayed for 20 min. Experiments carried out in quadruplicate.

[5] This can also be expressed as the equivalent of 0.6 μg bovine erythrocyte SOD/mg platelet protein.

TABLE IV
*Thrombin-Stimulated Platelets in the Presence of EDTA**

Conditions	O_2^--dependent NBT reduction	Non-O_2^- NBT reduction associated with aggregation process	[^{14}C]Serotonin release‡
	OD 515 nm	*OD 515 nm*	*%*
Thrombin (aggregation)	0.021	0.041	59
Thrombin plus EDTA (no aggregation)	0.023	0.022	37

* 4.1×10^{-3} M EDTA was added. This was in excess of the quantity required to chelate the calcium and magnesium present in the buffer.
‡ NBT was also present in these assays.

and unaggregated platelets were equal. This is in contrast to leukocytes, where metabolic events associated with phagocytosis and/or membrane stimulation result in a marked increase in O_2^- production (5, 27, 8, 9). The absence of an increase of superoxide production in platelets after stimulation may account for the findings of Clawson and White (45), who showed that bacteria remained viable after being sequestered in platelet aggregates.

The 'Delayed NBT Experiments' (Table II) were designed to test the following hypotheses: in response to stimulation by an aggregating agent platelets produce O_2^- radicals in a 'burst' and no additional O_2^- thereafter. In contrast unaggregated platelet suspensions generate O_2^- continuously. Thus under certain experimental conditions an 'apparently' equal production of O_2^- by aggregated and unaggregated platelets might be detected. The results of these experiments indicated that aggregating platelets did not produce superoxide in a 'burst.' If a 'burst' of O_2^- had occurred it would have been undetectable when NBT was added 5 or 12 min later, since superoxide radicals dismutate in a matter of seconds (46). It was therefore confirmed that the rate of O_2^- production in aggregated and resting platelets is constant.

With regard to the electron source for the formation of the superoxide radical, one can postulate a system whereby a substance 'S_{red}' is produced by platelets and univalently reduces molecular oxygen to O_2^-. In the process 'S_{red}' is oxidized to 'S_{oxid}.' Since the amount of O_2^- detected by a 20-min incubation with NBT commencing at any time in the course of an experiment was constant (Tables I and II, Figs. 4 and 5), it follows that there exists a small, steady-state concentration of 'S_{red}.' If 'S_{red}' were released by the platelets into the medium, it should be possible to detect O_2^- production even after removal of the platelets. Assays of the supernates of stimulated and unstimulated platelets revealed that as much as 25% of the superoxide radicals of the uncentrifuged controls were detected in the supernates (Table III). It is to be expected that the O_2^- generated by the platelet suspensions during the preincubation period before the time of supernatant separation would not be detected by the NBT assay, since it would have rapidly dismutated. What could be detected in the supernate is the amount of O_2^- produced by the 'S_{red}' present at the moment of separation, i.e., the steady-state concentration. It is also possible that platelets continued to generate 'S_{red}' in the cold before and during centrifugation, in which case the amount of 'S_{red}' could exceed the steady-state concentration. Although the duration of storage in the cold varied for different samples, the results of these experiments, obtained in quadruplicate, were in excellent agreement. This indicates that the concentration of 'S_{red}' was not altered, by reaction with oxygen or otherwise, when the supernates were maintained at 4°C. We conclude from these experiments that 'S_{red}' is elaborated by platelets into the surrounding medium where it reacts with oxygen to yield O_2^-.

It is apparent that there is a superoxide generating system in platelets which is unaffected by the aggregation process. Although the O_2^- flux produced by platelets is lower than that of phagocytizing leukocytes (5, 6), its importance should not be underestimated. In the course of hemostatic or thrombotic reactions there can be a local accumulation of O_2^- which could cause or augment tissue damage. Handin et al. (47) reported that externally generated. O_2^-, in concentrations insufficient to aggregate platelets, did so synergistically in the presence of subthreshold quantities of thrombin.

As can be seen in Figs. 4 and 6, platelet aggregation always resulted in an increase in non-O_2^--related reduction. When aggregation did not occur, as shown in Figs. 5 and 7, this increase in non-O_2^- reduction was absent. Further insight into the nature of this reducing activity was provided by the experiments shown in Table II. In contrast to the usual assay procedure, NBT was not immediately available for reduction. In the controls, reduction observed when NBT was initially present (time delay = 0) was greater than that detected after a 5- or 12-min delay. Data obtained in the two experiments could be compared when expressed in percentage of the '0 time' values (Table II, column 5). When NBT was added after 5 or 12 min there was a 31 and 55% decrease, respectively, in non-O_2^- reducing activity associated with aggregation. The interpretation could therefore be made that in the absence of NBT this reducing substance(s), which we shall call 'A,' was labile and that unlike superoxide it was produced in a finite quantity and in a 'burst.' A plot of these values versus delay time yields a curve suggesting first order kinetics. This was confirmed by a graphic solution for first order reaction rate constant yielding $k_1 = 0.067$ min^{-1} with a half life ($t_{1/2}$) for 'A' of 10.4 min.

Inspection of data from the supernatant experiments

(Table III) shows that 12 min after collagen stimulation we obtained an OD 515 nm value of 0.002. If 'A' had remained in the supernate, the OD should have been 45% of the control value, i.e., 0.028 (Table II, columns 4 and 5). An alternative possibility is that 'A' was indeed released into the supernate by the aggregating platelets but was completely oxidized at 4°C. However, in experiments not shown it was noted that non-O_2^--related reduction does not proceed in the cold. We thus postulate that the increased nonsuperoxide reduction elicited by platelet stimulation is platelet-bound. This membrane-associated transient reducing capacity may be due to platelet membrane sulfhydryl groups (48), whose spatial orientation is altered during the events leading to aggregation. The reduction may be membrane enzyme-mediated, as proposed by Baehner et al. (27, 49) for phagocytizing leukocytes. Since this reduction represents a surface alteration which was not present before the aggregation process, further biochemical and physical studies of the events responsible for this phenomenon may provide new insights into the role of the platelet membrane in platelet function. Finally, one could speculate about possible involvement of the process in platelet-blood vessel interactions, with particular reference to the maintenance of blood vessel integrity.

There are now reports in the literature concerning the use of SOD for therapeutic purposes (50–52). On the basis of current in vitro (53, 54) and in vivo (55) data, it can be speculated that inclusion of SOD in platelet and leukocyte concentrates would dismutate accumulating superoxide radicals and thereby prolong the functional capabilities of these components in the circulation of bleeding and (or) infected patients.

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