Hydrogen peroxide has a role in the aggregation of human platelets

D. Del Principe, A. Menichelli, W. De Matteis, M.L. Di Corpo, S. Di Giulio and A. Finazzi-Agro*

*Department of Pediatrics, University of Rome La Sapienza, Viale Regina Elena, 324, 00161 Rome, *Department of Experimental Medicine and Biochemical Sciences, II University of Rome, Via O. Raimondi, 00173 Rome,* and *CNR Center of Molecular Biology, Rome, Italy*

Received 20 February 1985

The aggregation of platelets induced by soluble and particulate stimuli is enhanced by the addition of minute amounts of H_2O_2. Externally added catalase strongly inhibits the aggregation induced by particulate stimuli and by phorbol myristate acetate (PMA). The addition of aminotriazole to stimulated platelets causes a significant inhibition of intracellular catalase. This indicates the formation of H_2O_2 inside the platelets during activation. No effects were observed when the platelets were stimulated by the ionophore A23187.

Human platelet *Platelet aggregation* *Catalase* *Hydrogen peroxide* *Kawasaki's disease*

1. INTRODUCTION

The interaction of H_2O_2 with platelets in vitro has been extensively studied because it is thought that platelets can be exposed in vivo to the H_2O_2 produced by PMN cells undergoing the respiratory burst [1]. However, the reports on the interaction of platelets with H_2O_2 appear to be conflicting. In fact, some authors found that H_2O_2 is a powerful inhibitor of platelet functions [2,3] while others found that H_2O_2 may induce aggregation of platelets [4]. Moreover, the aggregation is inhibited by a variety of antioxidants and scavengers of H_2O_2 [5–8]. Yoshikawa et al. [9] recently reported that catalase can prevent the intravascular coagulation in rats induced by endotoxin. It has also been reported that H_2O_2 alone or in the presence of myeloperoxidase stimulates various platelet activities, such as the hexose monophosphate shunt [4,10], and that platelets may consume large amounts of H_2O_2 added to the incubation medium [11]. These reports together with growing evidence that the generation of H_2O_2 by cells is indeed purposeful and can no longer be considered as a mere undesirable byproduct of cell respiration [12] prompted us to investigate in more detail the role of H_2O_2 in platelets. We previously reported that platelets carry a membrane-bound H_2O_2-generating system which is activated by immunological stimuli [13]. In fact human platelets have been shown to release minute amounts of H_2O_2 after stimulation with latex or opsonized zymosan [14].

Here we report that externally added catalase causes inhibition of platelet aggregation induced by several different stimuli. Moreover, almost all the platelet agonists investigated do induce H_2O_2 generation inside the platelets.

Abbreviations: AT, 3-amino-1,2,4-triazole; PMA, phorbol myristate acetate; U46619,15(S)-hydroxy-9α, 11α-methanoepoxyprostadienoic acid; PMN, polymorphonuclear cell; PBS, phosphate-buffered saline

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

The chemicals were from commercial sources and were used without further purification. Platelets were prepared from blood samples obtained from healthy donors not taking any drugs for at least 20 days, after informed consent. Blood, col-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

lected in 3.8% Na citrate or 5 mM EDTA, was centrifuged at $100 \times g$ for 15 min. Residual PMNs were eliminated by centrifuging the plasma through a Ficoll layer (23%, w/v) at $80 \times g$ for 20 min. This procedure gave the PRP used for the aggregation studies. A further centrifugation of PRP at $1200 \times g$ for 15 min gave a platelet-containing pellet and a supernatant platelet-free plasma. The pellet was resuspended in Dulbecco's PBS, pH 7.4, at a concentration of 3.3×10^8 platelets/ml. These platelet preparations contained less than 1 PMN/10^8 cells [14,15]. Platelets were activated with agonists and with opsonized zymosan as described [14]. Activation experiments were performed at least in triplicate in the presence and absence of crystalline bovine liver catalase (Sigma, St. Louis). Further controls with catalase inactivated by heat or by treatment with AT (Fluka AG, Switzerland) and peroxide [16] were also carried out. Platelet aggregation was monitored by the change in light transmission at 650 nm in a model 840 dual-channel Elvi aggregometer (Elvi Logos, Milan) according to Born [17]. Platelet catalase activity in the presence and absence of 1 mM AT was measured at 30°C, using an oxygen-sensitive electrode (Yellow Springs Instruments, OH) in a 3 ml vessel. The enzymic activity was measured in lysed platelet suspensions. At the end of incubation with agonists, the reaction was stopped with melting ice. After accurate removal of AT by at least 3 washings, the platelets were disrupted by homogenization in a teflon potter. The sample (200 μl) was diluted in the oxygraph vessel with 2 ml distilled water. When thermal equilibrium was reached, H_2O_2 was added to a final concentration of 8 mM, and catalase-catalyzed H_2O_2 degradation was recorded. The spontaneous degradation of H_2O_2 was also recorded and subtracted accordingly from the oxygen production in the presence of catalase. Specific activity of catalase was defined as nmol O_2 generated/min per mg platelet protein [18]. Peroxidase activity was determined spectrophotometrically in the presence of 10 mM H_2O_2 and o-dianisidine as in [19]. Protein was determined by the method of Lowry et al. [20].

3. RESULTS

The addition of catalase to a platelet suspension strongly reduced the extent of aggregation induced by particulate stimuli (latex particles, opsonized zymosan, collagen) (fig.1A–C) or by a membrane-perturbing agent such as PMA (fig.1D). Boiled catalase or catalse inactivated by AT and peroxide was without effect. The aggregation induced by soluble stimuli (ADP, thrombin, epinephrine) was instead insensitive to the presence of catalase (fig.1E–G). However, this enzyme strongly inhibited the ADP-induced aggregation of platelets from a patient bearing Kawasaki's disease (fig.2A). This effect was only observed during the acute phase of the disease, when a hyperaggregability of platelets was also present. In the remission stage,

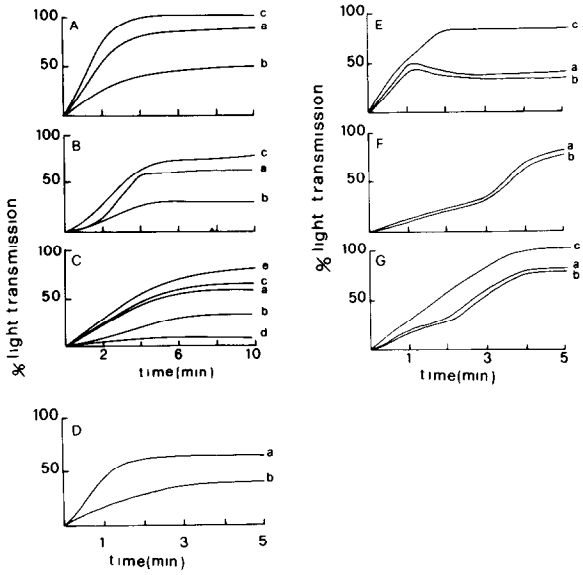

Fig.1. Effect of catalase and H_2O_2 on platelet aggregation. (A) Aggregation of PRP induced by collagen (2 μg/ml). a, no addition; b, + catalase; c, + H_2O_2. (B) Aggregation of PRP induced by latex (∅ 0.08 μm, 100 particles/platelet. a, no addition; b, + catalase; c, + H_2O_2. (C) Aggregation of PRP induced by zymosan (4 mg/ml). a, opsonized zymosan alone; b, opsonized zymosan + catalase; c, opsonized zymosan + H_2O_2; d, non-opsonized zymosan; e, non-opsonized zymosan + H_2O_2. (D) Aggregation of PRP induced by PMA (20 nM final concentration). a, no addition; b, + catalase. (E) Aggregation of PRP induced by ADP (1 μM). a, no addition; b, + catalase; c, + H_2O_2. (F) Aggregation of PRP induced by epinephrine (0.5 μM). a, no addition; b, + catalase. (G) Aggregation of a platelet suspension in Dulbecco's PBS (10^9/ml) induced by α-thrombin (0.05 U). a, no addition; b, + catalase; c, + H_2O_2. Where added catalase was 500 U;H_2O_2 was 200 nM.

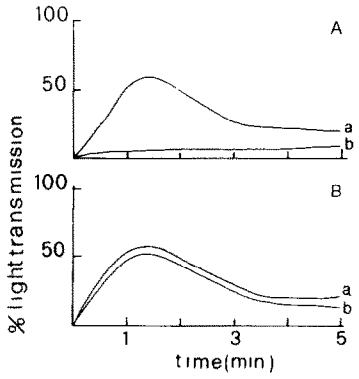

Fig.2. Aggregation of PRP from a patient with Kawasaki's disease. (A) During the acute phase. a, ADP (1 μM); b, + ADP and catalase. (B) During the remission phase. a, ADP (1 μM); b, + ADP and catalase. The patient was a 4-year-old girl (E.D.), who presented 6 out of the 6 symptoms of Kawasaki's disease [21]. The reversibility of aggregation is due to the fact that the patient was under treatment with acetylsalicylic acid.

catalase no longer affected ADP-induced aggregation, as found in healthy subjects (fig.2B). It is important to note that at this stage the patient also did not show platelet hyperaggregability. Non-opsonized zymosan is unable to elicit the aggregation of platelets except in the presence of 200 nM H_2O_2. The addition of peroxide also increased the aggregation induced by collagen, and to a lesser extent, that induced by ADP and thrombin. The aggregation induced by A23187 was not affected by the addition of catalase, while it was inhibited by exogenous H_2O_2.

Further evidence of the involvement of H_2O_2 in the aggregation process was obtained by poisoning the platelets with AT. This compound forms an irreversible adduct with catalase only in the presence of H_2O_2 [16]. The incubation of platelets with 1 mM AT and 1 mM H_2O_2 for 15 min produced 50% inhibition of the intracellular catalase. Greater inhibition of catalase was obtained when platelets were incubated with AT alone and then stimulated with collagen, ADP, opsonized zymosan, PMA, U46619, and low amounts (0.05 U/ml) of thrombin (table 1). Larger amounts ($\geq$2 U/ml) of thrombin and A23187 did not produce significant inactivation of platelet catalase. The activity of peroxidase was only slightly affected by AT.

Table 1

Catalase activity of platelets

Experiments	−AT (mean ± SE)	+AT (1 mM) (mean ± SE)	*n*
Resting platelets	3.70 ± 0.25	3.20 ± 0.8	7
Stimulated platelets			
+ A23187	3.60	3.1	3
+ U46619	3.60	1.5	3
+ latex	3.75 ± 0.15	1.5 ± 0.25	6
+ collagen	3.50 ± 0.15	1.0 ± 0.25	8
+ PMA	3.60 ± 0.26	1.2 ± 0.12	6
+ ADP (1 μM)	3.0	1.4	3
+ ADP (0.2 μM)	3.50	1.0	3
+ thrombin (0.05 U/ml)	3.0	1.3	3

Values expressed as nmol O_2 produced/mg protein

4. DISCUSSION

A recent review [12] has put into focus the widespread generation of H_2O_2 in cellular plasma membranes and its role in several cellular phenomena. Moreover, the production of H_2O_2 in mitochondria, microsomes, peroxisomes and nuclei is well established [12].

We have reported that platelets do release small amounts of H_2O_2 when challenged with particulate, immunological stimuli [14]. Here, we further show that the presence of externally added active catalase strongly decreases the aggregation of platelets triggered by particulate stimuli and by membrane-perturbing agents such as PMA. Soluble stimuli do not cause a detectable release of H_2O_2 from platelets. Accordingly, the aggregation of normal human platelets induced by these agents is not affected by the presence of catalase. One interesting exception is presented by the platelets drawn from a girl affected by the Kawasaki's disease [21]. During the acute phase of the disease, the aggregation induced by ADP was dramatically decreased by catalase. This effect was not seen to increase during the recovery period, when the platelets no longer showed hyperaggregability as in the acute phase.

The addition of minute amounts of H_2O_2 to a platelet suspension to some extent enhanced the aggregation induced by most stimuli (fig.1). The most spectacular effect of H_2O_2 was observed with

non-opsonized zymosan, which was unable to trigger the aggregation except in the presence of H_2O_2 as was the peroxide alone.

The experiments with AT clearly demonstrated that H_2O_2 is produced in the course of platelet activation with all stimuli used except the ionophore A23187 (table 1). Conversely, H_2O_2 was inhibitory with this stimulus only. This finding may suggest either that there is more than one mechanism of platelet activation, or that the production of H_2O_2 is an intermediate in a cascade process, perhaps occurring after the formation of arachidonic acid products but before the mobilization of Ca^{2+}. The latter hypothesis seems to be substantiated by the formation of H_2O_2 induced by the stable analogue of prostaglandin endoperoxides U46619, and by the experiments done with A23187. However, the differential sensitivity of platelets to external catalase depending on the type of stimulus (soluble or particulate) used seems to indicate that different transduction mechanisms of the external stimuli might be operating. Nishizuka [22] in fact suggested that the information can be brought inside from the platelet surface after the binding of a stimulatory agent either by the activation of protein kinase or by calcium mobilization [22]. The presence of alternative routes may allow finer control over different processes in the same cell. We suggest that H_2O_2 plays a role in such transduction. Similarly, H_2O_2 was found to mimick the insulin effect on adipocytes and to stimulate the lymphocyte blastization [23,24]. A relationship between oxidative agents and hypercoagulability has been suggested [25,26]. Furthermore, several lines of evidence have established a clear correlation between H_2O_2 and Ca^{2+} flux in mitochondria, microsomal vescicles and plasma membranes [27–29]. A physiological relevance of the peroxide-induced release of Ca^{2+} has been proposed [30].

It is conceivable that the level of H_2O_2 produced upon stimulation may vary as a function of physiological or pathological states of the platelets. Further study is required to ascertain whether the production of H_2O_2 is impaired when the aggregability of platelets is altered as in Kawasaki's disease, and to investigate the possible use of catalase as an anti-aggregating agent in vivo [9].

ACKNOWLEDGEMENT

This research was in part supported by Consiglio Nazionale delle Ricerche through Special Project 'Oncologica'.

REFERENCES

[1] Levine, P.H., Weinger, R.S., Simon, J., Scoon, K.L. and Krinsky, N.I. (1976) J. Clin. Invest. 57, 955–963.
[2] Stuart, M.J. and Holmsen, H. (1977) Am. J. Haematol. 2, 53–63.
[3] Rodvien, R., Lindon, J.N. and Levine, P.H. (1976) Br. J. Haematol. 33, 19–26.
[4] Clark, R.A. and Klebanoff, S.J. (1980) J. Immunol. 124, 399–405.
[5] Yoshikawa, T., Furukawa, Y., Murakami, M., Watanabe, K. Kondo, M. (1982) Thromb. Haemostas. 48, 235–237.
[6] Steiner, M. and Anastasi, J. (1976) J. Clin. Invest. 57, 732–737.
[7] Vargaftig, B.B., Trainer, Y. and Chignard, M. (1975) Eur. J. Pharmacol. 33, 19–29.
[8] Finazzi-Agrò, A., Menichelli, A., Di Giulio, S., Di Corpo, M.L., Cerrone, F., Todaro, D. and Del Principe, D. (1984) in: Oxidative Damage and Related Enzymes (Rotilio, G. and Bannister, J.V. eds) Life Chemistry Reports, Suppl. Ser. pp. 204–215, Harwood, London.
[9] Yoshikawa, T., Murakami, M., Yoshida, N., Seto, O. and Kondo, M. (1983) Thromb. Haemostas. 50, 869–872.
[10] Koufos, A. and Sagone, A.L. (1980) Blood 55, 835–840.
[11] Holmsen, H. and Robkin, L. (1977) J. Biol. Chem. 252m 1752–1757.
[12] Ramasarma, T. (1982) Biochim. Biophys. Acta 694, 69–93.
[13] Del Principe, D., Mancuso, G., Menichelli, A., Gabriotti, M., Cosmi, E.V. and Gherardi, G. (1980) Biol. Cell. 38, 135–140.
[14] Finazzi-Agrò, A., Menichelli, A., Persiani, M., Biancini, G. and Del Principe, D. (1982) Biochim. Biophys. Acta 718, 21–25.
[15] Ehrlich, H.P. and Gordon, J.L. (1976) in: Proteinases (Gordon, J.L. ed.) Platelets in Biology and Pathology, pp. 353–370, North-Holland, Amsterdam.
[16] Margoliash, E., Novogrodsky, A. and Scheyter, A. (1969) Biochem. J. 74, 339–350.
[17] Born, G.V.R. (1962) Nature 194, 927–929.
[18] Almagor, M., Yatziv, S. and Kahane, I. (1983) Infect. Immun. 41, 251–256.

[19] Worthington Enzyme Manual (1972) pp. 41–45, Worthington Biochemical Corp., Freehold, NJ.
[20] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Kawasaki, T., Kosaki, F., Okawa, S., Shigematsu, I. and Yanagawa, H. (1974) Pediatrics 54, 271–276.
[22] Nishizuka, Y. (1984) Trends Biochem. Sci. 9, 163–166.
[23] Mukherjee, S.P., Lane, R.H. and Lynn, W.S. (1978) Biochem. Pharmacol. 27, 2589–2594.
[24] Finazzi-Agrò, A., Conti, P., Hofman, M., Angeletti, P.U. and Rotilio, G. (1980) Agents Actions S 7, 220–223.
[25] Iatridis, S.G., Iatridis, P.G., Kyrkon, K.A., Markidou, S.G. and Iatridi, I.S. (1979) Thromb. Res. 15, 733–741.
[26] Niwa, Y. and Sohmiya, K. (1984) J. Pediatr. 104, 56–60.
[27] Satrustegni, J. and Richter, C. (1984) Arch. Biochem. Biophys. 233, 736–740.
[28] Jones, D.P., Thor, H., Smith, M.T., Jervell, S.A. and Orrenius, S. (1983) J. Biol. Chem. 258, 6390–6393.
[29] Bellomo, G., Mirabelli, F., Richelmi, P. and Orrenius, S. (1983) FEBS Lett. 163, 136–139.
[30] Baumhütter, S. and Richter, C. (1982) FEBS Lett. 148, 271–75.