Effects of Metabolic Inhibitors on the Platelet Aggregation Induced by Hydrogen Peroxide

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HIGASHI, O., KIKUCHI, Y. and KONNO, K. *Effects of Metabolic Inhibitors on the Platelet Aggregation Induced by Hydrogen Peroxide.* Tohoku J. exp. Med., 1973, 109 (3), 245-250 — In contrast to the adenosine diphosphate (ADP)-induced platelet aggregation, the hydrogen peroxide (H_2O_2)-induced platelet aggregation could be inhibited by either potassium cyanide or sodium azide or antimycin or oligomycin alone, suggesting that aerobic metabolism of glucose is essential to maintain a complete ability in aggregation of human platelets. — platelet aggregation by hydrogen peroxide; platelet metabolism; catalase

In the previous paper of our own, it was reported that normal platelets were readily aggregated by hydrogen peroxide (H_2O_2), while thrombasthenic platelets were not and that adenosine diphosphate (ADP) could be the final pathway as in cases of other aggregating agents (Higashi *et al.* 1972).

The purpose of the present paper is to disclose the unique characteristics, if any, of the platelet aggregation by this new agent, through the studies using various metabolic inhibitors.

MATERIALS AND METHODS

Blood was obtained from apparently healthy adolescent volunteers, 2 to 3 hours after breakfast.

Methods were as described in the previous paper (Higashi *et al.* 1971), unless otherwise indicated.

Deoxyglucose (DG), glucose, catalase, iodoacetic acid, potassium cyanide and sodium azide were dissolved in the imidazole buffer solution, pH 7.3 (IBS).

Seven mg of the beef liver catalase (Tokyo Kasei Kogyo Co.) was dissolved in 2 ml of the IBS. One portion of this enzyme solution was heated at 80°C for 15 minutes and precipitates were centrifuged at 3,000 r.p.m. for 5 minutes and the supernatant solution was used.

The catalase solution, unheated and heated, was added to the platelet rich plasma (PRP) just before addition of an aggregating agent by the use of the standard method described previously (Higashi *et al.* 1971).

Antimycin* (AM) and oligomycin† (OM) were used as alcoholic solutions. 3.5 mg of antimycin or oligomycin were dissolved in 0.1 ml of ethanol and 0.001-0.005 ml of the

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* Antimycin A (Kyowa Fermentation Company). † Oligomycin (Sigma).

solution was added to 0.6 ml of PRP by using a microsyringe (Hamilton 7005-N). In control tubes IBS or ethanol, of an amount corresponding to the inhibitor solution used, was added instead of the inhibitor solution.

The mixture was left at room temperature for one hour. Then, 0.029–0.025 ml of IBS was added to each tube respectively to make a total amount of mixture 0.63 ml.

After the incubation period of 1 minute at 37°C, 0.07 ml of the aggregating agent, namely 0.3% H_2O_2 or 0.2 mM ADP was added.

When the effect of AM or OM in combination with DG was studied, DG solution was added at about 5 minutes after AM or OM was mixed with PRP to avoid the possible precipitation of the antibiotics.

RESULTS

Effects of temperature on platelet aggregation induced by H_2O_2 or ADP

The incubation of PRP for 60 minutes at 37°C resulted in 50% reduction in platelet aggregation by H_2O_2 , while the incubation did not diminish platelet aggregation by ADP.

The storage of PRP at 4°C for one hour did not give rise to diminution of platelet aggregation by either H_2O_2 or ADP.

Effects of catalase on platelet aggregation induced by H_2O_2 or ADP

At the final concentration of 150 γ /ml, catalase prevented almost completely the platelet aggregation by H_2O_2 (cf. Fig. 1).

The heated catalase was ineffective, indicating that catalase was acting as an enzyme.

The platelet aggregation by ADP was unaffected by catalase.

Fig. 1. The effect of catalase on the platelet aggregation by H_2O_2 . The platelet aggregation by H_2O_2 is prevented by catalase, but not by the heated enzyme, as shown in these superimposed tracings of automatic recordings.

Effect of deoxyglucose on platelet aggregation induced by H_2O_2 or ADP

PRP was incubated with either glucose or DG, at a final concentration of 3 mM, for 60 minutes at either room temperature or 37°C.

No inhibitory effect of DG on H_2O_2 -induced platelet aggregation was apparent.

It was noticed, however, that when PRP was incubated at 37°C for 30 to 60 minutes with either glucose or DG added, the platelet aggregation by H_2O_2 was markedly diminished, while the platelet aggregation by ADP was not.

Effects of iodoacetic acid on platelet aggregation induced by H_2O_2 or ADP

The preincubation of PRP with iodoacetic acid, at a final concentration of 1 mM, at room temperature for 30 minutes resulted in a marked reduction of platelet aggregation induced by either H_2O_2 or ADP.

Effects of KCN and NaN_3 on platelet aggregation induced by H_2O_2 or ADP

When PRP was preincubated with either KCN or NaN_3 , at a final concentration of 1 mM, for 30 minutes at room temperature, either one of these agents had very marked effects upon the platelet aggregation induced by H_2O_2 but had no marked effects upon the platelet aggregation induced by ADP.

KCN, as well as NaN_3 , had dual effects upon the platelet aggregation by H_2O_2 . Namely, the aggregation time — which is required to obtain the maximum level of aggregation, — is shortened. The maximum level of aggregation, however, was decreased. An example is shown in Fig. 2.

Fig. 2. Effects of potassium cyanide and sodium azide on the platelet aggregation by H_2O_2 . Notice the dual effects, i.e. platelets start to aggregate faster but the maximum aggregation is less in the presence of the inhibitors than in their absence.

Effects of antimycin or oligomycin on platelet aggregation induced by H_2O_2 or ADP

The H_2O_2 -induced platelet aggregation was diminished by either antimycin or oligomycin alone at a final concentration of 50 to 250 γ /ml. Examples are shown in Figs. 3 and 4.

In a parallel study, it was observed that the ADP-induced platelet aggregation was not altered by the preincubation of PRP with AM or OM alone, of the same concentration as used in the H_2O_2 -induced platelet aggregation, while it was decisively inhibited when DG was added together with AM or OM.

Fig. 3. The inhibitory effect of antimycin on the platelet aggregation induced by H_2O_2 .

Fig. 4. The inhibitory effect of oligomycin on the platelet aggregation induced by H_2O_2 .

DISCUSSION

Comparative studies of H_2O_2 -induced and ADP-induced platelet aggregation in the present investigation disclosed that there were certain differences between these two distinct phenomena, especially with respect to the effects of various metabolic inhibitors.

The effect of catalase upon the platelet aggregation by H_2O_2 is self-explaining. It may prove that platelet aggregation is induced by H_2O_2 itself, not by any other substances contained in the H_2O_2 preparation used.

Both H_2O_2 -induced and ADP-induced platelet aggregation were inhibited by iodoacetic acid. In this case, however, whether the effect is due to the action on the intracellular glycolytic pathway is not clear as indicated by Skálhegg *et al.* (1964).

In literature it has been described that the ADP-induced platelet aggregation is not inhibited by KCN alone (O'Brien 1967), and we have confirmed this in the present study as described above. Here it is interesting to note that the H_2O_2 -induced platelet aggregation is inhibited by KCN or NaN_3 alone.

As described above, KCN as well as NaN_3 had dual effects upon the platelet aggregation by H_2O_2 . In the presence of KCN or NaN_3 , platelets began to aggregate more rapidly in response to H_2O_2 but the maximum degree of aggregation was less than that in the absence of these agents.

The former effect may be the result of inhibition by KCN of catalase in plasma, which may decompose H_2O_2 before it acts on platelets.

The mechanism which underlies the latter effect is not entirely clear, but it might be due to the known inhibitory effects of these agents on the respiratory enzymes (Hewitt and Nicholas 1963).

In supporting this hypothesis, it was demonstrated in the present investigation that the platelet aggregation by H_2O_2 could be inhibited by either AM or OM alone as described above. Mürer *et al.* (1967) reported that the ADP-induced platelet aggregation was not inhibited by AM or OM alone, but it could be blocked if DG was combined with AM or OM.

We have confirmed this in the present investigation. In addition, we observed that inhibitory effects of combined DG and AM upon the ADP-induced platelet aggregation were apparent not only in the case of preincubation at 37°C as shown by the previous investigators (Mürer *et al.* 1967), but also in the case of preincubation at room temperature as in the present experiment, although the degree of inhibition may be less than that in the case of preincubation at 37°C .

The exact mechanism of the suppression by AM or OM of H_2O_2 -induced platelet aggregation is not clear. However, it has been that AM blocks mitochondrial electron transport (Potter *et al.* 1952; Chance 1958) and OM inhibits oxidative phosphorylation (Huijing and Slater 1961).

It is generally accepted that platelets require a constant supply of ATP for the maintenance of their functions, including cell aggregation (Marcus 1969; Hellem 1967).

ATP is generated by either the anaerobic or the aerobic metabolism of glucose. And it has been suggested that only one of these pathways is necessary (Hellem 1967; Mürer *et al.* 1967). According to Gross, glycolysis is the most important energy source for platelets (Gross 1967).

However, the fact that the H_2O_2 -induced platelet aggregation was readily inhibited by either AM or OM alone, without addition of DG, at least under the experimental conditions described in the present paper suggests that not only the anaerobic but also the aerobic metabolism of glucose are required to supply the energy to maintain a complete ability in aggregation of human platelets.

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