A surface NAD-glycohydrolase of human platelets may influence their aggregation

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Human platelets may hydrolyze externally added NAD^+ yielding ADPR and nicotinamide. The extent of hydrolysis is significantly higher when the platelets are stimulated. The presence of external NAD^+ strongly inhibits the aggregation induced by every agonist used. It seems that adenosine or ADPR itself generated by NAD^+ hydrolysis may be responsible for the inhibition of aggregation. Evidence is given that some of the NAD^+ hydrolysis product is taken up by stimulated platelets.

(Human platelet) *Platelet function* *NADase* *Enzyme activity*

1. INTRODUCTION

NADase (NAD^+ glycohydrolase EC 3.2.2.5) is an enzyme which hydrolyzes NAD^+ at the nicotinamide-ribose bond. The presence of NADase in mitochondrial and plasma membranes of many cells has been reported. Several NADases have been identified as ADP-ribosyltransferase and poly(ADP-ribose) polymerase [1,2]. It has been suggested that the biochemical role of NADase is the regulation of certain metabolic processes through a control of the quantity of NAD^+ available to the NAD^+-dependent enzymes [3]. On the other hand it has been reported that NADases are ecto-enzymes in several cell types. For example, NADases from erythrocytes and macrophages are associated with the cell surface [4,5]. This evidence suggests a role of these enzymes in membrane-associated events, such as agonist-receptor interactions or in the transduction of external stimuli to the cell interior.

Deficiency of erythrocyte NADase has been reported in human subjects [6]. This deficiency was not apparently accompanied by clinical symptoms. Therefore the function of NADase in red cells is not yet known.

In the present paper we report on the presence of NADase in human blood platelets and its involvement in platelet function.

2. MATERIALS AND METHODS

The chemicals were from commercial sources and 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, collected in 5 mM EDTA, was centrifuged at $100 \times g$ for 15 min. Residual polymorphonuclear cells were eliminated by centrifuging plasma through a Ficoll layer (23%, w/v) at $80 \times g$ for 20 min. Further centrifugation of platelet-rich plasma at $1200 \times g$ for 15 min gave a platelet-containing pellet, which was resuspended in Dulbecco's PBS, pH 7.4, to a concentration of 3.3×10^8 platelets/ml. These platelet preparations contained less than 1 polymorphonuclear cell/10^8 platelets [7]. Fibrinogen (1 mg/ml) and $CaCl_2$ (1 mM) were added and then platelet aggregation was monitored after addition of appropriate stimuli by the change in light

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transmission at 650 nm in a standard platelet aggregometer (Aggrecoder PA-3210, Menarini, Firenze, Italy), according to the method of Born [8]. The agonists used were ADP (2 μM), collagen (2 μM/ml), adrenaline (0.5 μM), γ-thrombin (0.5 U/ml) and calcium ionophore A23187 (20 μM).

ADP-ribosylation was measured as the incorporation of [H 2,8-*adenine*-^{3}H]NAD^+ into intact cells, according to Berger et al. [9]. The disappearance of added NAD^+ was assayed using an enzymatic method based on alcohol dehydrogenase as described [10]. In some experiments the disappearance of NAD^+ was measured by isotachophoresis. The experimental conditions of this technique have been reported [11]. This method monitors both oxidized and reduced forms of pyridine nucleotides.

HPLC of supernatants from platelet suspensions was performed using a Perkin Elmer Series 3 liquid chromatograph, equipped with an LC 55 B spectrophotometric detector and an SC 55 S digital scanner and a Perkin Elmer 023 recorder. Integration of peak areas was achieved by means of a Hewlett-Packard mod. 3390 A integrating recorder. A Hibar-Merck RP-18 RT-250-4 10 μm column was used with the following solvent systems: solvent A, 0.01 M aqueous tetraethyl ammonium chloride (150 ml) and MeCN (350 ml); solvent B, 70 mM NaH_2PO_4 and 10 mM tetraethyl ammonium chloride in redistilled water. Solvents (HPLC grade) were filtered through Millipore HAW 0.45 μm filters and degassed under vacuum 10 min before use.

The following solvent program was used: (i) an isocratic step (2 min) at 3% solvent A: (ii) a linearly increasing gradient (10 min) from 3 to 10% solvent A; (iii) an isocratic step (8 min) at 10% solvent A; (iv) a purge step (5 min) at 50% solvent A (used every 8–10 injections); (v) an equilibration step (10 min) at the initial percentage of solvent A.

Sample elution was monitored at 260 nm and peak retention times neglecting the dead volume of the column are given. UV spectra of the single peaks were taken by means of a stop-flow technique with a scan rate of 120 nm/min, after background calibration. Identification of nucleotides and their degradation products was achieved by comparing them with authentic samples in the same eluting conditions or by using internal standards. The supernatant of platelet suspensions incubated in 100 mM phosphate buffer solution, pH 7.2, containing 500 μM NAD^+ (final concentration) obtained after centrifugation at $1200 \times g$ for 10 min, was analyzed on the chromatograph (sample volume size, 10 μl). An ethanolic extract of the lyophilized samples (1 ml four extractions with 5 ml of 95% EtOH) was made to enrich nicotinamide from the starting mixtures. EtOH was then evaporated under vacuum and the residue redissolved in 0.5 ml H_2O (sample volume size, 40 μl).

3. RESULTS

The addition of 500 μM NAD^+ to platelet suspensions strongly reduced the aggregation induced by agonists like ADP, collagen, adrenaline, thrombin and A23187 (fig.1). This finding induced us to ascertain whether NAD^+ was transformed during the process. In fact, the supernatant of stimulated platelets showed, by isotachophoresis, a reduction of the peak corresponding to NAD^+ when compared to the supernatant of resting platelets (not shown). The disappearance of added

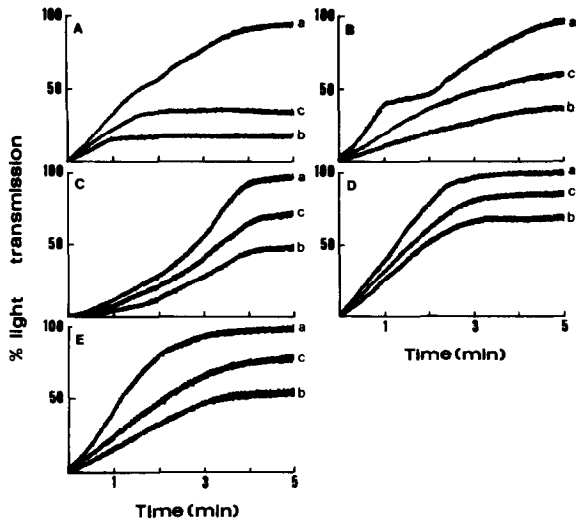

Fig.1. Effect of NAD^+ on the aggregation of platelets. Platelet suspensions (3.3×10^8 cells/ml) in PBS containing 1 mg/ml fibrinogen and 1 mM $CaCl_2$ were stimulated in the absence (a) and presence of 500 μM NAD^+ (b) or of 500 μM $NADP^+$ (c). The stimuli used were: (A) 2 μM ADP; (B) 0.5 μM epinephrine; (c) 2 μg/ml collagen; (D) 20 μM A23187; (E) 0.5 U/ml γ-thrombin.

NAD^+ from the supernatant of stimulated platelets was also found by testing it in the alcohol dehydrogenase reaction. Using [2,8-*adenine*-^{3}H]-NAD^+ it was found that some radioactivity was incorporated into platelets (fig.2). This radioactivity was acid labile, i.e., non-covalently bound to platelets. In fact trichloroacetic acid-treated and washed platelet pellet did not have significant

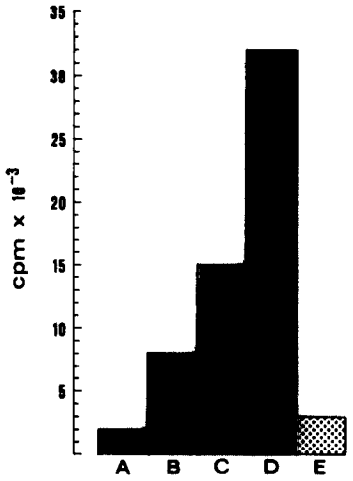

Fig.2. Uptake of [2,8-*adenine*-^{3}H]NAD^+ by stimulated platelets. The platelet suspension (3.3×10^8 cells/ml) was stimulated in the presence of 1 μCi of labeled NAD^+ diluted within 1 mM NAD^+. Bars: (A) resting platelets, time zero; (B) resting platelets after 20 min incubation at 37°C; (C) platelets stimulated with 2 μg/ml collagen after 20 min incubation at 37°C; (D) platelets stimulated with 2 μg/ml collagen in the presence of 0.01 U/ml phosphodiesterase; (E) trichloroacetic acid (10%) precipitate of C.

Table 1

Adenosine uptake by stimulated platelets

Incubation time (min)	Thrombin (0.5 U/ml)	Adenosine found in the supernatant (μM)
0	–	500 ± 10
0	+	470 ± 10
20	–	480 ± 20
20	+	450 ± 5

To platelet suspensions (3.3×10^8 cell/ml) 500 μM adenosine was added at zero time. Where indicated thrombin was added. Each determination made at least in triplicate. Values are expressed as mean ± SD. These values were determined from the area of the HPLC peaks

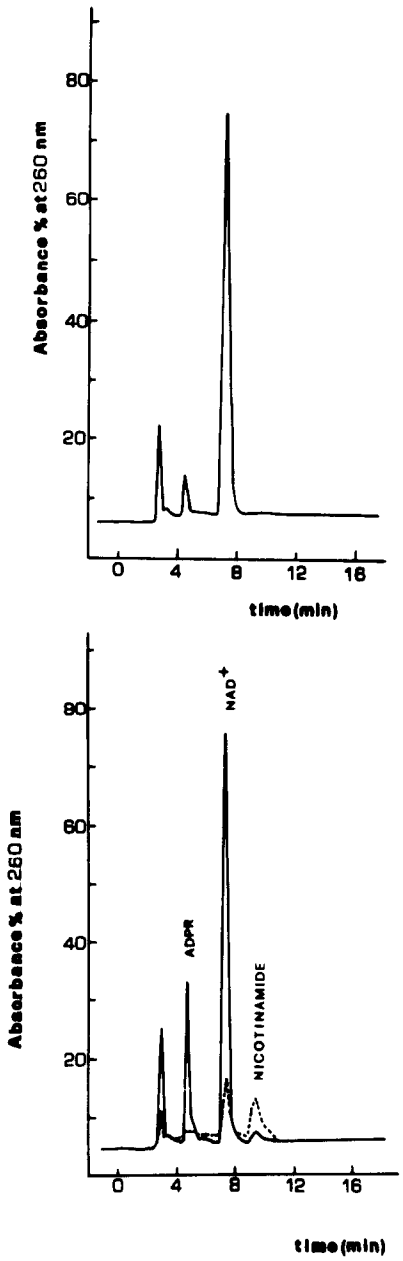

Fig.3. HPLC of NAD^+ in the presence of platelets. Upper panel: elution profile of a supernatant of platelet suspension (6×10^9 cells) in 0.1 M PBS, pH 7.2, containing 500 μM NAD^+, centrifuged at 1200 × *g* at 4°C, immediately after the addition of NAD^+. Absorbance is given in percent of full scale (see section 2). Lower panel: same as above, but after 30 min incubation at 37°C in the presence of 500 μM NAD^+ before centrifugation. Dashed line represents the corresponding elution profile of the ethanolic extract of a freeze-dried sample.

bound radioactivity. Instead, more radioactivity was taken up into platelets by adding snake venom phosphodiesterase to the incubation mixture.

Control experiments showed that stimulated platelets were also able to take up externally added adenosine. Table 1 shows that in 20 min about 10% of the adenosine initially present disappeared from the suspensions of platelets stimulated by ADP, but not from the suspensions of resting platelets.

The fate of NAD^+ incubated with stimulated platelets was checked by HPLC. Fig.3 shows the decrease of the peak corresponding to NAD^+ in the supernatant of stimulated platelets and the formation of new peaks. Two of them have been identified as nicotinamide and ADPR by comparison with authentic compounds. Thus NAD^+ appears to be hydrolyzed in an NADase reaction catalyzed by activated platelets.

The addition of 10 mM nicotinamide to the incubation mixture, containing activated platelets and NAD^+ reduced the extent of hydrolysis by 40–50%, further confirming the presence of an NADase activity [12]. The rate of NAD^+ hydrolysis was about 100 nmol/10^8 platelets per 20 min. This rate was about 60–70% lower than that obtained with the same amount of platelet homogenate. NADH and NADPH were not significantly hydrolyzed.

To check whether the inhibitory effect of NAD^+ on the aggregation was related to NAD^+ itself or rather to its hydrolysis products, the following controls were made. Nicotinamide did not influence the aggregation of platelets, but even counteracted the inhibitory effect of NAD^+ at the same concentrations which inhibited the hydrolysis of NAD^+. ADPR and adenosine, instead, were able to inhibit the aggregation induced by thrombin and by A23187 (fig.4).

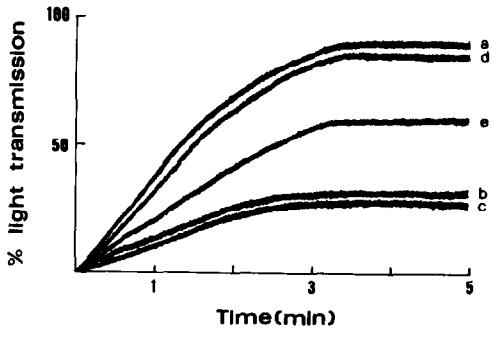

Fig.4. Effect of nicotinamide, ADPR and adenosine on platelet aggregation induced by γ-thrombin. Curves: (a) γ-thrombin alone (0.5 U/ml); (b) + NAD^+ (500 μM); (c) + adenosine (500 μM); (d) + nicotinamide (500 μM); (e) + ADPR (500 μM). Other conditions as in fig.1.

4. DISCUSSION

The above reported data show that stimulated human platelets can hydrolyze NAD^+ and to a lesser extent $NADP^+$, but not their reduced forms. Resting platelets show a much lower, if any, ability of hydrolyzing NAD^+. The control with resting platelets may give erroneous results due to unspecific stimulation by mechanical manipulation (e.g., pipetting, stirring, etc.). The hydrolysis of NAD^+ by platelets was inhibited in the presence of nicotinamide, which was identified as a product of the reaction. These two facts indicate that the hydrolytic enzyme is an NADase. As far as the present evidence goes, it appears that this activity is not related to ADP-ribosylation, since only free ADP-ribose has been detected. Using a radioactive NAD^+ some radioactivity was found inside platelets, but in soluble form. It is still conceivable that in vivo the platelets may indeed induce this ADP-ribosylation of some extracellular structure. Whatever the possible role of NADase toward exogenous substrates, it appears that this activity in the meantime strongly affects platelet aggregation. It is difficult to attribute this effect to a single chemical species since ADPR and adenosine, which may arise from the latter, both show an inhibitory effect when added to stimulated platelets. Adenosine has been already shown to inhibit platelet aggregation both in vitro and in vivo [13]. The present study has demonstrated that adenosine can be taken up by stimulated, but not by resting platelets. The greater radioactivity found inside the platelets incubated with NAD^+ and phosphodiesterase seems to confirm a role of adenosine. Furthermore it is also possible that the anti-aggregating effect is due to NAD^+. It should however be pointed out that the simultaneous addition of NAD^+ and nicotinamide, which strongly inhibits the NADase activity, reduces the effect of NAD^+ on platelet aggregation, then suggesting that the hydrolysis of NAD^+ is important in the

anti-aggregating effect. The presence of NADase activity on the cell surface has been reported for various cell types [3,14–18]. Though the physiological meaning of this enzyme is obscure, there are reports associating this activity with the cell function. The NADase activity of platelets is expressed only (or mainly) in stimulated platelets. This is clearly at variance with other cells, where the activity is always present or even lower after stimulation, like macrophages [14].

In conclusion it appears that platelets are sensitive to the presence of extracellular NAD^+. The nicotinamide formed is then methylated. It has been suggested that nicotinamide methylation and *N*-methylnicotinamide have growth regulation properties as their level is inversely correlated to cell proliferation [19]. This finding further stresses the regulatory role of this coenzyme in addition to the well known redox ability.

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