Guanylate cyclase in human platelets with different aggregability

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Summary. The activity of human platelet guanylate cyclase, and the activation of the enzyme by sodium nitroprusside were decreased in platelets with increased aggregability; these platelets were obtained from diabetes mellitus patients. Anomalies in guanylate cyclase activity and ADP-induced aggregation were more pronounced in platelets from subjects with type II than those with type I diabetes.

Key words. Guanylate cyclase; human platelets; platelet aggregation; diabetes mellitus.

Abnormal platelet aggregation accompanies a variety of human diseases; myocardial infarction, cerebral ischemia, atherosclerosis, diabetes, etc. A rise in platelet aggregability is the essential causative factor of diabetic angiopathies, which are the leading cause of invalidity and death in patients with diabetes mellitus[1-5]. Up to now there is no clarity with respect to the pathogenesis and pathobiochemistry of diabetic angiopathies. In this connection the biochemical regulation of platelet aggregation is of great experimental interest.

According to current ideas[6-11], platelet aggregation and disaggregation are under the control of the cyclic nucleotide (cAMP and cGMP) system. While the cAMP system mediates only the extracellular command to diminish the rate of aggregation (e.g. in response to prostacyclin), the cGMP system is assumed to act as an intracellular negative feedback mechanism, where overaggregation causes the stimulation of guanylate cyclase and the accumulation of cGMP, which initiates the disaggregation step. This concept is based on two main bodies of experimental data: 1) agents that elevate cGMP level in platelets are inhibitors of platelet aggregation[7-9]; 2) aggregation by itself leads to an increase in the cGMP content in platelets[10-11]. Nevertheless, there is no information about guanylate cyclase in platelets with increased or decreased aggregability.

Guanylate cyclase (EC 4.6.1.2.) catalyses formation of cyclic GMP from GTP in the presence of Mg^{2+} or Mn^{2+}. There are two forms of guanylate cyclase; soluble and particulate. In mammalian platelets, more than 95% of guanylate cyclase activity is soluble. Guanylate cyclase can be activated in vitro by various oxidizing agents, especially by nitric oxide (NO) and nitrogen-containing compounds capable of releasing or forming NO; sodium nitroprusside (SNP), NaN_3 or glyceryl trinitrate[12-16]. Moncado et al.[6] have demonstrated that NO is a biogenic agent capable of stimulating guanylate cyclase in vivo.

Here we used platelets with different states of aggregability; from healthy donors and from patients with diabetes mellitus type I or II, and compared their guanylate cyclase activities (basal and SNP-stimulated) with ADP-induced aggregation. Our results indicate that increased platelet aggregation may be due to disorders in guanylate cyclase function.

Materials and methods

The study was performed with diabetic patients of both sexes with normal body weight (mean: 69 kg) who had been diabetic for periods ranging from 5 to 20 years. They were in a state of compensation, with no apparent vascular complications. Their ages were in the range of 20 to 41 years (mean: 32 years) for type I, and 24 to 45 years (mean: 34 years) for type II diabetes. Healthy donors were also of both sexes, with normal body weight (mean: 71 kg) and aged 22 to 46 years (mean: 35 years). Fasting blood samples were collected in plastic bottles containing 1/10 volume of 3.8% sodium citrate and centrifuged at 450 g for 10 min at room temperature. Platelet rich plasma (PRP), usually with not less than $4.5 \cdot 10^8$ platelets/ml, was recovered and layered on Ficoll-Paque (Pharmacia, Sweden)[13]. Platelets were collected by sedimentation of PRP at 650 g for 30 min and washed twice, by resuspension in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 5 mM EDTA and centrifugation at 800 g for 10 min. The final platelet pellet was suspended in 1 ml of cold (4 °C) 50 mM Tris HCl (pH 7.6) containing 0.2 mM dithiothreitol. The cells were sonicated at 0 °C for 20 s on a sonicator MSE 5–78 (U.K.) and centrifuged at 105,000 × g for 60 min.

Guanylate cyclase activity in the supernatant was assayed by the method of Murad[15]. The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 1 mM GTP, 4 mM $MgCl_2$ or 4 mM $MnCl_2$, 4 mM creatine phosphate, 5 U of creatine phosphokinase, 10 mM theophylline, and enzyme preparation (5–10 µg of protein) in a total volume of 150 µl. Following a 10-min incubation at 37 °C the reaction was stopped by heating at 90 °C for 2 min. Suitable aliquots were removed and diluted (usually, 10–50 fold) to give concentrations of 1–2 pmol cGMP/100 µl. Samples were assayed for cGMP using a 'cGMP RIA kit' (Amersham, U.K.). Dilution prevented any nonspecific interference with the radioimmunoassay by components of the reaction mixture (data not shown).

Platelet aggregation in PRP was induced by ADP and studied according to the turbidimetric method of

Born[17]. The appropriate platelet count in PRP was adjusted with platelet-poor plasma (PPP). PPP was obtained by centrifugation of PRP at 650 g for 30 min. Maximum of light transmission (100% platelet aggregation) on photometer (FEC-56 M, USSR) and recorder (KSP-4, USSR) was measured with PPP, then PPP was replaced by PRP.

Statistical processing of the data was performed according to Student's t-test; figures show mean values ± SE.

Results and discussion

Many authors have reported hyper-aggregability of platelets in diabetic disease[1-5], nevertheless none of them have compared the rates of aggregation in platelets from type I and type II diabetic patients. We had to start our experiments with both types, and we noticed that platelet guanylate cyclase activities from patients with different types of diabetes were different. Basal activity, measured in the presence or 4 mM Mg^{2+}, was decreased by 30 and 50% below normal in patients with type I and type II diabetes, respectively (table). It is known that guanylate cyclase activity measured in the presence of Mn^{2+} is higher than the basal activity, owing to the oxidative effect of manganese as a transition metal[12-16]. We confirmed this fact and detected a decrease in the Mn-dependent activity, too, although it was not so pronounced as the decrease in the basal activity.

Soluble guanylate cyclase is an enzyme whose activity is controlled by redox mechanisms within the cell[12,15]. The ratio, Mn-activity to Mg-activity, reflects the redox response of guanylate cyclase[13]. This parameter was increased in platelets from diabetic subjects (table). Responsiveness of guanylate cyclase to SNP is an important criterion of the functional state of this enzyme[8,14]. In our experiments with donor platelets, 0.1 mM SNP raised the guanylate cyclase activity by a factor of approximately 12. In platelets from diabetic patients we observed a great fall in SNP-stimulation. This decrement was 2-fold in type I and 3-fold in type II diabetes (table).

Finally, activity of guanylate cyclase and its response to NO-containing activator were both diminished in platelets from diabetic patients, i.e. in platelets known to show an increased aggregability.

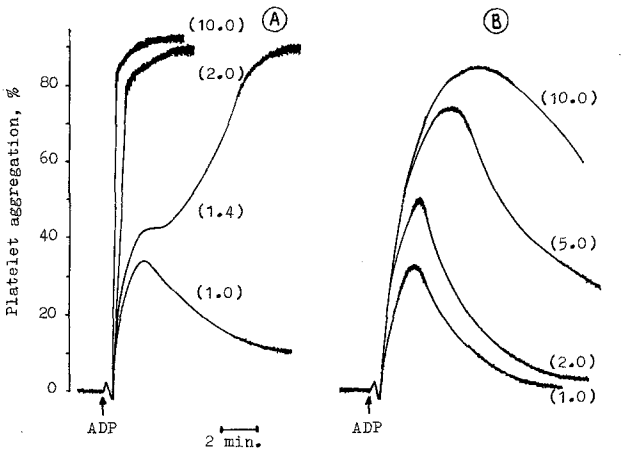

Figure 1. ADP-induced aggregation in normal PRP containing $4.5 \cdot 10^8$ (*A*) or $2.5 \cdot 10^8$ (*B*) platelets/ml.

Simultaneously, with enzymatic experiments, we checked ADP-induced aggregation in PRP. The reversible phase of ADP-induced aggregation was our main interest. The concentration level of platelets in PRP that allows the manifestation of the reversible phase in response to ADP (from 0.5 to 10 µM) was found to be $2.5 \cdot 10^8$/ml (fig. 1). We have shown that platelets of diabetic patients exhibited a more pronounced response to ADP than donor platelets did (fig. 2). These results are in accordance with the data obtained by other authors describing the irreversible ADP-aggregation[1-3,5]. It is worthwhile to note here that we were able to discriminate between the types of platelet aggregability in the two types of diabetes. 50% aggregation in platelets from type I patients was observed after the addition of 1.3 µM ADP, whereas for type II patients the concentration required was 0.9 µM. For normal controls it was 2 µM (fig. 2). In other words, platelets from diabetic patients were 1.62-fold (type I) or 2.33-fold (type II) more sensitive to the aggregating agent than normal platelets.

One might speculate that the less pronounced anomalies in platelets from patients with type I than in those with type II are due to insulin administration. Conflicting

Guanylate cyclase activities in platelets from healthy donors and patients with type I or type II diabetes mellitus

Subjects	Activity of guanylate cyclase, pmol of cGMP formed $min^{-1} \cdot mg\ protein^{-1}$			Ratio of guanylate cyclase activities	
	Mn^{2+}	Mg^{2+} − SNP	+ SNP	Mn^{2+}/Mg^{2+}	+ SNP/ − SNP
Donors (18)	409 ± 34	187 ± 12	2115 ± 174	2.16 ± 0.12	11.56 ± 1.07
Type I diabetes (10)	326 ± 29	128 ± 9*	859 ± 62*	2.55 ± 0.15	6.70 ± 0.38*
Type II diabetes (8)	272 ± 20*	92 ± 4**	336 ± 29**	2.98 ± 0.22*	3.61 ± 0.30**

Guanylate cyclase was preincubated with 0.1 mM SNP at 0 °C for 50 min[14] and the activity measured in the presence of 4 mM Mg^{2+} or 4 mM Mn^{2+}. The number of subjects observed is given in parentheses. *$p < 0.05$ (with reference to donors); **$p < 0.05$ (with reference to type I patients).

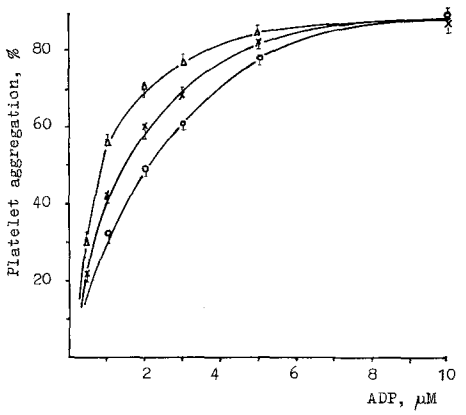

Figure 2. ADP-induced aggregation in PRP from healthy donors (o—o, 7 subjects) and patients with type I (×—×), 7 subjects) and type II (△—△), 5 subjects) diabetes mellitus.
Each concentration-response curve represents the maximum values of reversible aggregation induced by ADP in corresponding concentrations. Platelet count in PRP was $2.5 \cdot 10^8$/ml.

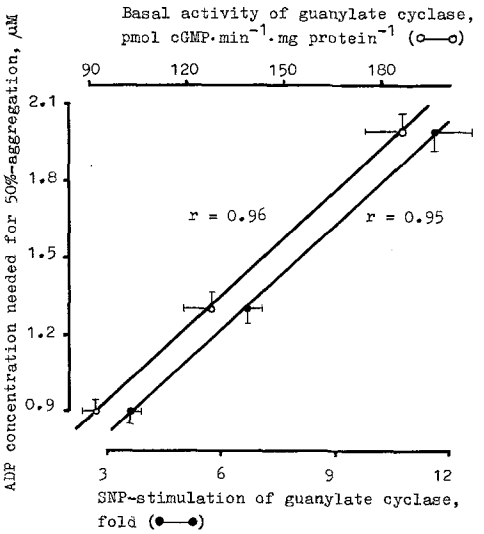

Figure 3. Correlation between platelet aggregability and guanylate cyclase activities.

reports exist in the literature concerning the role of insulin in the correction of abnormalities of platelet function in insulin-dependent diabetics. Some authors consider the manifestation of hyperaggregability to be independent of insulin[4]; others observed a complete normalization 24 h after a single injection of insulin[18], or a slight compensation after a long period of treatment[5]. In seems reasonable to propose that observed dissimilarities between type I and type II reflect different states of abnormality accumulated in the whole organism, not only in circulating platelets, during the genesis of this complicated disease. A study of biochemical regulatory systems will be needed to clarify the nature of hyperaggregability and the ways in which it might be normalized.

In our experiments, comparison of the platelet aggregability with the functional characters of platelet guanylate cyclase in three observed groups (healthy donors, patients with type I, and patients with type II diabetes) revealed a regularity; the increase in aggregability is linearly related to the decrease in guanylate cyclase activity and reactivity (fig. 3). The strong correlation between these parameter supports the concept of the down-regulation of platelet aggregation by the cGMP system[6–11]. A fall in guanylate cyclase activity and sensitivity to stimulator might disturb the regulative capacity of the cGMP system. It is tempting to suggest that a decrease in the platelet guanylate cyclase activities in diabetes causes disorders in intracellular control over the platelet aggregation-deaggregation process, and this results in the formation of platelet aggregates in blood vessels that provokes diabetic angiopathies. The molecular mechanism of guanylate cyclase disfunction in diabetes mellitus remains to be understood.

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