Thrombin-Induced Breakdown of Phosphoinositides in Platelets from Patients with NIDDM

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Abstract. We have examined thrombin-induced metabolism of phosphoinositides in the platelets from fifteen NIDDM (non-insulin-dependent diabetes mellitus) patients and fifteen healthy subjects (control). The diabetic patients were divided into two groups. One group (group I) had diabetic retinopathy (microangiopathy) and the other group (group II) had atherosclerosis of great vessels (macroangiopathy). In platelets incubated with [32P] orthophosphate for 80 min, the incorporation of 32P radioactivity into phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) was significantly lower in the group II than in the control. The addition of thrombin induced a marked decrease in PIP2 radioactivity at 10 sec in platelets from group I compared with that from the control. These results suggest that the breakdown of polyphosphoinositides is increased in platelets from diabetic subjects with retinopathy, and also that the formation of polyphosphoinositides is decreased in the platelets from diabetic subjects with macroangiopathy.

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THE ADDITION of agonists such as thrombin to resting human platelets initiates the processes of intracellular Ca++ mobilization, secretion, fibrinogen receptor exposure, and aggregation that are the features of normal platelet function. Several laboratories have suggested that phospholipid-derived messenger molecules are important participants in these events. Thrombin causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C with a concomitant formation of diacylglycerol and inositol 1,4,5-trisphosphate (IP3) [1-5] and activates A2-type phospholipase to release arachidonic acid. IP3 induces the rapid discharge of Ca++ from the dense tubular system [5]. Diacylglycerol is known to activate protein kinase C [6, 7]. Arachidonic acid is oxygenated to produce thromboxane A2. On the other hand, diacylglycerol is effectively phosphorylated to phosphatidic acid (PA) [8], which can then be redirected to phosphatidylinositol (PI) through CDP-diacylglycerol. PI is phosphorylated to phosphatidylinositol 4-monophosphate (PIP) and PIP2 by PI kinase and PIP kinase, respectively [9].

Platelet responsiveness to aggregating agents has been shown to be increased in both man and animals with diabetes mellitus [10, 11]. On the other hand, the alteration of platelet aggregation and arachidonic acid metabolism, as well as endothelial functions such as von Willebrand factor activity, prostacyclin release and fibrinolytic activity, and elevated low-density lipoprotein (LDL) cholesterol and low high-density lipoprotein (HDL) cholesterol levels in uncontrolled diabetes mellitus [11], participate in the pathogenesis of accelerated atherosclerosis complicated with diabetes mellitus as macroangiopathy. Platelet
cholesterol and phospholipids were highest in patients with macroangiopathy [12].

Kalofoutis & Lekakis [13] reported that the arachidonate content of platelet phospholipids was increased in diabetes. In addition, enhanced thromboxane production in response to thrombin has also been observed in platelets from diabetic patients [14, 15]. Recently attention has focussed on the breakdown of polyphosphoinositides as being the earliest event underlying the reactivity of platelets. A change in platelet shape induced by thrombin is closely linked to phosphoinositide hydrolysis and may be independent of the liberation and metabolism of arachidonic acid [16]. Bastyr et al. [17] reported that decreased phosphoinositide turnover may play a role in abnormal platelet function in IDDM. The alteration of phosphoinositide metabolism in sciatic nerve from fatty diabetic rats which have been identified with potential genetic models for non-insulin-dependent diabetes mellitus (NIDDM) was also reported [18]. For many years, investigators interested in the vascular complications of diabetes have focussed their attention on microvascular disease. Such classic microvascular complications as retinopathy and nephropathy are major problems in diabetes mellitus. However, recently there has been an upsurge in interest in diabetes as a primary risk factor for macrovascular disease. No investigator has indicated platelet phosphoinositide hydrolysis in diabetes complicated with microangiopathy or with macroangiopathy.

We therefore investigated whether abnormalities of phosphoinositide turnover and hydrolysis are different in NIDDM with microangiopathy from that with macroangiopathy. However, the mechanism of thrombin-induced activation of platelets might be complicated. Thus, this study was undertaken to examine thrombin-induced hydrolysis of phosphoinositides by phospholipase C in platelets from patients with NIDDM complicated with microangiopathy and with macroangiopathy.

Materials and Methods

Subjects

The patients with NIDDM included in the study were eight men and seven women at age ranging between 29 and 72. The control subjects had no history of diabetes or any other disease that would affect platelet aggregation. Ten patients (group I) had diabetic retinopathy (3 with background retinopathy and 7 with proliferative retinopathy; 8 also had persistent proteinuria). Five patients (group II) had diabetic macroangiopathy such as calcification of the aorta and great vessels and atherosclerosis obliterans diagnosed by computerized tomography or angiography, but had no retinopathy. Neuropathy was diagnosed by motor nerve conduction velocity. Nephropathy was estimated by the presence or absence of proteinuria. There were no statistical differences between diabetic patients with retinopathy and macroangiopathy in the levels of cholesterol, triglyceride, fasting plasma glucose or glycosylated hemoglobin. No subject had taken aspirin or any other drugs which might alter prostaglandin metabolism during the previous 2 weeks. All the patients were treated with diet therapy and were not obese (less than 10% above ideal body weight). Seven patients in group I and three patients in group II were treated with sulfonylureas. Clinical details of the control and the diabetic subjects are shown in Table 1.

Preparation of [32P] labelled human platelets

Fresh blood was obtained from the control subjects and diabetic patients and then centrifuged at 164 × g for 10 min as described previously [19]. The resultant supernatant (platelet-rich plasma; PRP) was suspended in buffer I (15.4 mM Tris, 140 mM NaCl and 5.6 mM glucose, pH 7.4) and incubated with [32P] orthophosphate (200 μCi) for 80 min at 37°C. The labelled platelets were washed and resuspended in buffer I containing 1 mM CaCl2 to a final concentration of 10⁹/ml.

Lipid analysis

After the platelet suspension (490 μl) was incubated with thrombin (1 unit/ml), 2 ml of chloroform/methanol/HCl (20:40:1, v/v) [20] was added and lipid was then extracted by a modification of the Bligh-Dyer method [21]. Briefly, to the resultant mixture were added and mixed 0.5 ml chloroform and 0.5 ml 0.2 M KCl-5 mM EDTA. The phases were separated by centrifugation, and the lower phase was collected and dried under nitrogen. The phosphoinositides were separated on high performance thin layer chromatography (HPTLC) plates, impregnated with potassium oxalate (1%), in a solvent system of chloroform/
Table 1. Clinical and biochemical characteristics of subjects examined

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Sex M</th>
<th>F</th>
<th>Age (years)</th>
<th>Duration of diabetes (years)</th>
<th>Smoking (%)</th>
<th>Neuropathy (%)</th>
<th>Nephropathy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinopathy (group I)</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>50±13</td>
<td>6.3±5.2</td>
<td>30</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>Macroangiopathy without retinopathy (group II)</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>52±18</td>
<td>7.2±6.0</td>
<td>40</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>8</td>
<td>7</td>
<td>45±20</td>
<td>/</td>
<td>33</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HbA1C (%)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Glucose (mg/dl)</th>
<th>Medications with sulfonylurea (%)</th>
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<tbody>
<tr>
<td>Retinopathy (group I)</td>
<td>8.5±1.9</td>
<td>180±45</td>
<td>99±48</td>
<td>180±90*</td>
<td>70</td>
</tr>
<tr>
<td>Macroangiopathy without retinopathy (group II)</td>
<td>8.0±2.0*</td>
<td>160±22</td>
<td>110±91</td>
<td>140±20</td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>5.0±0.6</td>
<td>190±40</td>
<td>105±55</td>
<td>86±5</td>
<td>/</td>
</tr>
</tbody>
</table>

The values are the mean ± SD. * P<0.001

Fig. 1. Incorporation of $^{32}$P radioactivity into PI, PIP, PIP$_2$ and PA in platelets from diabetic patients with retinopathy and macroangiopathy and control subjects.
Platelets were incubated with $^{32}$P orthophosphate for 80 min at 37°C. Data represent mean ± SEM. Asterisks show statistically significant difference (* p<0.05, ** p<0.01). Abbreviations used; PI: phosphatidylinositol, PIP: phosphatidylinositol 4-monophosphate, PIP$_2$: Phosphatidylinositol 4,5-bisphosphate, PA: phosphatidic acid, N: control subjects, R: diabetic patients with retinopathy, M: diabetic patients with macroangiopathy.
Fig. 2. Time-dependent changes in $^{32}$P radioactivity in PI, PIP, PIP$_2$ and PA of platelets from diabetic patients with retinopathy (●) and macroangiopathy (●) and control subjects (○).

Data represent mean ± SEM. Asterisks show statistically significant difference from the control values (* p<0.05, ** p<0.01). Abbreviations used; See Fig. 1.

Fig. 3. The rate of transient decrease in PIP$_2$ radioactivity in thrombin-stimulated platelets from diabetic patients with retinopathy and macroangiopathy and control subjects.

Data represent mean ± SEM. Asterisks show statistically significant difference from the control values (* p<0.05). Abbreviations and symbols used; See Fig. 1 and 2, respectively.

Materials

HPTLC plates were purchased from E. Merck. $[^{32}P]$ Orthophosphate was obtained from New England Nuclear. PIP and PIP$_2$ as the standards were from Sigma. Thrombin (bovine) was supplied by Mochida Pharmaceutical Co. X-O matic Medical X-ray films used for autoradiographs were obtained from Eastman Kodak Co. All other chemicals were of reagent grade.

Results

When platelets were incubated with $[^{32}P]$ Orthophosphate for 80 min at 37°C, $^{32}$P radioactivity was incorporated into the phospholipid fraction. The $^{32}$P radioactivity incorporated into PIP and PIP$_2$ was significantly lower in the platelets from the group II than in those from the control (PI: 48.2±23.8 versus 34.5±4.8 (%), p<0.05, PIP: 17.5±8.2 versus 31.0±7.0 (%), p<0.01, PIP$_2$: 15.2±5.5 versus 23.6±4.3 (%), p<0.01) (Fig. 1). On the other hand, there were no
significant differences between the platelets from group I and the control in $^{32}$P radioactivity in PI, PIP, PIP$_2$ and PA.

As shown in Fig. 2, after stimulation with thrombin, the level of $^{32}$P radioactivity in PIP was lower in the platelets from group II than in those from the control (at 10, 20, 30, 60, and 90 sec, p<0.05-0.01, respectively). The $^{32}$P radioactivity in PIP$_2$ was also lower in the platelets from the group II than in those from the control (at 10, 20, 30, 60 and 90 sec, p<0.05-0.01, respectively). In the platelets from group I, a thrombin-induced decrease in PIP$_2$ radioactivity was observed at 10 sec (13.6±6.1 versus 19.9±4.2 (%), at 10 sec, p<0.01) (Fig. 2). The extent of thrombin-induced increase in PA radioactivity appear to be similar in the platelets from group I, group II and the control. We found no significant difference between platelets from group I and the control in thrombin-induced change of PI, PIP or PA radioactivity except for the lower PIP radioactivity at 90 sec in the platelets from group I (p<0.01) (Fig. 2). Thrombin-induced PIP$_2$ radioactivity at 60 and 90 sec in the platelets from group II was lower than in the platelets from group I (at 20, 60 and 90 sec, p<0.05-0.01, respectively).

The basal level of PIP$_2$ was different in each group. To exclude the effect of different basal values on the thrombin-induced changes, the percent change in the basal values in PIP$_2$ was replotted in Fig. 3. A marked thrombin-induced decrease in radioactivity was observed in the platelets from group I compared to the control, with the maximum decrease being approximately 35% from the unstimulated level (Fig. 3).

**Discussion**

Several studies have been made to explore the mechanism(s) responsible for the enhanced platelet responsiveness to aggregating and release-inducing stimuli in both man and animals with diabetes [10, 11]. Most of these studies have been directed toward examining whether arachidonate metabolism of platelets is altered in diabetes. Recently, decrease platelet phosphoinositide turnover in patients with IDDM [17] and the alteration of phosphoinositide metabolism in sciatic nerve from fatty diabetic rats [18] have been reported. In this study, we have examined $^{32}$P incorporation into phosphoinositides [19] and thrombin-induced hydrolysis of phosphoinositides in platelets from patients with NIDDM.

As shown in Fig. 1, an increase in PI radioactivity and decrease in both PIP and PIP$_2$ radioactivity in the platelets from group II were observed in comparison with those from the control when the platelets were incubated with $^{32}$P-orthophosphate, suggesting a decrease in the activity of PI kinase and PIP kinase. Howthorn et al. [23] reported that PIP kinase was less active in the sciatic nerve of diabetic animals, and suspected that diabetic neuropathy was associated with a decrease in PIP kinase activity. Our data showed that the subjects in group II were highly complicated with neuropathy (80%) as indicated in Table 1. Therefore, phosphoinositide analysis of the platelets in group II revealed an increase in PI radioactivity and decreases in PIP and PIP$_2$ radioactivity. Another possible reason for the increase in PI in the platelets from patients with NIDDM complicated with macroangiopathy is the acceleration of de novo phospholipid synthesis [24] in atherosclerosis.

As shown in Fig. 2 and Fig. 3, the thrombin-induced decrease in PIP$_2$ radioactivity at 10 sec was greater in the diabetic platelets with retinopathy (Group I) than in the control platelets. The thrombin-induced PIP$_2$ breakdown is known to be transient and PIP is rapidly phosphorylated to PIP$_2$ by PIP kinase. These results indicate that the hydrolysis of PIP$_2$ by phospholipase C may be enhanced in diabetic platelets with retinopathy. However, the results are different from those of a previous study [17] which indicated decreased platelet phosphoinositide turnover in IDDM. These IDDM patients were probably complicated with retinopathy, neuropathy, or macroangiopathy. Moreover, these patients with IDDM had been treated with insulin for 15 years. Exogenous insulin possibly provokes dynamic changes in second messenger molecules such as tyrosine kinase, diacylglycerol and protein kinase C [25-27]. Therefore, phosphoinositide turnover in platelets from NIDDM and IDDM has been modified by endogenous and exogenous insulin-induced activation of messenger molecules. Our data showed that PIP$_2$ hydrolysis was markedly increased in the platelets from diabetics with retinopathy but did not increase at all in the platelets from diabetics with macroangiopathy.
compared with the control. These results lead us to suspect that atherosclerosis in diabetes mellitus might not be due to an increase in phospholipase C activity in platelets, but to the other factors such as an increase in phospholipase A2 activity which resulted in an increase in thromboxane A2 production, a decrease in prostacyclin release, increased LDL cholesterol and the other factors.

In summary, the alteration of phosphoinositide metabolism in platelets might be different in patients with NIDDM complicated with microangiopathy and with macroangiopathy.

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References


