Increased Binding of Fibrinogen to Platelets in Diabetes: The Role of Prostaglandins and Thromboxane

By Giovanni DiMinno, Melvin J. Silver, Anna M. Cerbone, Gabriele Riccardi, Angela Rivellese, Mario Mancini, and Perumal Thiagarajan

Platelets from diabetic patients are known to be more sensitive to several aggregating agents and to produce more prostaglandins and thromboxane than platelets from normal subjects. We compared fibrinogen binding to platelets from diabetic subjects with binding to platelets from normal subjects and determined whether aspirin (which inhibits the formation of prostaglandins and thromboxane) would inhibit the binding of fibrinogen to platelets from diabetic subjects and whether this correlated with its effects on platelet aggregation. We found the following: Aspirin suppressed thromboxane formation and rendered the platelets less sensitive to the induction of aggregation by adenosine diphosphate (ADP) or collagen. The amount of U-46619 ([15s]-hydroxy-11-alpha, 9-alpha [epoxy-methano]-prosta[5Z,13E]-dienoic acid, a stable analog of prostaglandin endoperoxide/thromboxane A2) necessary to induce aggregation, was similar in normal and diabetic subjects and was unchanged after ingestion of aspirin. Binding of ¹²⁵I-fibrinogen following stimulation of platelets by ADP or collagen was greater in diabetic (because more binding sites were exposed) than in normal subjects. However, following stimulation by U-46619, binding was similar in diabetic and normal subjects. Aspirin caused a reduction in the exposure of binding sites on both platelets from diabetic and normal subjects, so that (in this respect) platelets from diabetic subjects became more like those from normal subjects. Effects of the monoclonal antibody B59.2, which is specific for the platelet glycoprotein Ib-IIIa complex (the presumed receptor for fibrinogen on the platelet surface) were also studied. The amount of this antibody that bound to platelets was the same for normal and diabetic subjects both before and after aspirin and with or without stimulation by ADP or collagen. In addition, B59.2 inhibited aggregation and fibrinogen binding in both platelets from diabetic and normal subjects. The combined data suggest that the glycoprotein Ib-IIIa complex of platelets from diabetic subjects is similar to that of platelets from normal subjects and that the increased fibrinogen binding and aggregation of platelets from diabetic subjects in response to ADP or collagen is mediated by increased formation of prostaglandin endoperoxide or thromboxane A2, or both.

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Materials and Methods

Subjects

Fifteen type I diabetic patients (eight men, seven women, 16 to 20 years old) and ten control subjects, matched for sex and weight, were studied. Diagnosis of type I diabetes was based, in ten of 15 patients, on the occurrence of at least two episodes of ketoacidosis; in the other five it was based on an abnormal C-peptide response to arginine (less than 0.06 pmol/mL C-peptide following intravenous administration of 0.5 g/kg body wt of arginine over a 30-minute period; sensitivity of the method was 0.06 pmol/mL). None of the controls had a history of other diseases known to alter platelet aggregation. Neither patients nor controls had taken any medication (other than insulin by the patients) for at least ten days before donating blood. There was no difference between diabetic (156.9 ± 10.6 mg/dL, SEM) and control (168.3 ± 12.5 mg/dL, SEM) subjects in serum cholesterol levels or triglyceride levels (79.5 ± 6.5 mg/dL for patients and 79.2 ± 11.5 mg/dL for normals.) The body mass index for controls was 25.7, and for patients, 26.2. The fasting plasma glucose concentrations of the patients ranged between 144 mg/dL and 350 mg/dL (mean, 215 ± 36); glycosylated hemoglobin, determined by the method of Rahbar, ranged between 6.1% and 10.1% (mean, 8.5% ± 0.8% SEM).

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INCREASED FIBRINOGEN BINDING TO PLATELETS

8.4 ± 1.2% with normal values in our laboratory being 7.1 ± 0.6%. The average duration of diabetes was 3.5 years (range, three to ten years). All diabetic subjects were on twice-daily injections of regular plus intermediate insulin (35 to 88 U/d; mean, 40.4 ± 10.3). None of the patients had retinopathy (ie, none exhibited hemorrhage, microaneurysms, exudates, or new vessels) as determined by fluorangiography; and none showed advanced vascular diseases as judged by normal electrocardiograms, no history of angina pectoris or myocardial infarction, no claudication, and normal peripheral pulses. Neuropathy and nephropathy could be excluded since in all patients beat-to-beat variation and microalbuminuria were found within normal ranges. Blood was collected from the diabetic and the control subjects on two occasions, before and 12 to 15 hours after ingestion of 1 g of aspirin (Bayer Italia, Milan). After overnight fasting and before the morning dose of insulin, nine volumes of blood (90 ml) were collected into a plastic tube containing 1 volume (10 ml) 3.7% trisodium citrate from an antecubital vein via a 19-gauge scalp vein needle.

Informed consent was obtained from all patients and volunteers after approval of the local Human Investigation Committee, and the studies were carried out according to the principles of the Declaration of Helsinki.

Materials

Silicone oils (methyl silicone 1.0 DC 200 and Hi phenyl silicone 125 DC 550) were purchased from W.F. Nye Inc, Specialty Lubricants, New Bedford, Mass. Mixtures of DC 200 and DC 550 were prepared as described previously. Disodium ethylenediamine-tetra-acetate (EDTA) was obtained from Fisher Scientific Co, King of Prussia, Pa. Bovine serum albumin (Pentex, fraction V) was purchased from Miles Laboratories Inc, Elkhart, Ind. ADP (adenosine-5'-diphosphate, sodium salt), was obtained from Sigma Chemical Co, St Louis. Collagen (Kollagen Reagents Horm) was purchased from Hormon Chemie (Munich). U-46619, [15 5]-hydroxy-11α, 9α(epoxymethano)-prosta(5Z,13E)dienoic acid] was kindly provided by Dr J. Pike (The Upjohn Company, Kalamazoo, Mich). Solutions of U-46619 were prepared as described elsewhere. Dilutions of ADP or collagen were in TRIS buffer 0.154 mol/L, pH 7.4. (H)-thromboxane B2 was from New England Nuclear, Boston.

Methods

Suspensions of washed platelets in Tyrode's buffer (pH 7.4) containing 1 mmol/L CaCl2, 5 mmol/L glucose, and 3.5 mg/mL bovine serum albumin were prepared as previously described. Platelet aggregation tests were performed (between one and three hours after completion of the washing procedure) in plastic cuvettes in an ELVI-840 aggregometer (ELVI-LOGOS, Milan, Italy). The apparatus was adjusted so that the platelet suspension and the buffer produced 10% and 90% light transmittance, respectively. AC50 was defined as the lowest concentration of an aggregating agent added to a platelet suspension that caused 50% light transmittance within three minutes.

Platelet secretion of ATP was determined in a Lumi-Aggregometer (Mascia-Brunelli, Milan, Italy) as previously described. In some cases, ATP secretion was determined without stirring. ATP as well as TXB2 levels were determined three minutes after the aggregating agent was added. TXB2 levels (in aliquots of the supernatant solution taken from tests for platelet aggregation) were measured by radioimmunoassay. The sensitivity of the assay was such that as little as 0.5 pmol/mL TXB2 could be detected. The TXB2 levels reported were corrected for 3 x 108 platelets per milliliter.

For time-course studies, 0.5-mL aliquots of unstirred platelet suspensions (5 x 109/mL) were incubated at 22 °C (room temperature) or at 37 °C with ADP (10 μmol/L), collagen (1 μg/mL), or U-46619 (1 μmol/L). After a three-minute incubation, [125I]-fibrinogen (250 nmol/L, final concentration) was added and the amount of fibrinogen bound to platelets was measured at time intervals between one and 180 minutes. At the end of each interval, 0.4 mL of the platelet suspension was layered onto 50 μL of silicone oil in a 0.5-mL micro-Eppendorf tube and free and platelet-bound fibrinogen were separated by centrifugation for two minutes at 12,000 g at room temperature, and counted separately in a DP 5500 γ counter (Beckman Instruments, Milan, Italy). For all the agents tested and at each time point, there was no difference in the binding observed when the tests were run at 37 °C or 22 °C. All further experiments, therefore, were conducted at 22 °C. The effect of increasing concentrations of ADP, collagen, or U-46619 on the extent of fibrinogen binding to platelets was assessed by incubating unstirred platelet suspensions with ADP (0.16 to 100.00 μmol/L), collagen (0.12 to 50.00 μg/mL) or U-46619 (0.06 to 12.00 μmol/L). After three minutes, [125I]-labeled fibrinogen (200 nmol/L final concentration) was added. The extent of binding was determined 30 minutes later. All values for fibrinogen binding are given as specific binding, ie, total binding minus nonspecific binding. Nonspecific binding was measured in the presence of a 20-fold excess of unlabeled fibrinogen and was always less than 13% of total binding. The affinity of fibrinogen for normal and diabetic platelets both before and after ingestion of aspirin was studied by incubating aliquots of platelet suspensions with 10 μmol/L ADP, 1 μg/mL collagen, or 1 μmol/L U-46619. Three minutes later, increasing amounts of [125I]-fibrinogen (from 3 to 1,200 nmol/L) were added and, after 30 minutes, free and platelet-bound fibrinogen was separated in silicone oils and counted. Statistical analysis was performed using Student's t test for paired or unpaired comparison. Analysis of the binding data was performed as recommended by Klotz.

RESULTS

Aggregation of Platelets From Diabetic Subjects in Response to ADP. Collagen, or U-46619

In the absence of added fibrinogen, washed platelet suspensions did not aggregate in response to ADP (100 μmol/L or to collagen (0.2 to 1.0 μg/mL). When fibrinogen (200 nmol/L) was added, aggregation occurred and the minimal concentrations of ADP or collagen required to cause 50% light transmittance was significantly lower (P < .01) in platelet suspensions from diabetic subjects than in those from normal subjects (Table 1). In contrast, the AC50 for U-46619 was similar in platelet suspensions from normal and diabetic subjects. Ingestion of aspirin did not affect the sensitivity of platelets to U-46619, while in both normal subjects and diabetics the AC50 for ADP as well as collagen became elevated. After ingestion of aspirin, the minimal concentrations of each agent required to cause 50% light transmittance in platelet suspensions from normal and diabetic subjects were not significantly different from each other (P > .1, Table 1).

Secretion of ATP by Platelets From Diabetic Subjects in Response to ADP. Collagen, or U-46619

Secretion of ATP in response to ADP (10 μmol/L), collagen (1 μg/mL), or U-46619 (1 μmol/L) was
Table 1. AC50 of ADP, Collagen, and U-46619 for Aggregation of Platelet Suspensions From Normal and Diabetic Subjects in the Presence of 200 nmol/L Fibrinogen (Mean ± SEM)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Normal Subjects (N = 10)</th>
<th>Diabetic Subjects (N = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Aspirin</td>
<td>After Aspirin</td>
</tr>
<tr>
<td>ADP (μmol/L)</td>
<td>1.87 ± 0.30</td>
<td>4.73 ± 0.81†</td>
</tr>
<tr>
<td>Collagen (μg/mL)</td>
<td>0.52 ± 0.12</td>
<td>2.69 ± 0.30†</td>
</tr>
<tr>
<td>U-46619 (μmol/L)</td>
<td>0.42 ± 0.13</td>
<td>0.39 ± 0.08</td>
</tr>
</tbody>
</table>

N is the number of subjects tested.
*P < .01, normals v diabetics (both before aspirin). Student’s t test for unpaired comparisons.
†P < .01, before v after aspirin. Student’s t test for paired comparisons.

Table 2. Secretion of ATP in Platelet Suspensions From Normal and Diabetic Subjects in the Presence of 200 nmol/L Fibrinogen

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Normal Subjects (N = 4)</th>
<th>Diabetic Subjects (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Aspirin</td>
<td>After Aspirin</td>
</tr>
<tr>
<td>ADP (10 μmol/L)</td>
<td>1.00 ± 0.09</td>
<td>0.37 ± 0.43†</td>
</tr>
<tr>
<td>Collagen (1 μg/mL)</td>
<td>1.75 ± 0.06</td>
<td>0.67 ± 0.16†</td>
</tr>
<tr>
<td>U-46619 (1 μmol/L)</td>
<td>1.57 ± 0.06</td>
<td>1.77 ± 0.22</td>
</tr>
</tbody>
</table>

N is the number of subjects tested. Values are given as means ± SEM.
*P < .05, normals v diabetics (both before aspirin). Student’s t test for unpaired comparisons.
†P < .01, before v after aspirin. Student’s t test for paired comparisons.

AC50 of ADP, Collagen, and U-46619 for Aggregation of Platelet Suspensions From Normal and Diabetic Subjects in the Presence of 200 nmol/L Fibrinogen.

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N is the number of subjects tested. Values are given as means ± SEM.
*P < .01, normals v diabetics (both before aspirin). Student’s t test for unpaired comparisons.
†P < .01, before v after aspirin. Student’s t test for paired comparisons.

Significantly greater (P < .05) in platelet suspensions from diabetics than in those from normal subjects (Table 2). Ingestion of aspirin did not affect secretion in response to U-46619, while it significantly reduced that in response to ADP or collagen. Under these conditions, platelets from patients secreted as much ATP as those from normal subjects when exposed to ADP or collagen (P > .1, Table 2).

When similar studies were done without stirring, secretion of ATP could not be detected in response to ADP or collagen at one or three minutes, while, as expected, secretion in response to U-46619 was unchanged (data not shown).

**TXB2 Formation in Platelet Suspensions From Normal and Diabetic Subjects**

At the concentrations of collagen and ADP tested, there was significantly (P < .05) greater TXB2 formation in platelet suspensions from diabetics than in those from normal subjects (Fig 1). After ingestion of aspirin (data not shown), TXB2 formation (< 0.5 pmol/mL) could not be detected in response to U-46619 (0.1 to 1.2 μmol/L) or in response to collagen or ADP in platelets from either normal or diabetic subjects.

**Binding of 125I-Fibrinogen to Platelets From Diabetic Subjects**

Figure 2 shows the binding of fibrinogen to platelets from normal or diabetic subjects in response to increasing concentrations of ADP or collagen. Binding in response to U-46619 reached a plateau, both in platelets from normal and diabetic subjects, at 1 μmol/L (data not shown). For all the agents tested and for platelet suspensions from both normal and diabetic subjects, the binding reaction was complete within 30 minutes (Fig 3) and was inhibited more than 85% by EDTA (10 mmol/L). When the affinity of fibrinogen for platelets was evaluated, it appeared that in diabetes as in normals, saturation of specific binding to platelets was reached at a concentration of fibrinogen.
ADP or collagen and the amount of TXB₂ formed, as well as between TXB₂ and fibrinogen binding to platelets \((r = .74)\). In contrast, no correlation could be demonstrated between glycosylated hemoglobin and TXB₂ formed \((r = .39)\) or between glycosylated hemoglobin and AC₅₀ \((r = .41)\) or fibrinogen binding \((r = .40)\) or between insulin dose and fibrinogen binding \((r = .31)\).

**Binding of Monoclonal Antibody B59.2 to Platelets From Normal and Diabetic Subjects**

Binding of ¹²⁵I-B59.2 to unstimulated platelets was complete in one minute. The amount of binding was similar for platelets from normal or diabetic subjects \((23,416 ± 3,912 \text{ molecules bound per platelet in normal subjects} v 26,312 ± 5,613 \text{ in diabetics} \(P > .1\)). Similar binding was also observed in platelets from normal and diabetic subjects obtained after ingestion of aspirin (data not shown). The number of molecules bound was not increased by pretreating platelet suspensions from normal or diabetic subjects with ADP \((10 \mu\text{mol/L})\), collagen \((1 \mu\text{g/mL})\), or U-46619 \((1 \mu\text{mol/L})\).

**Inhibition of Platelet Aggregation and Fibrinogen Binding by B59.2**

It has previously been shown that antibody B59.2 inhibited aggregation, secretion, and binding of fibrinogen to normal platelets stimulated by ADP or collagen.\(^7\) Similarly, B59.2 inhibited aggregation and fibrinogen binding to platelets from diabetic subjects (Fig 5) or normal subjects (not shown) stimulated by \(1 \mu\text{mol/L} \text{ U-46619} \) in a concentration-dependent fashion. Inhibition of fibrinogen binding to platelets from diabetic subjects stimulated by ADP, collagen, or U-46619 was similar to that seen in suspensions of normal platelets (Fig 6).

**DISCUSSION**

It had been previously shown that platelets from retinopathic diabetic subjects exhibit increased binding of fibrinogen after stimulation by ADP.\(^3\) We have now observed increased binding of fibrinogen to platelets, stimulated with collagen or ADP, from nonretinopathic diabetic patients. Furthermore, we found that in response to several different aggregating agents, the patterns of fibrinogen binding to platelets from diabetic patients were similar to those previously reported for normal platelets.\(^15,17,31,41\) While we found no difference in fibrinogen binding at 22 °C or 37 °C as did Niewiarowski et al.,\(^42\) Marguerie and Plouw\(^43\) did find a difference. The reason for this discrepancy is not presently known. The kinetics of fibrinogen binding in response to increasing concentrations of ADP, colla-
gen, or U-46619 were similar for platelets from normal and diabetic subjects. However, the number of molecules of fibrinogen bound to platelets from diabetic subjects stimulated by ADP or collagen was found to be significantly higher than the number bound to normal platelets (Table 3). Ingestion of aspirin tended to normalize the increased binding in response to ADP or collagen. Thus, we have begun to clarify the mechanisms involved in the increased binding of fibrinogen to platelets from diabetic subjects by studying the effects of aspirin. Regardless of aspirin ingestion, we found that fibrinogen binding, as well as aggregation in response to U-46619, was similar in platelets from both normal and diabetic subjects. These results suggest that aspirin does not cause quantitative or qualitative changes in platelet receptors for fibrinogen. U-46619 does not induce TX formation. Thus, the normal binding in response to U-46619 in platelets from diabetic subjects indicates that these platelets behave like those from normal subjects in the absence of TX formation. U-46619 exposed fibrinogen binding sites by secreting intraplatelet ADP. In spite of the increased secretion of nucleotides that occurs in platelets from diabetic patients exposed to U-46619, these platelets bound as much fibrinogen as normal platelets when challenged by this agent. This apparent discrepancy may be explained by assuming that only small quantities of released ADP are required to maximally expose binding sites for fibrinogen on platelets, and that amounts of ADP in excess of these quantities would be without effect. The data also suggest that the receptor for ADP is similar in platelets from normal and diabetic subjects.

Under conditions normally employed for measuring secretion of nucleotides (ie with stirring and aggregation), platelets from our diabetic patients secreted more ATP than normal platelets (Table 2). However, when studies on the secretion of ATP were done under conditions similar to those employed for fibrinogen binding studies (ie, without stirring), secretion of ATP could not be detected at one or three minutes in response to collagen or ADP, while platelets from diabetic subjects bound significantly more fibrinogen than those from normal subjects. It can be hypothesized that, without stirring, concentrations of nucleotides are present in the microenvironment of platelets that are not high enough to be detected by the luciferin–luciferase system but are sufficiently high to enhance binding and aggregation in response to other agents. The observation that aspirin rendered platelets from diabetic subjects less sensitive to this potential synergistic effect, however, points to a major role for the synthesis of prostaglandin endoperoxides/TXA₂ in the abnormalities observed. Together, the data are consistent with the possibility that in nonretinopathic diabetic subjects, the increased synthesis of cyclic endoperoxides/TXA₂ occurring in response to ADP or collagen plays a dominant role in the increased binding

Table 3. Binding of ¹²⁵I-Fibrinogen to Platelets From Normal Subjects and Diabetic Subjects Before and After Ingestion of Aspirin

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Kd (μmol/L)</th>
<th>Molecules Bound/Platelet</th>
<th>Kd (μmol/L)</th>
<th>Molecules Bound/Platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Diabetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP (10 μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.32 ± 0.04</td>
<td>31.276 ± 5,948</td>
<td>0.25 ± 0.03</td>
<td>49.612 ± 2.319*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.37 ± 0.07</td>
<td>18.316 ± 3,878†</td>
<td>0.30 ± 0.06</td>
<td>25.397 ± 6.314†</td>
</tr>
<tr>
<td>Collagen (1 μg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.26 ± 0.06</td>
<td>42.948 ± 6,998</td>
<td>0.26 ± 0.04</td>
<td>69.361 ± 5.319*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.26 ± 0.05</td>
<td>23.989 ± 6,724†</td>
<td>0.29 ± 0.07</td>
<td>32.648 ± 6.791†</td>
</tr>
<tr>
<td>U-46619 (1 μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.30 ± 0.06</td>
<td>61.378 ± 7,428</td>
<td>0.33 ± 0.05</td>
<td>53.748 ± 7.234</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.30 ± 0.04</td>
<td>59.865 ± 5,229</td>
<td>0.27 ± 0.05</td>
<td>50.439 ± 5.988</td>
</tr>
</tbody>
</table>

N is the number of subjects tested. Values are given as means ± SEM.

*P < .05, normal vs diabetic subjects (for each stimulus employed).
†P < .01, before vs after aspirin (for each stimulus employed).
Increased fibrinogen binding to platelets

Fig 5. Concentration-dependent inhibition, by antibody B59.2, of platelet aggregation and fibrinogen binding of platelet suspensions from diabetic patients stimulated with 1 μmol/L U-46619. Increasing concentrations of B59.2 were incubated with 0.5 mL of platelet suspensions (5 × 10^5/mL) which had been stirred at 37°C for one minute at 1,000 rpm. Unlabeled fibrinogen (200 nmol/L final concentration) was then added, followed by 1 μmol/L U-46619. Aggregation was measured as described in Materials and Methods. In parallel, aliquots of 0.5 mL of unstimulated platelet suspensions (5 × 10^5/mL) were incubated at room temperature with U-46619 (1 μmol/L). After three minutes, increasing concentrations of B59.2 were added, and one minute later 125I-fibrinogen (200 nmol/L) was added. After 30 minutes, free and platelet-bound fibrinogen were separated on silicone oils and counted. The data reported are means of 15 determinations. The inhibition of fibrinogen binding is underestimated, because at 15 minutes, 14% of the bound labeled fibrinogen could not be displaced by a 100-fold excess of the cold protein.

Fig 6. Inhibition by B59.2 of fibrinogen binding to normal or diabetic platelets stimulated by ADP, collagen, or U-46619. Means ± SEM for ten normals and 15 diabetics are shown. Experiments were carried out in a manner similar to the studies on concentration-dependent inhibition (see Fig 5) except that only one concentration of B59.2 was employed (2.1 μmol/L). *P < .01 for B59.2-treated platelets v control (buffer-treated) platelets.

Our experiments show that the hyperaggregable platelets from type-I diabetic subjects bind significantly more fibrinogen than normal platelets and, after ingestion of aspirin, their platelets aggregate and bind fibrinogen like those from normal control subjects who have taken aspirin. In addition, studies with a monoclonal antibody to the glycoprotein IIb-IIIa complex of platelets indicate that this complex is similar in platelets from diabetic and normal subjects. Thus, increased fibrinogen binding and hyperaggregability of platelets from these diabetic subjects appears to be related to their capacity to form more PG endoperoxide/TX than normal platelets. It appears that the present nonretinopathic diabetic population contains the seeds for the future retinopathic population of diabetic subjects.

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Increased binding of fibrinogen to platelets in diabetes: the role of prostaglandins and thromboxane

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