Proaggregatory Effect of Fasting on Platelet Aggregation in the Microcirculation of Mice with Streptozotocin Diabetes

William I. Rosenblum, Farouk El-Sabban, and Roger M. Loria

This study investigates whether experimental diabetes alters the ease with which platelet aggregation can be initiated in pial and mesenteric microvessels of the mouse. Aggregation was elicited by exposing microvessels to radiant energy from a mercury lamp in the presence of sodium fluorescein. The time required for this noxious stimulus to initiate aggregation was similar in fed or fasted alloxan diabetics and their controls, and in fed streptozotocin diabetics and their controls, but was significantly shortened in streptozotocin mice fasted for 18 to 24 hours when these animals were compared with either fed or fasted controls. Aggregation was also elicited by puncture of microvessels or by micropuncture plus locally applied adenosine 5'-diphosphate. No differences in aggregability were found between either fed or fasted diabetics and their respective controls. In the light plus dye model of injury, the capacity to enhance aggregation at will by fasting streptozotocin diabetics may provide a means by which some of the factors controlling aggregation in this model of diabetes can be identified, and this in turn may provide insights into the reasons for the variable occurrence of enhanced aggregation in other species or in other types of diabetes.

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Platelet aggregation in diabetes is of interest for many reasons, including the possible relationship between aggregation and enhanced atherosclerosis in diabetics. There are many reports of enhanced platelet aggregation in diabetic humans, but this phenomenon has not been observed in all studies or in all groups of diabetics.1-7 Moreover, the variables responsible for the presence or absence of enhanced aggregation have not been determined, although an increased production of thromboxane A2 by diabetic platelets has been suggested as a possible cause of increased aggregability.7,6 All studies of platelets from human diabetic subjects have utilized in vitro tests of aggregation. To gain insight into the factors determining aggregability of human diabetic platelets, in vitro studies of rat platelets have also been performed. Unlike the situation in humans, diabetic rat platelets have shown reduced rather than enhanced aggregability when suspended in diabetic plasma.9,10 When tested in the absence of the plasma factor, however, they showed enhanced aggregation.9,10 The relevance of these in vitro observations to in vivo phenomena has not been established, and in vivo studies of platelet aggregation in diabetic animals have been limited.11-13 These few published studies indicate enhanced aggregability in microvessels on the cerebral surface (pial vessels) of the rabbit.

The following report describes our in vivo findings in diabetic mice. We demonstrate that fasting enhances platelet aggregation in pial and mesenteric microvessels of mice rendered diabetic with streptozotocin.14 Fed streptozotocin mice failed to display abnormal platelet aggregability. Fasting streptozotocin mice displayed...
enhanced aggregation whether they were compared with fasted or fed nondiabetic controls. These observations could not be duplicated in mice rendered diabetic with alloxan rather than streptozotocin. Furthermore, enhanced aggregation in fasted streptozotocin mice could only be demonstrated with one method of inducing platelet aggregation, but not with another.

Methods

Animals

Male mice, ICR strain, were anesthetized with urethane, and a tracheotomy performed. This was followed either by a craniotomy with exposure of the pial vessels or by a linear abdominal incision through which a loop of intestine was extruded and draped over a pedestal. Microvascular observation began approximately 15 minutes after induction of anesthesia. After preliminary observations with a dissecting microscope, the pial or mesenteric vessels were observed with a Leitz Ultropak microscope equipped with a “dipping cone” (immersion attachment). Observations were made with a tungsten lamp except during production of aggregates when a mercury lamp was used. A dual lamp housing permitted instantaneous shifting between the tungsten and mercury light source.

Induction of Aggregation

Two techniques were used. The first employed light plus dye and has been described extensively in previous publications. Briefly, light from an appropriately filtered mercury lamp injures vascular endothelium if, and only if, an intravascular energy-absorbing target such as sodium fluorescein is circulating. We selected a single field containing an arteriole and venule for continuous microscopic observation. We then injected 0.2 ml of 2% sodium fluorescein into the tail vein and immediately exposed the field, via the Ultropak optical system, to the incident rays from the mercury lamp. We measured the time elapsing from the onset of this noxious stimulus to the appearance of the first platelet aggregate, recognized microscopically as a fluorescent mass adherent to the endothelium. Control values differed widely from one time period to another, precluding comparisons between the control and experimental groups studied at different times. We do not know what factors determined the variability, but it appeared in other in vivo models of platelet aggregation or clot formation and was indicated by standard deviations exceeding the mean value of the parameter being measured. For this reason, we continued to follow our initial design, which entailed examining a control animal immediately before or after an experimental (diabetic) mouse, so that uncontrolled factors present over any period of time were randomly distributed between the two groups.

The second technique for inducing aggregation employed injury with a micropipette and exposure of the vessel to adenosine 5'-diphosphate (ADP). A micropipette puller was used to produce a glass micropipette with a tip approximately 2 μ in diameter. With the aid of a micromanipulator and dissecting microscope, a vessel was punctured just once with the pipette, and the animal was transferred in a few seconds to the stage of the Leitz Ultropak for further observation. If no platelet aggregate (“white body”) appeared at the puncture site, the entire microscopic field was covered with a drop of ADP beginning with 0.086 μM, and platelet aggregates were again sought. ADP was used because local application to microvessels either in the absence of or following injury is known to produce platelet aggregation and because ADP is a substance that is released from platelets and thought to play an important role in stimulating platelet aggregation during nonexperimental situations. The drop of ADP was held between the tip of the immersion lens (“dipping cone”) and the tissue. If no aggregate appeared within 3 minutes, the drop was replaced by one with a higher concentration of ADP (0.86 μM) and observation was continued for another 3 minutes. If no aggregates appeared, 8.6 μM ADP was used, and in studies of mesenteric vessels (but not of brain vessels), if 8.6 μM ADP was ineffective, 86 μM ADP was used. If aggregates appeared either after puncture alone or following puncture plus addition of ADP, we measured the latency between the initiating stimulus and first aggregate and counted the number of emboli breaking off the aggregate over a 6-minute period following aggregation. In addition, we recorded whether aggregation was produced by puncture alone, whether ADP was required, and if so what concentration was sufficient to induce aggregation after puncture. We also recorded failure to produce aggregation even after ADP. In any mouse only a single arteriole or venule was punctured. When produced either by puncture or puncture plus ADP, aggregates almost always occurred at the puncture site but sometimes were a small distance from the site (e.g., 40 μ away). If ADP was required, additional aggregates might appear at other sites or even in other vessels, particularly venules. However, only the aggregate at or next to the puncture site was used in compiling our data. It seemed unlikely that aggregates were only coincidentally located at the puncture sites and were related instead to nonspecific factors such as operative trauma.
Nevertheless, to rule this out, every field was observed in the presence of 8.6 μM ADP prior to puncture. Any vessel displaying aggregates under these circumstances was discarded.

Production of Diabetes

Two well-known techniques were used\textsuperscript{14} employing intraperitoneal injection of alloxan (100 mg/kg) or of streptozotocin (200 mg/kg). These agents destroy pancreatic beta cells, producing an insulin-deficient model of diabetes.\textsuperscript{14} All animals used had sufficient hyperglycemia to produce glycosuria as determined with Combi-stix (Ames Laboratories, Elkhart, Indiana).

Measurement of Blood Glucose, Lipids, and Insulin

Plasma glucose measurements were made on tail vein blood using a glucose oxidase technique modified for use in mice.\textsuperscript{25} Plasma cholesterol, triglycerides, and fatty acids were measured by thin layer chromatography as previously described.\textsuperscript{26} Plasma insulin was determined by radioimmunoassay using an \textsuperscript{125}I insulin kit (Immuno Nuclear Corporation, Stillwater, Minnesota) modified to use 50 μl plasma samples.

Results

Platelet Aggregation In Pial and Mesenteric Vessels Induced by Light plus Dye

Streptozotocin Diabetes

Table 1 summarizes the data from one study of pial arterioles in fed mice and two studies of mesenteric arterioles in fed mice. In no study did streptozotocin diabetes in fed mice influence the time required to initiate aggregation.

Table 1 also illustrates the effect of streptozotocin diabetes in four studies of fasted mice. In the three studies of pial vessels and the one study of mesenteric vessels, streptozotocin diabetics displayed enhanced platelet aggregation, as indicated by a significant shortening of the time required by the noxious stimulus to initiate aggregation ($p < 0.05$ in each study, Mann Whitney Test).\textsuperscript{27} The fasted mice were without food for 18 to 24 hours but received water ad libitum.

Because the preceding data showed an effect of streptozotocin only in studies of fasted mice, we carried out an additional investigation in which fed and fasted mice were examined at the same time. Animals had diabetes for 7 to 9 weeks. On each day, four mice were observed: a fed diabetic, a fed control, a fasted diabetic, and a fasted control. The results are summarized in Table 1.

<table>
<thead>
<tr>
<th>Duration of diabetes</th>
<th>Pial arterioles (Fed mice)</th>
<th>Pial arterioles (Fasted mice)</th>
<th>Mesenteric arterioles (Fed mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Second to initiate aggregation (M ± SD)</td>
<td>Arteriolar diameter ($\mu$m) M ± SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic*</td>
<td>Control*</td>
<td>Diabetic*</td>
</tr>
<tr>
<td>Pial arterioles:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed mice 3-4 wks</td>
<td>98 ± 48 (10)</td>
<td>96 ± 44 (10)</td>
<td>32 ± 3 (10)</td>
</tr>
<tr>
<td>Fasted mice 2-3 wks</td>
<td>41 ± 18 (10)</td>
<td>62 ± 36 (10)</td>
<td>35 ± 3 (10)</td>
</tr>
<tr>
<td></td>
<td>$p &lt; 0.02$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-5 wks</td>
<td>38 ± 15 (10)</td>
<td>74 ± 38 (10)</td>
<td>34 ± 2 (10)</td>
</tr>
<tr>
<td></td>
<td>$p &lt; 0.05$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-9 wks</td>
<td>44 ± 15 (10)</td>
<td>85 ± 39 (10)</td>
<td>33 ± 2 (10)</td>
</tr>
<tr>
<td></td>
<td>$p &lt; 0.02$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric arterioles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed mice 2-3 wks</td>
<td>87 ± 69 (10)</td>
<td>109 ± 71 (10)</td>
<td>101 ± 20 (10)</td>
</tr>
<tr>
<td>5 wks</td>
<td>152 ± 90 (10)</td>
<td>144 ± 88 (10)</td>
<td>98 ± 29 (10)</td>
</tr>
<tr>
<td>Fasted mice 7-8 wks</td>
<td>135 ± 55 (10)</td>
<td>298 ± 67 (10)</td>
<td>114 ± 32 (10)</td>
</tr>
</tbody>
</table>

*Number in parentheses indicates the number of mice in each group.
a fasted control. Again, a significant reduction in
the time to initiate aggregation was found only in
the fasted streptozotocin diabetics (fed diabetic,
98 ± 66 seconds; fed control, 114 ± 41 seconds;
fasted diabetic, 40 ± 11 seconds; fasted control,
158 ± 125 seconds; M ± so, Kruskal-Wallace
analysis of variance, p < 0.01; fasted diabetic less
than each of the other groups, p < 0.05, Mann
Whitney Test).

Alloxan Diabetes

As shown in table 2, platelet aggregation in
alloxan diabetics was not different from that of
age-matched controls. This was true whether
animals were fed or fasted, and whether
aggregation was monitored in pial or mesenteric
arterioles (Mann-Whitney test, all p > 0.05).

Platelet Aggregation In Pial and Mesenteric
Vessels Induced by Puncture and ADP

As indicated under Methods, we asked the fol-
lowing questions: Was puncture alone sufficient
to induce aggregation or was ADP required? If
ADP was required, what was the threshold dose
producing aggregation? Did any mice fail to
display aggregation even after the highest dose
of ADP? How many seconds elapsed between
application of an effective stimulus and aggrega-
tion? What was the frequency of embolization
from aggregates? We were not able to detect any
significant differences between alloxan diabetics
and their controls or between streptozotocin
animals and their controls in studies of animals
that were diabetic for 1 to 11 weeks.

Sixteen studies were performed, eight with
alloxan and eight with streptozotocin mice. Each
study utilized 10 diabetics and 10 age-matched
controls. In alloxan diabetes, pial arterioles were
observed in two studies (one of fasted mice and
one of fed mice), pial venules were observed in
two studies (one of fasted, one of fed mice), and
four studies of mesenteric vessels were carried
out paralleling the four studies of pial vessels. An
identical series of eight investigations was per-
formed utilizing the streptozotocin animals.

Since no differences were found between the
diabetics and controls, we show only four repre-
sentative studies in table 3. To simplify the
display we show only whether aggregation was
finally produced, but do not show whether it was
produced by puncture alone or only after the
addition of ADP. We have also omitted the
tabulation of the threshold doses required to
elicit aggregation in those animals where ADP
was used. The incidence of aggregation in
diabetes and controls was compared in each
study using the Fisher Test of Exact Probability;
the latency of aggregation and numbers of
emboli were analyzed with the Mann-Whitney
Test. No effect of diabetes was observed on
these parameters.

Glucose, Lipid, and Insulin Levels

The plasma glucose and lipid levels of repre-
sentative groups of mice diabetic for 5 to 10
weeks are shown in table 4 together with those of
age-matched controls. Each fed animal also was
studied in the fasted state. The insulin values for
10 fasted alloxan mice were 5.3 ± 3.4 μIU/ml
(M ± so), and for ten fasted streptozotocin mice,
5.6 ± 3.8 μIU/ml.

### Table 2. Platelet Aggregation In Arterioles of Alloxan Diabetic Mice Is Like That of Controls

<table>
<thead>
<tr>
<th>Duration of diabetes</th>
<th>Seconds to initiate aggregation (M ± sd)</th>
<th>Arteriolar diameter (μ) M ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetic*</td>
<td>Control*</td>
</tr>
<tr>
<td>Pial arterioles:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>52 ± 21(10)</td>
<td>67 ± 33(10)</td>
</tr>
<tr>
<td>1-2 wks</td>
<td>45 ± 19(16)</td>
<td>58 ± 21(16)</td>
</tr>
<tr>
<td>2-3 wks</td>
<td>70 ± 41(8)</td>
<td>64 ± 16(8)</td>
</tr>
<tr>
<td>Fasted mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-6 wks</td>
<td>35 ± 8(15)</td>
<td>48 ± 33(15)</td>
</tr>
<tr>
<td>Mesenteric arterioles:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3 wks</td>
<td>121 ± 126(10)</td>
<td>136 ± 97(10)</td>
</tr>
<tr>
<td>Fasted mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3 wks</td>
<td>81 ± 65(10)</td>
<td>114 ± 94(10)</td>
</tr>
<tr>
<td>3-5 wks</td>
<td>65 ± 52(10)</td>
<td>87 ± 82(10)</td>
</tr>
</tbody>
</table>

*Number in parentheses indicates the number of mice in each group.
Table 3. Platelet Aggregation and Embolization Following Puncture or Puncture Plus ADP

<table>
<thead>
<tr>
<th>Vascular bed and treatment</th>
<th>Aggregation observed</th>
<th>Seconds to aggregation (M ± sd)</th>
<th>Emboli in 6 minutes (M ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pial arterioles:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptozotocin (fed)</td>
<td>Yes: 6, No: 3</td>
<td>29 ± 31</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>Control (fed)</td>
<td>Yes: 9, No: 1</td>
<td>40 ± 58</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Mesentery venules:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptozotocin (fasted)</td>
<td>Yes: 8, No: 2</td>
<td>30 ± 38</td>
<td>6 ± 7</td>
</tr>
<tr>
<td>Control (fasted)</td>
<td>Yes: 9, No: 1</td>
<td>18 ± 11</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Pial arterioles:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alloxan (fasted)</td>
<td>Yes: 6, No: 4</td>
<td>58 ± 64</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Control (fasted)</td>
<td>Yes: 4, No: 6</td>
<td>57 ± 48</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Mesentery venules:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alloxan (fed)</td>
<td>Yes: 10, No: 0</td>
<td>14 ± 9</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Control (fed)</td>
<td>Yes: 10, No: 0</td>
<td>19 ± 17</td>
<td>7 ± 9</td>
</tr>
</tbody>
</table>

Table 4. Plasma Glucose and Lipid Values Before and After Fasting

<table>
<thead>
<tr>
<th></th>
<th>Glucose (M ± sd)</th>
<th>Total cholesterol (M ± sd)</th>
<th>Fatty acids (M ± sd)</th>
<th>Triglycerides (M ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptozotocin (n = 10)</td>
<td>460 ± 56*</td>
<td>322 ± 133</td>
<td>114 ± 15</td>
<td>102 ± 27</td>
</tr>
<tr>
<td>(mg%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>133 ± 11*</td>
<td>103 ± 13</td>
<td>92 ± 35</td>
<td>87 ± 13</td>
</tr>
<tr>
<td>(mg%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alloxan (n = 10)</td>
<td>550 ± 51*</td>
<td>444 ± 106</td>
<td>86 ± 22</td>
<td>81 ± 20</td>
</tr>
<tr>
<td>(mg%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05, paired t test, fed vs fasting. All data expressed as mg %.

Discussion

We were able to demonstrate enhanced aggregation of platelets in vivo in mice with streptozotocin-induced diabetes only by depriving them of food for 18 to 24 hours. We were not able to demonstrate enhanced aggregation in mice with alloxan diabetes, even in fasted animals. Moreover, the enhancing effect of fasting on aggregation in streptozotocin mice could only be demonstrated when microvessels were injured by radiant energy, not by mechanical puncture or puncture plus ADP.

We have no explanation for our results. We could not relate differences in the effect of fasting on streptozotocin and alloxan mice to differences in glucose, triglyceride, or free fatty acid levels since these levels were similarly diminished after fasting in both types of diabetes. Nor could we relate the results of fasting to an effect on insulin levels since the insulin levels were the same in fasted streptozotocin and fasted alloxan groups.

The results are interesting for several reasons. First, they deal with platelet aggregation in vivo, an area in which there are relatively few studies of diabetic animals.11-13 While in vivo studies may not distinguish between differences in platelet adhesiveness and differences in the capacity of platelets to undergo the release reaction or to aggregate, such studies are generally interpreted as reflecting aggregability, at least in part, and provide unique information concerning the rapidity with which aggregates may be induced in the microcirculation.

Second, because we can control aggregation in streptozotocin mice, we may be able to gain insight into the factors regulating aggregation in this diabetic model. With respect to the effect of
fasting on platelet function, it has recently been reported that platelets from fasted rats have a greater uptake of arachidonic acid than control platelets. It may be that an increase in arachidonate uptake by platelets in fasted mice provides increased substrate for cyclooxygenase-dependent metabolic reactions leading to platelet aggregation. Why this should be so only in streptozotocin diabetics and not in control mice or in alloxan diabetics, and why the effect is not seen with both models of vessel injury, remains to be explained.

Our results are also of interest because they may seem at variance with often cited in vitro studies of human platelets showing enhanced aggregation in diabetics, and with in vivo studies of rabbits. Of course, species differences, aggregation in diabetics, and with in vivo studies of human platelets showing enhanced aggregation in several studies of diabetic mice is accounted for by the vessel wall itself. Diminished production of prostacyclin, an anti-aggregant, has been observed in blood vessels from human diabetics and from rats with streptozotocin diabetes. Moreover, enhanced aggregation in fasted streptozotocin diabetics could reflect greater vascular damage in those mice compared with fed animals. However, it is difficult to understand why damage produced by light plus dye would be enhanced by fasting only in streptozotocin diabetics, or indeed, why fasting should enhance damage only when light plus dye was used but not puncture. It is equally difficult to imagine that prostacyclin synthesis by the vessel wall would be altered only in the fasted streptozotocin mouse.

References

rabbit mesentery and rabbit ear chamber. Thromb Res 1973:3:75–85


Index Terms: pial arterioles, mesenteric arterioles, microvascular injury, light plus dye, micropuncture plus ADP, alloxan
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