Platelets Derived From the Bone Marrow of Diabetic Animals Show Dysregulated Endoplasmic Reticulum Stress Proteins That Contribute to Increased Thrombosis

Rodrigo Hernández Vera, Gemma Vilahur, Raquel Ferrer-Lorente, Esther Peña, Lina Badimon

Objective—Patients with diabetes mellitus have an increased risk of suffering atherothrombotic syndromes and are prone to clustering cardiovascular risk factors. However, despite their dysregulated glucose metabolism, intensive glycemic control has proven insufficient to reduce thrombotic complications. Therefore, we aimed to elucidate the determinants of thrombosis in a model of type 2 diabetes mellitus with cardiovascular risk factors clustering.

Methods and Results—Intravitral microscopy was used to analyze thrombosis in vivo in Zucker diabetic fatty rats (ZD) and lean normoglycemic controls. Bone marrow (BM) transplants were performed to test the contribution of each compartment (blood or vessel wall) to thrombogenicity. ZD showed significantly increased thrombosis compared with lean normoglycemic controls. BM transplants demonstrated the key contribution of the hematopoietic compartment to increased thrombogenicity. Indeed, lean normoglycemic controls transplanted with ZD-BM showed increased thrombosis with normal glucose levels, whereas ZD transplanted with lean normoglycemic controls—BM showed reduced thrombosis despite presenting hyperglycemia. Significant alterations in megakaryopoiesis and platelet–endoplasmic reticulum stress proteins, protein disulfide isomerase and 78-kDa glucose-regulated protein expression that can contribute to increase thrombotic risk independently of blood glucose levels. (Arterioscler Thromb Vasc Biol. 2012;32:2141-2148.)

Key Words: thrombosis ■ diabetes mellitus ■ glucose-related protein 78 ■ protein disulfide isomerase ■ tissue factor

The link between diabetes mellitus and cardiovascular disease is well established, as patients with diabetes mellitus have an increased risk of developing ischemic syndromes1-7 and are particularly prone to clustering various cardiovascular risk factors, such as obesity,8-11 hypercholesterolemia, and hypertension, and with a stronger negative impact than that observed in patients without diabetes mellitus.7 Although hyperglycemia has been associated with cardiovascular disease and acute event occurrence,12-14 recent clinical trials (ie, Action to Control Cardiovascular Risk in Diabetes [ACCORD], Action in Diabetes and Vascular Disease [ADVANCE], and Veterans Affairs Diabetes Trial [VADT])15-18 have shown that intensive glycemic control is insufficient to reduce cardiovascular event presentation.

Numerous alterations have been previously described in hemostasis19-23 and platelet activity16-21,24-27 and in the vascular endothelial function19-21,26-30 among patients with diabetes mellitus, altogether contributing to the development of a characteristic proatherothrombotic state. However, the degree of contribution of each compartment (blood or vessel wall) to the increased thrombotic risk has not been elucidated. Thus, in the present study we aimed to (1) evaluate, in a rat model of type 2 diabetes mellitus with cardiovascular risk factor clustering, whether the presence of hyperglycemia plays a role in the increased thrombotic risk; and (2) evaluate the contribution of platelets and the vessel wall to the increased prothrombotic risk.

Our results indicate that megakaryopoiesis is altered in the bone marrow (BM) of diabetic animals, giving rise to hyperreactive platelets with altered expression of endoplasmic reticulum (ER) stress proteins, 78-kDa glucose-regulated protein (GRP78) and protein disulfide isomerase (PDI), which can increase the amount of active tissue factor (TF), thus contributing to increased thrombotic risk in diabetic animals independently of blood glucose levels.

Materials and Methods

Animals
The study was developed in 3 phases: (1) 8-week-old Zucker diabetic fatty rats (ZD) and their corresponding lean normoglycemic controls (ZC) were fed with Lab Diet 5008 (PMI Nutrition International,
St. Louis, MO) chow for 5 weeks, and thrombotic risk was evaluated in vivo by photochemically induced thrombosis monitored by intravital microscopy; (2) BM from ZD donors was transplanted to age-matched ZC (ZC-BM/ZC) and ZD (ZC-BM/ZD) recipients, and animals were tested for thrombotic risk; and (3) BM from ZD donors that had already developed type 2 diabetes mellitus was transplanted to age-matched ZC hosts (ZD-BM/ZC). As a proof of concept, Wistar rats received BM from age-matched green fluorescent protein–transgenic Wistar rats.

All procedures fulfilled the criteria established by the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Internal Animal Committee Review Board.

**In Vivo Real-Time Thrombosis Evaluated by Intravital Microscopy**

Rose Bengal was administered to anesthetized animals through the femoral vein, and a portion of the mesentery was extended flat on the microscope stage. Within 30 minutes of Rose Bengal administration, mesenteric vessels were irradiated to produce photochemical endothelial injury until a full thrombotic occlusion was achieved. Irradiation times necessary to produce a full occlusion (occlusion time [OT]) were determined.

**Bone Marrow Transplants**

Recipient mice received combined immunosuppressive therapy (cyclophosphamide and busulfan) before transplantation. Femurs and tibias were isolated from the donors and BM was flushed with Dulbecco Modified Eagle Media. 3×10^7 BM nucleated cells were injected to immunosuppressed recipients through the lateral tail vein. Blood cell counts, were routinely analyzed in all animals including red blood cell counts, hematocrit levels, platelet (PLT) counts, and white blood cell counts, were routinely analyzed in all animals (Table I in the online-only Data Supplement), revealing that ZD had a higher thrombotic risk than that of ZC, as their OTs were significantly shorter (Figure 1B; 1.00±0.05 minutes and 3.19±0.21 minutes, respectively; P<0.0001). Moreover, ZD also showed a hypercoagulable state reflected by significantly shorter prothrombin times and significantly increased fibrinogen levels (Table 1) compared with those of ZC.

Hematological parameters, including red blood cell counts, hematocrit levels, platelet (PLT) counts, and white blood cell counts, were routinely analyzed in all animals (Table I in the online-only Data Supplement), revealing that ZD had significantly increased platelet counts compared with ZC (626.4±40.5×10^3 per mm^3 and 510.0±22.2×10^3 per mm^3, respectively; P<0.05). No significant differences were observed in red blood cells, hematocrit, or white blood cell levels.

**Hematopoietic Versus Vessel Wall Contribution to Thrombogenicity**

BM transplantation experiments between ZC and ZD animals allowed us to determine the relative contribution of each compartment to thrombogenicity. The behavior of platelets shed by ZD-BM in a normoglycemic environment was analyzed in contrast to that of ZC-BM–derived platelets dyslipidemia with significantly increased non–high density lipoprotein cholesterol levels and hypertriglyceridemia (Table 1). Insulin levels of ZD were also significantly increased compared with those of ZC (Table 1), reflecting the existence of insulin resistance. Fasting blood glucose levels of ZD were nearly 5-fold higher than those of ZC.

**Thrombosis and Coagulation**

In vivo photochemically induced thrombus formation (Figure 1A) was monitored by real-time intravital microscopy, and time needed to produce vessel occlusion was determined. Short OTs reflected the existence of a prothrombotic tendency, whereas prolonged OTs showed a reduced thrombotic risk in the presence of vascular damage.

ZD had a higher thrombotic risk than that of ZC, as their OTs were significantly shorter (Figure 1B; 1.00±0.05 minutes and 3.19±0.21 minutes, respectively; P<0.0001). Moreover, ZD also showed a hypercoagulable state reflected by significantly shorter prothrombin times and significantly increased fibrinogen levels (Table 1) compared with those of ZC.

**Statistical Analysis**

Results were expressed as mean±SEM. Student t test or 1-way ANOVA, as appropriate, were used to determine statistical significance between the groups. A P<0.05 was considered significant. An extended Methods section is available in the online-only Data Supplement.

**Results**

**Metabolic Profile and Coagulation Parameters**

Table 1. Metabolic Profile and Coagulation Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ZC</th>
<th>ZD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid Profile</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>82.7±1.7</td>
<td>169.9±5.9*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>47.7±2.9</td>
<td>95.9±4.9*</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mg/dL</td>
<td>34.9±2.5</td>
<td>74.1±7.2*</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
<td>11.9±0.4</td>
<td>19.8±2.9*</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>74.8±9.4</td>
<td>292.8±75.8*</td>
</tr>
<tr>
<td>Weight, g</td>
<td>339.8±3.0</td>
<td>358.9±6.1*</td>
</tr>
<tr>
<td><strong>Glycemic Status</strong></td>
<td></td>
<td></td>
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<tr>
<td>Fasting blood glucose, mg/dL</td>
<td>87.4±2.2</td>
<td>457.8±18.1*</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>0.39±0.04</td>
<td>0.87±0.17*</td>
</tr>
<tr>
<td><strong>Coagulation Parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin time, s</td>
<td>18.8±0.5</td>
<td>13.8±0.3*</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>201.2±8.0</td>
<td>287.1±6.4*</td>
</tr>
</tbody>
</table>

ZC indicates lean normoglycemic controls; ZD, Zucker diabetic fatty rats; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

*P<0.05 vs ZC.
in a milieu of hyperglycemia with other cardiovascular risk factors. Indeed, BM transplantation showed no effect over the metabolic profile of the recipients (Table 2) as, in line with nontransplanted groups, ZD recipients transplanted with ZC-BM (ZC-BM/ZC) were obese and showed significantly increased fasting blood glucose, triglycerides, total, non–high density lipoprotein, and low-density lipoprotein cholesterol levels compared with ZC recipients to those of nontransplanted ZD and ZC-BM significantly increased OTs in ZD recipients. *P<0.001 vs ZC.

### Table 2. Metabolic Profile and Coagulation Parameters in Transplanted Animals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ZC-BM/ZC</th>
<th>ZD-BM/ZC</th>
<th>ZC-BM/ZD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group Characteristics</strong></td>
<td></td>
<td></td>
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<tr>
<td>Donor</td>
<td>ZC</td>
<td>ZD</td>
<td>ZC</td>
</tr>
<tr>
<td>Recipient</td>
<td>ZC</td>
<td>ZC</td>
<td>ZD</td>
</tr>
<tr>
<td><strong>Lipid Profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>86.9±2.9</td>
<td>101.5±2.1</td>
<td>142.0±7.8*†</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>40.4±2.8</td>
<td>83.0±9.6*†</td>
<td>34.2±7.8</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mg/dL</td>
<td>46.5±3.1</td>
<td>18.5±8.7* †</td>
<td>107.8±8.5*†</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>28.4±2.0</td>
<td>18.6±2.2</td>
<td>39.6±5.2†</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>80.4±10.8</td>
<td>91.9±7.9</td>
<td>514.9±64.1*†</td>
</tr>
<tr>
<td>Weight, g</td>
<td>310.8±6.3</td>
<td>365.4±6.7*†</td>
<td>404.8±9.0*†</td>
</tr>
<tr>
<td><strong>Glycemic Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dL</td>
<td>94.3±1.7</td>
<td>96.2±2.6</td>
<td>323.1±35.9*†</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>0.35±0.06</td>
<td>0.30±0.05</td>
<td>1.27±0.46</td>
</tr>
<tr>
<td><strong>Coagulation Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin time, s</td>
<td>13.8±0.5</td>
<td>17.4±0.9*†</td>
<td>13.4±0.2</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>199.2±26.0</td>
<td>195.8±14.4</td>
<td>213.3±22.8</td>
</tr>
</tbody>
</table>

ZC indicates lean normoglycemic controls; BM, bone marrow; ZD, Zucker diabetic fatty rats; HDL, high-density lipoprotein; LDL, low-density lipoprotein. *P<0.05 vs ZC-BM/ZC; †P<0.05 vs ZD-BM/ZC; ‡P<0.05 vs ZC-BM/ZD.

Interestingly, when ZD-BM was transplanted to ZC recipients (ZD-BM/ZC), animals became ZD-like, as their OTs were significantly reduced compared with those of nontransplanted ZC (Figure 1C; ZD-BM/ZC, 0.93±0.17 minutes; *P<0.001 vs ZC and ZC-BM/ZC) and became similar to those of nontransplanted ZD rats (Figure 1B; ZD, 1.00±0.05 minutes) despite showing normal blood glucose levels (Table 2). Conversely, transplantation with ZC-BM transformed ZD recipients (ZC-BM/ZD) into ZC-like by significantly increasing their OTs compared with those of nontransplanted ZD (Figure 1C; ZC-BM/ZD, 5.94±0.61 minutes; *P<0.0001) despite their increased glucose levels (Table 2). Thus, these results highlight the greater contribution of platelets produced by the BM to the increased thrombogenicity observed in ZD animals compared with that of the vessel wall and show a lack of effect of blood glucose levels over the thrombotic response.

Donor BM engraftment in the recipients was confirmed by the presence of green fluorescent protein–positive cells in the femur of wild-type recipients 5 weeks after receiving BM from green fluorescent protein–transgenic Wistar rat donors (Figure I in the online-only Data Supplement). Routine controls to assure the effectiveness of the immunosuppressive protocol before transplantation, as well as to confirm posttransplantation recovery of all hematological parameters (red blood cells, hematocrit, platelet, and white blood cell) were performed in all transplanted animals (Table I in the online-only Data Supplement). Such analyses revealed that the immunosuppressive treatment led to a significant reduction in white blood cell counts in all transplanted groups at day 0 (transplant) compared with the corresponding nontransplanted groups and a gradual recovery of all the hematological parameters. In fact, ZC-BM/ZD and ZD-BM/ZC showed no significant differences at day 28 with the corresponding nontransplanted groups.
Platelet Size and Activation
The fact that thrombotic risk was transferred by BM transplantation allowed us to conclude that the main modification affecting thrombogenicity in ZD animals was in the hematopoietic compartment. Therefore, we analyzed numerous factors that could contribute to such increased thrombotic risk.

First, mean platelet volume analysis showed that ZD platelets were significantly larger (Figure 2A; 6.78±0.09 μm³) than those of ZC (Figure 2A; 6.24±0.03 μm³; P<0.0001), and the flow cytometric analysis of CD62P expression (Figure 2B) indicated that platelet susceptibility to activation was significantly increased in ZD animals compared with ZC. Moreover, despite the fact that platelets from both groups responded in a dose-dependent manner to thrombin, the percentage of CD62P+ platelets in ZD animals was significantly higher than that of ZC at all tested doses (Figure 2B).

Reticulated Platelets and Platelet Turnover
ZD showed significantly more reticulated platelets (Figure 2C) than ZC, indicating an increase in the platelet production of ZD at all doses tested (Figure 2B).

Platelet TF and ER Stress Proteins Expression
TF protein expression was analyzed in platelets from ZD and ZC (Figure 4A) and ER stress proteins previously associated with active TF regulation, GRP78 and PDI, were then investigated.

No differences were observed in the platelet TF expression between ZD and ZC. However, GRP78 (found to exert an antithrombotic effect by inhibiting active TF by direct binding)33–35 expression was found to be significantly reduced in platelets from ZD animals compared with those of ZC (Figure 4B; 1.65±0.37 AU and 2.81±0.37 AU, respectively; P<0.05), whereas PDI (a thiol isomerase and oxidoreductase that contributes to TF decryption and increases TF procoagulant activity) expression was significantly increased in ZD compared with ZC (Figure 4C; 0.47±0.04 AU and 1.05±0.06 AU; P<0.0001).

Plasma TF Procoagulant Activity and Coagulation Pathway Analysis
TF procoagulant activity in plasma of ZD animals was significantly increased compared with ZC (Figure 4D; 0.15±0.01 U/mL and 0.08±0.00 U/mL, respectively; P<0.0004). Thromboelastometry confirmed an increase in the activity of the extrinsic (TF-dependent) coagulation pathway (Figure 4E) in ZD rats, reflected by a significantly reduced extrinsically activated assay clotting time (extrinsically activated assay Ct=42.0±1.7 seconds) compared with ZC (54.0±0.0 seconds; P<0.05). However, no significant differences were observed in the activity of the intrinsic coagulation pathway between ZD and ZC (intrinsically activated assay Ct=80.4±4.7 seconds and 86.0±12.0 seconds, respectively; P>0.05). No significant differences were observed between groups in clot properties (clot formation time or maximum clot firmness; data not shown) in either the extrinsically activated or intrinsically activated assays.

Discussion
In this study, we have shown that the increased thrombotic risk observed in diabetes mellitus with clustering of cardiovascular risk factors is independent of blood glucose levels and associated with a modification in platelets produced by diabetic BM megakaryocytes. Indeed, we demonstrate for the
first time by a transplant crossover study that hematopoietic-derived blood alterations, rather than those of the vessel wall, greatly contribute to the detected increased thrombotic risk and that alterations in platelet expression of ER stress proteins, PDI and GRP78, likely contribute to the overall detected prothrombotic phenotype.

Various clinical trials have shown that intensive glycemic control, despite achieving near-normal blood glucose levels (≈6.0%–6.5% HbA1c), does not reduce acute ischemic events. In fact, our results show that, despite the nearly 3.5-fold difference in blood glucose levels, ZC animals transplanted with ZD-BM present the same thrombotic risk as nontransplanted ZD animals (reflected by short OTs upon injury), confirming that hyperglycemia has no direct effect over thrombogenicity. Crossed BM transplantation experiments have allowed us to discriminate the contribution of the hematopoietic compartment and the vessel wall to the prothrombotic behavior observed in the ZD animals. Transplantation per se showed no effect over the thrombotic behavior as no significant differences were observed between ZC transplanted with ZC-BM and those of nontransplanted ZC. However, ZC recipients transplanted with ZD-BM were transformed in ZD-like animals with short OTs despite their normal blood glucose levels, whereas ZD recipients of ZC-BM dramatically increased their OTs behaving as ZC-like animals despite showing hyperglycemia. Therefore, our results highlight the contribution of the hematopoietic compartment to the increased thrombotic risk observed in diabetes mellitus. Interestingly, platelets produced by ZC-BM were insensitive to the hyperglycemic environment of ZD, raising the question of whether ZC animals might be resistant to hyperglycemia because of their genetic background. Further analyses are required to clarify this point.

ZD animals showed an increased platelet turnover compared with ZC, possibly because of the combination of accelerated death with increased production of new platelets. According to this observation, ZD showed an increased number of reticulated platelets, the youngest and more immature platelets. 

Figure 3. Megakaryocyte number and ploidy. A, Gate R1 created to include only viable cells in the analysis. B, No differences were observed in the number of megakaryocytes (CD61+ cells) present in the bone marrow (BM) of Zucker diabetic fatty rats (ZD) and lean normoglycemic controls (ZC). C, No differences were observed in megakaryocyte ploidy between the groups. Representative plots of megakaryocyte ploidy in ZC and ZD are shown in D and E, respectively. PI indicates propidium iodide; SS LOG, side scatter in logarithmic scale; FS LOG, forward scatter in logarithmic scale; N, haploid number; BMC, bone marrow cells.
However, no differences were observed in megakaryocyte number or ploidy between ZD and ZC in contrast to previous observations where diabetic subjects showed an increased mean ploidy compared with controls. Yet, the biological significance of megakaryocyte ploidy remains unclear, and it has previously been described that ploidy and platelet size are independent. Thus, our results suggest that alterations observed in ZD probably occur during platelet formation or maturation. The observed alterations in platelet turnover might contribute to explain the fact that ZD showed increased platelet number and size compared with ZC as immature platelets are larger in size. 

Interleukin-6 likely contributes to enhance megakaryopoiesis in ZD, as increased interleukin-6 levels have been previously observed in these animals (in line with that observed in obese and patients with diabetes mellitus), and interleukin-6 has been proven to increase platelet count by inducing hepatic synthesis of thrombopoietin, the main regulator of megakaryopoiesis. Moreover, ZD platelets also showed an increased activation in response to thrombin, which together with their increased size reflects a hyperreactive state, as large platelets are more reactive and aggregable because of the fact that they contain denser granules, secrete more serotonin and β-thromboglobulin, and produce more thromboxane A2. In fact, mean platelet volume is clinically considered as an index of platelet activity, as increased platelet size has been linked to increased cardiovascular risk in both diabetic and nondiabetic subjects.

Diabetes mellitus, hypercholesterolemia, and obesity (all present in ZD animals) have all been described to induce ER stress in various cell types. When such stress is sensed, cells activate the unfolded protein response (UPR) to restore normal ER function. GRP78 is a multifunctional protein usually found in the lumen of the ER that has also been described to exert an atheroprotective function in platelets and endotherial cells, inhibiting TF’s procoagulant activity by direct binding. On the other hand, PDI is a ubiquitously expressed thiol isomerase and oxidoreductase that has been described to contribute to TF’s decryption and to increase TF procoagulant activity. Interestingly, our data show that diabetes mellitus with cardiovascular risk factor clustering induces alterations in ER stress proteins in the BM and that platelets produced by such marrow have expression levels of GRP78 and PDI that confer an increased thrombogenicity to ZD animals. Indeed, both GRP78 and PDI have been associated with the regulation of TF activation.

The origin of platelet TF is currently under debate. Traditionally, it was assumed that platelets did not synthesize TF and only bound blood-borne TF-bearing microparticles (mostly originating from leucocytes) in their membrane when they became activated. However, various studies have now reported the presence of TF in platelets and TF-mRNA has also been detected in unstimulated platelets. Nevertheless, the mere presence of TF does not imply a prothrombotic behavior as TF procoagulant activity is tightly regulated. Indeed, it has previously been described that only a fraction of TF is functionally active in coagulation, whereas the vast majority of it remains encrypted. In fact, we have detected no differences in TF protein content between ZD and ZC but significantly different procoagulant activity.

GRP78 is a multifunctional protein usually found in the lumen of the ER that has also been described to be present in the cell surface. In fact, cell-surface GRP78 has been proven to exert an atheroprotective function in platelets and endothelial cells, inhibiting TF’s procoagulant activity by direct binding. On the other hand, PDI is a ubiquitously expressed thiol isomerase and oxidoreductase that has been described to contribute to TF’s decryption and to increase TF procoagulant activity even though the mechanism still remains unclear. Thus, the decreased levels of GRP78 observed in platelets from ZD animals together with the increased levels of PDI seem to contribute to the increased TF procoagulant activity observed in such animals.

In summary, our data indicate that thrombotic risk in diabetic animals with cardiovascular risk factor clustering remains high.
even when blood glucose levels are reduced to normal levels and, hence, seems independent of glycemia. Although many regulatory factors may be involved in the increased thrombotic response of diabetic subjects (including the mechanisms controlling thrombopoeisis), our data show that platelet thrombotic tendency is enhanced by disturbances in ER stress proteins induced in their parent cells in the BM. Particularly, decreased GRP78 and increased PDI expression may contribute to the higher thrombotic risk observed in diabetes mellitus because both PDI and GRP78 have been shown to regulate TF activity and, hence, thrombogenicity. However, further studies in human diabetic platelets are needed to translate these experimental studies into the clinical scenario.

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We thank Dr Laura Casaní, Dr Javier Crespo, and Dr Oriol Juan-Babot for their technical help. We also thank the National Bio Resource Project for the Rat in Japan for providing the green-crease protein–transgenic rats.

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Disclosures
None.

References


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Platelets derived from the bone marrow of diabetic animals show dysregulated ER stress proteins that contribute to increased thrombosis

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EXTENDED METHODS

Animals

Zucker Diabetic Fatty (ZD) and their corresponding lean normoglycemic controls (ZC) were fed with LabDiet® 5008 chow and water given *ad libitum*. The study was developed in 3 phases: I) 8 week-old ZD and ZC rats were kept in diet for 5 weeks and thrombotic risk was evaluated *in vivo* by photochemically induced thrombosis monitored by intravital microscopy; II) bone marrow (BM) from 8-week old ZC donors was transplanted to age-matched ZC (ZC-BM/ZC) and ZD (ZC-BM/ZD) recipients and 5 weeks after transplantation animals were tested for thrombotic risk; and III) 12-week old ZD BM (to assure that donors had already developed type 2 diabetes) was transplanted to age-matched ZC hosts (ZD-BM/ZC). As a proof of concept, 8 week-old Wistar rats received bone marrow from age-matched green fluorescent protein (GFP) transgenic Wistar rats (Wistar-GFP). Engraftment of GFP positive (GFP+) cells in the bone marrow was analyzed by confocal microscopy 5 weeks after transplantation.

ZD, ZC, and Wistar rats were purchased from Charles River Laboratories. Wistar-GFP rats (Wistar-Tg [CAG-GFP] 184Ys) were obtained from the National Bio Resource Project for the Rat (Japan). All procedures fulfilled the criteria established by the “*Guide for the Care and Use of Laboratory Animals*” published by the United States National Institutes of Health (NIH Publication No.85-23, revised 1996) and were approved by the Internal Animal Committee Review Board.

Blood samples

A 1-cm portion of the carotid artery was isolated, catheterized, and blood was gently withdrawn, discarding the first drops to minimize platelet activation. Blood was collected in either 3.8% sodium citrate or serum-separation tubes. Anticoagulated blood samples were centrifuged for 10min at 300g to obtain platelet rich plasma (PRP) and red cells were discarded. Platelets were then isolated from PRP by centrifugation.
(15min at 3500g) after which platelet poor plasma (PPP) and platelet pellets were immediately frozen in liquid nitrogen and stored at -80°C to perform further analysis. Blood collected in serum-separation tubes was incubated at 37°C to clot and then centrifuged 15min at 1800g at 4°C. Serum samples were immediately frozen and stored for further analysis.

Hematological, biochemical, and coagulation parameters

Hematological parameters from whole blood samples were analyzed using a Medonic CA620 analyzer (Boule Medical AB, Sweden). Biochemical analyses of serum samples were performed with a CLIMA MC-15 analyzer (RAL, Spain). Control evaluation of coagulation parameters (prothrombin time and fibrinogen levels) was performed with a STart® 4 Coagulometer (Diagnostica Stago, France).

Blood glucose and insulin determination

Animals were fasted for 4h after which a blood sample was extracted through the lateral tail vein and analyzed with a Glucocard™ Memory 2 meter (A. Menarini Diagnostics). A rat-specific insulin enzyme immunoassay (A05105 SPI-Bio, France) was used to measure insulin levels.

In vivo real-time thrombosis evaluated by intravital microscopy

Animals were anesthetized with a combination of ketamine and medetomidine (75mg/Kg and 0.5mg/Kg; i.m.). Buprenorfine (0.075mg/Kg) was administered as analgesic. Once anesthetized, a 1-cm segment of the right femoral vein was isolated and catheterized. 50mg/Kg of Rose Bengal (RB; Sigma) were then administered in a 1mL bolus followed by 0.5mL of saline. After RB administration, a portion of the small intestine was exteriorized through a lateral (right) incision in the abdominal wall. Then, animals were placed on a lateral position and the mesentery was extended flat on the microscope stage (Nikon Eclipse TE2000) where it was kept at 37°C in saline.
throughout the duration of the experiment. Within 30min of RB administration, mesenteric vessels were irradiated with a 540nm light (Hamamatsu Photonics) coupled to the microscope through an optical fiber and a Spot Illumination module (Rapp OptoElectronic) to produce endothelial damage and continuously observed until a full occlusion was achieved. Irradiation times necessary to produce a full occlusion were determined (occlusion time; OT) and real-time images of the process were recorded using a CCD camera (Hamamatsu Photonics). Short OTs indicated prothrombotic tendency while prolonged OTs showed a reduced thrombotic tendency.

**Bone marrow transplants**

**A) Recipient conditioning.** Animals received combined immunosuppressive therapy consistent of 60mg/Kg of cyclophosphamide (i.p.; Genoxal® Baxter Oncology GmbH) 2 days prior to transplantation (day -2) and 20mg/Kg of busulfan (Allen Farmaceútica S.A.) on day -1 administered orally through a gastric gavage diluted in 1mL saline.

**B) Bone marrow extraction.** On day 0, donors were anesthetized with a combination of ketamine and medetomidine (75mg/Kg and 0.5mg/Kg; i.p.) and then sacrificed with an overdose of sodium pentobarbital (i.c.). Femurs and tibias were isolated from both hind legs and bone marrow was flushed with Dulbecco’s Modified Eagle Media (DMEM; Gibco) and filtered through a 100µm mesh to eliminate remaining bone fragments.

**C) Bone marrow transplantation.** On day 0, previously immunosuppressed recipients were anesthetized with ketamine-medetomidine (i.p.) and buprenorphine was administered as an analgesic. 3x10⁷ bone marrow nucleated cells suspended in DMEM (6x10⁷cells/mL) were injected through the lateral tail vein followed by 0.5mL of saline. Transplanted animals received antibiotic treatment for 5 days following transplantation (enrofloxacine; 20mg/Kg every 24h; s.c.). Blood cell counts were performed on days -2, 0, 7, 14, 21, and 28 to assess the effect of the immunosuppressive treatment as well as post-transplantation recovery.
Bone marrow engraftment assessment

Wistar rats that received Wistar-GFP bone marrow were sacrificed 5 weeks after transplantation and femurs were extracted and fixed with 4% paraformaldehyde for 24h. After fixation, bones were submerged in a 10% formic acid solution containing Amberlite IR-120 (plus) ion-exchange resin (Aldrich) for 5 days to decalcify. Then, femurs were cut in 4 sections (head, 2 mid-sections, and end) and embedded in optimal cutting temperature compound (OCT) before freezing. 5 consecutive 5-µm cuts from one of the mid-sections were obtained and nuclei were stained with Hoechst (Sigma). Finally, samples were analyzed by confocal laser scanning microscopy (Leica TCS SP2) using an APO 20X objective.

Activated platelets flow cytometry

For flow cytometry analysis of platelet activation, citrate-anticoagulated whole blood was diluted with Tyrode's Buffer (134mM NaCl, 0.34mM Na2HPO4x12H2O, 2.9mM KCl, 12mM NaHCO3, 1mM MgCl2x6H2O, 20mM Hapes) to obtain 1x10⁸ platelets/mL. 10µL aliquots were activated with escalating doses of thrombin (0.2, 0.5, and 1.0U/mL) and 2.5mM Gly-Pro-Arg-Pro (Sigma) was added to all activated aliquots. A fluorescein isothiocyanate (FITC) conjugated monoclonal antibody against CD61 (554952; BD Biosciences) was used as an activation-independent marker of platelets and platelet activation was assessed with a phycoerytrin (PE) conjugated monoclonal antibody against P-selectin (CD62P; MA1-81632; Pierce). The reaction mixture was incubated in the dark at room temperature for 15 minutes. To assess non-specific association of proteins with platelets, diluted blood was added to control tubes with FITC-labeled and PE-labeled non-immune immunoglobulins. Platelet population was identified based on its forward and side scatter and the association with CD61 antibody. A total of 10,000 events were analyzed for percentage of CD62P positive platelets using Expo32 ADC XL 4 Color software. Fluorescence was measured with a Beckman Coulter Epics XL instrument.
Reticulated platelets quantification

2µl of citrate-anticoagulated whole blood were resuspended in 1ml of thiazole orange solution (Retic-Count®, Beckton Dickinson) and incubated for 30min at room temperature in the dark. As a negative control, 2µl of citrate-anticoagulated whole blood were resuspended in 1ml of PBS. Platelets were identified based on their logarithmic forward and side scatter. Fluorescence was measured with a Beckman Coulter Epics XL instrument and 15,000 events were analyzed for thiazole orange positive platelets using Expo32 ADC XL 4 Color software.

Platelet turnover analysis

Biotin N-Hydroxysuccinimide ester (biotin-NHS) was dissolved in dimethyl sulfoxide (40mg/mL) and 20µg/g were administered by the lateral tail vein. Blood samples were extracted 3h after biotin-NHS administration and daily for the following 6 days. Citrate-anticoagulated blood samples were diluted with Tyrode’s Buffer and 1x10⁶ platelets were incubated with PE-conjugated streptavidin (BD Pharmingen) and an antibody against CD61. Platelets were identified based on forward and side scatter and association with CD61. 10,000 events were analyzed for streptavidin positive platelets with a Beckman Coulter Epics XL instrument using Expo32 ADC XL 4 Color software.

Megakaryocyte ploidy

Bone marrow cells were isolated from femurs and tibias as previously described. Cells were fixed with 70% ethanol for 2h at 4°C and then washed with PBS. Fixed cells were then incubated with an antibody against CD61. Afterwards cells were resuspended in PBS containing 0.1% Triton X-100, 20µg/mL RNAse A, and 20µg/mL propidium iodide (PI) and incubated for 2h. Gate R1 (Figure 4.A) was created to analyze propidium iodide content in CD61 positive viable cells. 10,000
events were analyzed with a Beckman Coulter Epics XL instrument using Expo32 ADC XL 4 Color software.

Western blot analysis

Platelet protein extracts were obtained using a lysis buffer containing: 0.01M Tris-HCl, 0.15M KCl, 1µg/mL leupeptin, 3µg/mL aprotinin, and 2.9mM phenylmethylsulfonyl fluoride. Protein concentration was determined by the BCA protein assay. Samples containing 15µg of protein were subjected to SDS-PAGE. Detection was performed with a rabbit polyclonal antibody against TF (Santa Cruz Biotechnology), a goat polyclonal antibody against GRP78 (Santa Cruz Biotechnology), and a rabbit polyclonal antibody against PDI (Enzo Life Sciences). Band densities were determined with the ChemiDoc™ XRS system (Bio-Rad) in chemiluminescence detection mode and analyzed with Image Lab™ software (Bio-Rad). β-actin was used as a loading control.

Tissue factor procoagulant activity (TF-PCA) determination

TF procoagulant activity was measured in platelet poor plasma obtained from citrate anticoagulated blood by using a factor Xa generation test as previously described. Briefly, samples were added to a solution containing 100µM liposomes of phosphatidylserine (30%)-phosphatidylcholine (70%), 4nM factor VIIa and 5mM CaCl$_2$. The mixture was incubated for 15min and then 300nM factor X was added. After 15min of incubation, EDTA buffer (TBS, 0.1% BSA, 300mM EDTA, pH 7.5) was added to stop factor Xa production. Finally, a chromogenic substrate (factor Xa chromogenic substrate; Sigma; 25µL, 0.5mM), was added and absorbance was measured at 405nm for 15min using a kinetic ELISA reader at 37°C (SpectraMax).

Thromboelastometry

Citrate-anticoagulated blood was used to analyze dynamic whole blood clot formation with the ROTEM® coagulation analyzer (Pentapharm, Munich, Germany) using the
intrinsically activated assay (INTEM; containing 20μl CaCl2 0.2M, 20μl thromboplastin–phospholipid, and 300μL whole blood) and the extrinsically activated assay (EXTEM; containing 20μl CaCl2 0.2M, 20μl TF, and 300μl whole blood). Clotting time (CT; s), clot formation time (CFT; s), and maximum clot firmness (MCF; mm) were analyzed. Tests were performed using ROTEM® cups and pins. All reagents were purchased from Pentapharm GmbH (Munich, Germany).
**SUPPLEMENTAL TABLE**

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<th>PARAMETER</th>
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<td>ZC</td>
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**RBC (10^6/mm³)**

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<td>10.7±0.7‡</td>
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**HCT (%)**

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**PLT (10³/mm³)**

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**WBC (10³/mm³)**

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Supplemental Table. Hematological levels of wild type and transplanted animals.

* p<0.05 vs. ZD; † p<0.05 vs. ZC-BM/ZC; ‡ p<0.05 vs. ZC; § p<0.05 vs. ZC-BM/ZD.
Supplemental Figure. Bone marrow engraftment assessment. Femur sections of Wistar rats transplanted with Wistar-GFP bone marrow were analyzed by confocal microscopy 5 weeks after transplantation. Bone marrow cells of recipient rats were GFP\(^+\). 

A) Nuclei stained with Hoechst. B) GFP expression in marrow cells. C) Merge. Scale bar = 20µm.