Enhanced platelet activity and thrombosis in a murine model of type I diabetes are partially insulin-like growth factor 1-dependent and phosphoinositide 3-kinase-dependent

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Summary. Objectives: To determine whether dysregulation of platelet signaling mechanisms contributes to the increased risk of thrombosis associated with diabetes, using a type I diabetes mouse model. Methods and Results: Type I diabetes was induced in C57Bl6 mice following streptozotocin injection. Arterial thrombosis, platelet signaling and function were assessed 4 weeks later in comparison with non-diabetic control mice. Fifty-seven per cent of diabetic mice (glucose level of > 250 mg dL⁻¹) developed stable occlusive thrombi after FeCl₃ injury, as compared with 5% of their non-diabetic counterparts, suggesting that diabetic mice are more sensitive to arterial injury (P ≤ 0.02). Platelets from diabetic mice were more sensitive to protease-activated receptor 4 (PAR4) agonist-induced fibrinogen binding than platelets from non-diabetic mice, and the average Akt phosphorylation induced by PAR4 agonist peptide was greater (P ≤ 0.01) in platelets from diabetic mice. Recent studies suggest that insulin-like growth factor 1 (IGF-1) potentiates Akt phosphorylation in platelets. To determine whether IGF-1 signaling contributes to the increase in PAR4 sensitivity in platelets from diabetic mice, platelet signaling and function were evaluated in the presence of inhibitors of the IGF-1 receptor. IGF-1 receptor inhibition reduced Akt phosphorylation and fibrinogen binding in platelets from diabetic mice to levels consistent with those seen in normoglycemic platelets, but had no significant effect on platelets from non-diabetic mice. Conclusions: The results suggest that platelets from mice with streptozotocin-induced diabetes show enhanced platelet Akt phosphorylation and activity resulting from IGF-1-dependent mechanisms. Increases in platelet Akt activation may explain the enhanced sensitivity to thrombotic insult seen in diabetic mice.

Introduction

Persons with type I or type II diabetes are at increased risk of arterial thrombosis, manifested as myocardial infarction and coronary artery disease [1–3]. The causes are multifactorial, and may involve regulation of tissue factor expression [4] and plasminogen activator inhibitor-1 [5] (particularly in patients with type II diabetes), but part of this risk may be attributable to increased platelet activity. There is increasing evidence that platelets are hyperactivated in patients with both type I and type II diabetes, and platelet hyperactivity may therefore play a contributory role in the increased risk of arterial thrombosis associated with diabetes [3,6,7]. In addition, diabetic patients frequently show resistance to conventional antiplatelet drugs used as antithrombotic therapies, such as aspirin and clopidogrel [8]. Therefore, finding novel means to reduce cardiovascular risk that will maintain efficacy in diabetic populations is an important area of research. However, the mechanisms by which hyperglycemia or other diabetic sequelae contribute to arterial thrombosis remain poorly understood.

Mouse models are frequently used to assess factors affecting thrombosis in vivo [9]. Generally, injury-induced vascular occlusion of large or small vessels is measured, but these models have only infrequently been used to assess the impact of diabetes on thrombosis [10]. Accordingly, we assessed the impacts of streptozotocin-induced type I diabetes on thrombus formation in the carotid artery and on platelet function in vitro, so that we may begin to determine whether platelet-dependent mechanisms may contribute to enhanced thrombosis risk.

In this article, we describe enhanced thrombosis in a carotid artery injury model and enhanced platelet sensitivity to platelet agonists in a mouse streptozotocin-induced type I diabetes model. The causal events leading to
increased platelet activity under diabetic conditions are themselves multifactorial [1], but it is likely that dysregulated insulin and growth factor axes play a contributory role. Because we and others have previously reported that Akt contributes to platelet activation and thrombosis [11–13], and because Akt responses can be altered by hyperglycemia [14], and affect responses to insulin [15] and insulin-like growth factor 1 (IGF-1) [16,17], we hypothesized that dysregulation of platelet Akt activation because of hyperglycemia or dysregulated growth factor axes might contribute to enhanced platelet reactivity. We found enhanced sensitivity of platelet Akt phosphorylation to agonist and enhanced fibrinogen binding in platelets from diabetic mice relative to those from non-diabetic controls. We also found that the enhanced Akt phosphorylation found in platelets from diabetic mice was reduced to levels at or below those seen in non-diabetic control mice by inhibitors of the IGF-1 receptor (IGF-1R) or phosphoinositide 3-kinase (PI3K) isoforms. These results suggest that dysregulation of the IGF-1 signaling pathway, with consequent PI3K-dependent Akt activation, is at least partially responsible for the enhanced platelet activity of type I diabetic animals. Inhibition of target proteins along this pathway may be a feasible approach to limiting the risk of thrombosis in diabetic patients.

Materials and methods

Materials

Streptozotocin, IGF-1, Tyrode’s buffer and HEN reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). The following antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA). AYPGKF was synthesized by New England Peptide (Gardner, MA, USA).

Platelet preparation

All protocols and procedures involving mice were approved by the University of Delaware Institutional Animal Care and Use Committee. Blood was collected from the inferior vena cava of anesthetized mice (100 mg kg⁻¹ pentobarbital) in a 1-mL syringe containing 15 units of heparin at a ratio of 1 : 10. Anticoagulated blood was diluted 1 : 1 with Tyrode’s buffer (pH 7.4, 137 mM NaCl, 20 mM Hepes, 5.6 mM glucose, 1 g L⁻¹ bovine serum albumin, 1 mM MgCl₂, 2.7 mM KCl, 3.3 mM NaH₂PO₄) and treated with 0.1 U mL⁻¹ apyrase prior to centrifugation at 250 × g. Platelet-rich plasma (PRP) was isolated from the supernatant, and platelets were washed by diluting PRP 1 : 10 in HEN buffer (10 mM Hepes, pH 6.5, 1 mM EDTA, 150 mM NaCl) and centrifugation at 750 × g for 10 min. The platelet pellet was then resuspended in Tyrode’s buffer and 1 mM calcium prior to use. For fibrinogen binding studies, platelets were isolated from PRP by gel filtration over a Sepharose CL-2B (GE Healthcare, Mickleton, NJ, USA) column, as previously described [11].

Diabetic mouse model

Type I diabetes was induced in 5–6-week-old C57Bl/6 mice by five consecutive intraperitoneal injections of streptozotocin (50 mg kg⁻¹). Glucose levels were measured 4 weeks postinjection, and blood glucose levels of > 250 mg dL⁻¹ were considered to indicate diabetes. Age-matched non-diabetic control mice were housed under the same conditions, and had blood glucose levels of < 250 mg dL⁻¹.

FeCl₃-induced carotid artery thrombosis

The right carotid artery of an anesthetized adult mouse (9–11 weeks of age, 20–25 kg, anesthetized with 100 mg kg⁻¹ pentobarbital) was exposed to a filter paper strip saturated with 10% FeCl₃ for 2 min, and then rinsed with phosphate-buffered saline (PBS). The arterial flow rate was monitored with a Doppler flow probe for 30 min, as previously described [11]. Mice in which complete cessation of blood flow was recorded were scored as having stable occlusive thrombi. Those mice with unstable thrombi recovered at least 80% of the initial flow rate after cessation of blood flow within the 30-min assay time.

Immunoblotting

Washed platelets were resuspended in Tyrode’s buffer, and 100-μL aliquots containing 0.25 × 10⁶ platelets were stimulated with 50 μM AYPGKF for 5 min at 37 °C, and lysed by addition of 5 × Laemmli buffer. Lysates were resolved by 7.5% SDS-PAGE, and immunoblotted with antibody against phospho-Akt Ser473 or phospho-Akt Thr308 (Cell Signaling Technology, Beverly, MA, USA) at a 1 : 1000 dilution in LiCor blocking buffer. Membranes were washed in Tris-buffered saline-Tween, and
then incubated with anti-rabbit AlexaFluor 680 (Molecular Probes) secondary antibody for visualization on a LiCor Fluorescence Imager. Membranes were stripped with 1 × Re-Blot Plus solution (Millipore, Billerica, MA, USA) for 10 min at room temperature, blocked as described above, and incubated with antibody against pan-actin (Cell Signaling, Boston, MA, USA) to verify equal loading.

Immunoprecipitation

Washed mouse platelets (8 × 10^8 platelets mL^−1) were treated for 5 min with or without IGF-1 at 37 °C, and then lysed by addition of 2 × IP buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM Na3VO4, 5 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, pH 7.4) plus protease inhibitor cocktail (Sigma-Aldrich), and rotated at 4 °C for 30 min. Antibodies or control IgG were added to lysates (2 μg per sample), and rotation was performed at 4 °C overnight; this was followed by incubation with 15 μL mL^−1 protein A/G-agarose at 4 °C for 2 h. Samples were washed with 1 × IP buffer three times, and applied in Laemmli buffer to a 10% SDS-PAGE gel for immunoblotting.

Fibrinogen binding/flow cytometry

Fibrinogen binding to the platelet surface was measured by incubating 0.4 × 10^8 platelets mL^−1 simultaneously with 25 μg mL^−1 fibrinogen–AlexaFluor 488 and the indicated concentration of the agonist peptide AYPGKF for 10 min at 37 °C. Samples were fixed with 1% paraformaldehyde, diluted 1 : 5 with PBS, and evaluated on a FACS Coulter XL. Quantification was performed similarly (in the absence of agonist), but platelets were incubated with rat FITC-conjugated anti-mouse CD41 (BD Pharmingen).

IGF-1 and insulin ELISA

IGF-1 was measured with an IGF-1 mouse Quantikine ELISA kit (R&D Systems), and insulin was measured with an anti-mouse insulin kit (EMDMillipore). Samples were collected by drawing 0.8–1 mL of blood per mouse into heparin, as described for platelet isolation. Blood plasma samples were diluted 500-fold with calibrator diluent, and assayed immediately. The optical density was measured on a BioRad 680 microplate reader (BioRad, Hercules, CA, USA), and was compared with a standard curve for each set of samples assayed.

Complete blood counts (CBCs)

CBCs were obtained by retro-orbital puncture to collect blood into heparinized capillary tubes, and blood was immediately analyzed with a Hemvet950 (Drew Scientific, Waterbury, CT, USA).

Results

Persons with type I diabetes are at increased risk of arterial thrombosis, but there is sparse evidence from animal models that diabetic conditions exacerbate thrombosis following arterial injury [10]. Furthermore, the mechanisms contributing to thrombotic risk under diabetic conditions remain incompletely defined. Accordingly, we assessed the impact of streptozotocin-induced type I diabetes on thrombus formation in the carotid artery and on platelet function in vitro, so that we may begin to determine whether platelet-dependent mechanisms may contribute to enhanced thrombosis risk. To determine whether mice showing symptoms of type I diabetes have increased susceptibility to arterial thrombosis, diabetes was induced in C57/B16 mice following five consecutive days of streptozotocin injection, and sensitivity to arterial injury-induced thrombosis was assessed 4 weeks later in comparison with age-matched control mice. Glucose concentrations were assessed in the mice prior to thrombosis assay. All mice injected with streptozotocin developed hyperglycemia, defined here as a blood glucose level of > 250 mg mL^−1. Diabetic mice also developed low insulin concentrations (Fig. S1), as expected for this induced type I diabetes model. Age-matched control mice housed under the same conditions were normoglycemic (defined here as a glucose level of <250 mg mL^−1). Thrombus formation was induced by FeCl3-induced injury of the carotid artery, and occlusion was measured with a Doppler flow probe. When thrombotic insult was induced under the same conditions, as shown in Fig. 1A, only 5% of non-diabetic mice developed complete stable occlusions of the artery, whereas 58% of the mice with type I diabetes did so. Some thrombi formed after injury were unstable and dissipated prior to the end of the 30-min assay time; these were characterized as unstable thrombi. Only 28% of all non-diabetic mice formed thrombi, 22% of which were unstable, whereas 64% of all diabetic mice assayed developed thrombi, the majority of which were stable. In Fig. 1B, the plasma glucose concentrations of the mice are plotted for those forming no occlusive thrombi, unstable thrombi, or stable occlusive thrombi. The glucose concentration of mice forming no thrombi differed significantly from that of those forming stable occlusions (P = 0.0035, two-tailed unpaired t-test), although there was no significant difference between the glucose concentration of mice forming unstable thrombi and that of those forming no thrombi. Thus, we can conclude that mice with streptozotocin-induced type I diabetes are more sensitive to the formation of stable arterial thrombi than their non-diabetic counterparts.

To determine whether the platelets from diabetic mice were more reactive to agonist than those from non-diabetic controls, we compared thrombin-dependent fibrinogen binding to surface αIIbβ3 integrin in platelets from both groups of mice. Platelets from diabetic mice were
Diabetic mice are more susceptible to occlusive thrombus formation (diabetic mice, unstable thrombi or stable occlusive thrombi). The number of mice forming no thrombi, unstable thrombi or stable occlusive thrombi were plotted for diabetic vs. non-diabetic mice (A) and as a function of glucose concentration (B). Diabetic mice are more susceptible to occlusive thrombus formation (diabetic mice, $n = 14$; non-diabetic controls, $n = 18$; $P \leq 0.05$, unpaired, two-tailed Student’s $t$-test). (C) Gel-filtered platelets (0.4 × 10⁷ platelets mL⁻¹) were incubated with increasing protease-activated receptor 4 (PAR4) agonist (AYPEGKF) concentrations for 10 min at 37 °C and fixed, and the mean fluorescence intensity (MFI) of bound fibrinogen (AlexaFluor 488) was measured by flow cytometry. Responses to PAR4 agonist were greater in platelets from diabetic mice ($EC_{50} = 39.55 \mu M$) than in those from non-diabetic mice ($EC_{50} = 107.5 \mu M$) (unpaired two-tailed Student’s $t$-test: *$P \leq 0.05$, **$P \leq 0.005$. (Data shown are mean ± standard error of the mean, $n = 9$).

**Fig. 1.** Mice with type 1 diabetes are more sensitive to injury-induced arterial thrombus formation in vivo, and platelets from diabetic mice are more sensitive to agonist-induced fibrinogen binding in vitro. (A, B) Carotid arteries of pentobarbital-anesthetized diabetic and non-diabetic mice were exposed to 10% FeCl₃ for 2 min. The flow rate was monitored for 30 min postinjury, and thrombi were scored as stable (■), unstable (hatched bars), or no occlusion (○). The number of mice forming no thrombi, unstable thrombi or stable occlusive thrombi were plotted for diabetic vs. non-diabetic mice (A) and as a function of glucose concentration (B). Diabetic mice are more susceptible to occlusive thrombus formation (diabetic mice, $n = 14$; non-diabetic controls, $n = 18$; $P \leq 0.05$, unpaired, two-tailed Student’s $t$-test). (C) Gel-filtered platelets (0.4 × 10⁷ platelets mL⁻¹) were incubated with increasing protease-activated receptor 4 (PAR4) agonist (AYPEGKF) concentrations for 10 min at 37 °C and fixed, and the mean fluorescence intensity (MFI) of bound fibrinogen (AlexaFluor 488) was measured by flow cytometry. Responses to PAR4 agonist were greater in platelets from diabetic mice ($EC_{50} = 39.55 \mu M$) than in those from non-diabetic mice ($EC_{50} = 107.5 \mu M$) (unpaired two-tailed Student’s $t$-test: *$P \leq 0.05$, **$P \leq 0.005$. (Data shown are mean ± standard error of the mean, $n = 9$).

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hypersensitivity of platelets isolated from hyperglycemic mice was attenuated with inhibitors of either PI3Kα or PI3Kβ, the platelets were still capable of agonist-induced fibrinogen binding. Thus, PI3K inhibition in platelets may be a viable way to reduce thrombosis caused by platelet hyperactivation, while allowing agonist-induced responses to remain viable for hemostatic needs.

It has been reported that activation of IGF-IR can contribute to enhanced Akt phosphorylation in platelets and that PI3Kα contributes more selectively to the potentiation of Akt phosphorylation induced by IGF-I than PI3Kβ. To determine whether an IGF-IR-dependent pathway may contribute to the enhanced Akt response in platelets from the diabetic mice, Akt phosphorylation and fibrinogen binding were evaluated in platelets isolated from diabetic and non-diabetic mice in the presence and absence of either pharmacologic inhibitors of IGF-IR (PPP, AG1024) or anti-IGF-IR antibody (α-IR3). PAR4-stimulated Akt phosphorylation in diabetic mice was reduced by inhibition of IGF-IR (α-IR3, PPP, and AG1024; Fig. 5A,B); however, Akt phosphorylation in non-diabetic platelets was not significantly inhibited by IGF-IR or antibody. These results suggest that IGF-IR-dependent pathways contribute to Akt activation in platelets exposed to diabetic conditions. Platelet fibrinogen binding was reduced in both normoglycemic and hyperglycemic mice by incubation with IGF-IR inhibitor, suggesting that IGF-I may reinforce platelet signaling even under normal conditions, but that this effect is more pronounced under diabetic conditions (PPP; Fig. 5C). Similar results were obtained with AG1024 (additional data not...
shown). Consistent with this hypothesis, exogenous application of IGF-1 to non-diabetic platelets potentiated PAR4-induced fibrinogen binding to a level that was non-significantly different from that of diabetic mice (Fig. S2B).

These results, taken together, may suggest that platelets from diabetic mice are hypersensitive to IGF-1, as has been reported for other cells under diabetic conditions [18-20]. To evaluate this, washed platelets from diabetic and non-diabetic mice were exposed to varying concentrations of IGF-1, and Akt phosphorylation was assessed by immunoblot. The results indicated that platelets from the diabetic mice were more sensitive to IGF-1-stimulated Akt phosphorylation (Fig. 6A,B). In addition, we detected a constitutive level of IGF-1R phosphorylation in platelets from diabetic mice and an increased sensitivity to IGF-1, suggesting that potentially high concentrations of circulating IGF-1 had preactivated this receptor in diabetic mice (Fig. 6C). To determine whether IGF-1 can potentiate Akt phosphorylation in AYPGKF-stimulated platelets, we assessed Akt phosphorylation stimulated by AYPGKF, IGF-1 or both together in both diabetic and control mice. We found that IGF-1 potentiated AYPGKF-stimulated Akt phosphorylation in control mice (Fig. 7A).
mice to a level that was not significantly different from that seen in diabetic mice stimulated with AYPGKF alone (Fig. 6D,E). In addition, IGF-1-dependent Akt phosphorylation was blocked by the inhibitors PPP or AG1024 (Fig. 6F), suggesting that the reduction in AYPGKF-dependent Akt phosphorylation caused by these inhibitors was, indeed, attributable to blocking of IGF-1-dependent signaling. Increased levels of phospho-Src after IGF-1 stimulation were also detected in diabetic mice (Fig. S3). However, increased levels of IGF-1R did not appear to be responsible for the increased IGF-1 sensitivity, because no differences in the expression level of IGF-1R were detected between diabetic and non-diabetic mice (Fig. S4). Because diabetic platelets seem to have an increased level of IGF-1R phosphorylation even in the absence of exogenous stimulus, we considered that the diabetic mice in this model may have had more circulating IGF-1. To address this, plasma levels of IGF-1 were assessed in both sets of mice. Circulating levels of IGF-1 were significantly higher in mice with streptozotocin-induced diabetes (Fig. 7A). In addition, the serum levels of IGF-1 correlated significantly with plasma glucose concentrations (Fig. 7B). Taken together, these results may suggest that platelets become hyper-reactive under diabetic conditions, at least in part because of dysregulation of IGF-1 levels and signaling responses, resulting in enhanced responses to PI3K-dependent fibrinogen binding in platelets. Thus, inhibition of platelet IGF-1Rs or PI3Ks may be efficacious for reducing thrombosis under diabetic conditions.

Discussion

The increased risk of cardiovascular disease associated with type I and type II diabetes is multifactorial: part of the risk is attributable to dysregulation of circulating coagulation factors and fibrinolysis, and another part to increased platelet activity. Multiple studies have
demonstrated that markers of platelet activation are elevated in patients with type I diabetes and in those with type II diabetes [6,7]. The causal events leading to increased platelet activity under diabetic conditions are themselves multifactorial [1], and may involve dysregulated cholesterol composition in platelet membranes [21], hyperglycemia-dependent regulation of mean platelet volume [6] and advanced glycation endproducts [10], dysregulated calcium regulation (e.g. calpain [22]/calsequestrin regulation [23], and dysregulated insulin and growth factor axes [24].

The effects of insulin, IGF-I and growth hormone (GH) on platelet activation are just beginning to be elucidated. Insulin action, in particular, is the subject of some controversy: Insulin was proposed by Ferreira et al. [25] to dampen platelet activation through the sequestration of platelet-activating Gαi family members. However, more recently, Hunter and Hers reported that insulin potentiated responses to classic platelet agonists through its interaction with heterodimerized insulin receptors/IGF-1Rs [26]. IGF-1 alone is insufficient to induce platelet aggregation, but potentiates platelet responses to classic agonists [27,28]. GH has also been shown to have a potentiating effect on platelet activation stimulated by classic agonists [29]. Both IGF-1 and GH stimulate Akt phosphorylation [30] and potentiate platelet activation [27,29]. Taking these findings together with evidence that deletion or inhibition of Akt reduces platelet activation and thrombosis [11–13], it is likely that the ability of these hormones to enhance platelet activity is attributable to activation of Akt-dependent pathways in platelets. Thus, we hypothesized that dysregulation of the IGF-1–GH axis in diabetic subjects may trigger enhanced platelet activity through enhanced stimulation of platelet Akt. If this is the case, as our data suggest, then inhibiting these pathways in platelets may be a viable solution for reducing the cardiovascular risk associated with diabetes.

Thrombosis occurs more frequently in patients with type I diabetes and in those with type II diabetes than in the general population, but there is evidence that patients with type I diabetes experience poorer outcomes after receiving drug-eluting stents than patients with type II diabetes [31]. Thus, in this study, we addressed the mechanism contributing to high platelet reactivity and thrombosis in a drug-induced mouse model of type I diabetes. In an FeCl3-induced arterial injury model, thrombosis was evident in twice as many diabetic mice as normoglycemic mice (Fig. 1A). Platelets from diabetic mice were also more sensitive to the ability of PAR4 peptide (a thrombin mimetic activator of the thrombin receptor in mice) to induce fibrinogen binding (Fig 1B) and Akt phosphorylation (Fig. 2). These results are consistent with those of a prior study demonstrating that platelets from diabetic pigs were hypersensitive to stimulation with thrombin [32].

Akt activation in platelets, as in other cells, is largely PI3K-dependent. However, different agonists use different PI3K isoforms to effect Akt activation. Specifically, thrombin, thromboxane A2 and collagen appear to induce Akt activation predominantly through PI3Kβ [33]. In contrast, ADP requires both PI3Kβ and PI3Kγ to attain maximal Akt phosphorylation [34,35]. Finally, a number of soluble factors that potentiates platelet aggregation stimulate Akt phosphorylation by activating PI3Kα. These include IGF-1 [27], CD40L [27], and Gas6 [36]. Each of these is incapable of inducing platelet aggregation on its own, but potentiates platelet aggregation in response to classic agonists such as ADP and thrombin. At least part of the mechanism is presumably attributable to Akt activation, as loss of any of the three mammalian isoforms of Akt reduces platelet aggregation in response to multiple agonists in vitro and reduces thrombosis in vivo [11–13]. Thus, interfering with pathways that are specifically elevated under diabetic conditions may limit prothrombotic consequences, while leaving hemostatic
function intact. In particular, our results suggest that platelet Akt phosphorylation is elevated in a mouse model of streptozotocin-induced diabetes, and that the elevation in Akt phosphorylation, as well as associated increases in fibrinogen binding to the platelets, is reduced by inhibitors of PI3Kα or IGF-1R (Figs. 3–5). These data are consistent with those of Hers, who showed that inhibition of PI3Kα reduced IGF-1-potiated increases in platelet aggregation, but not aggregation stimulated by thrombin receptor agonists alone [27] (see also [28] for IGF-1-stimulated potentiation of the epinephrine response). In contrast, Akt activation stimulated by thrombin, collagen or other platelet agonists takes place predominantly through activation of PI3Kβ (with some contribution from PI3Kγ, particularly for ADP-dependent stimulation) [33,35,37].

These data imply that, in this diabetic model, either IGF-1 is elevated or platelet responses to this growth factor are elevated. In fact, our data show that both effects appear to be true (Figs. 6 and 7). The elevation that we observed in IGF-1 levels is not consistently seen in human diabetic patients, although, interestingly, a recent study observed a significant correlation of high concentrations of IGF-1 (above the 90th percentile) with risk of incident diabetes mellitus [38]. Recent studies have also shown that hyperglycemia can enhance IGF-1-mediated signaling responses, including Src phosphorylation, which is consistent with our results (Fig. S3) [39]. Studies evaluating vascular responses to IGF-1 in human diabetic patients are somewhat variable: some have demonstrated increased sensitivity to IGF-1 signaling in some of the vascular complications associated with diabetes, including diabetic retinopathy and renal hypertrophy [20,40,41], whereas other studies have shown that high levels of IGF-1 inversely correlate with cardiovascular disease and diabetes, leading to a few suggestions that it may be cardioprotective [42]. However, administration of IGF-1 gave mixed results in various models of heart failure or reperfusion injury [43–45], and a more recent study suggests that cardioprotective agents that raise the level of IGF-1 afford their protection through IGF-1-independent mechanisms [46]. The conflicting data may suggest that IGF-1, like Akt activation itself, has cell-type-specific effects that contribute to different aspects of cardiovascular disease. In the heart, for example, activation of Akt is cardiomyocyte-protective [47], and deletion of Akt1 exacerbates ischemic injury and peripheral vascular disease in mouse models [48]. Conversely, deletion of Akt isoforms is protective in assays of acute thrombosis after arterial injury—a platelet-dependent event [11,13]. Thus, inhibiting IGF-1R-dependent stimulation of PI3K and Akt specifically in platelets may be of particular benefit for limiting thrombosis in the context of diabetes. Future studies will explore the role of platelet-specific inhibition or deletion of IGF-1/PI3K-dependent increases in thrombosis.

Disclosure of Conflict of Interests

This work was supported by R01 HL081241 and R01 HL106009 (to D. S. Woulfe). The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Plasma glucose and insulin levels were measured in mice induced to develop diabetes following injection with streptozotocin (open circles) and in untreated mice.

Figure S2. Gel-filtered platelets (0.4 × 10⁸ platelets mL⁻¹) were incubated with a combination of PI3Kα (0.6 μM PIK-75) and PI3Kβ (1 μM TGX-221) inhibitors for 10 min at room temperature.

Figure S3. Platelets from diabetic or non-diabetic mice were incubated with the indicated concentrations of AY-PGKf and IGF-1⁻¹⁻¹, a combination of PPP (120 nm) and AG1024 (100 μM).

Figure S4. IGF-1R expression was assessed by flow cytometry in platelets from diabetic and non-diabetic mice.

References


