Journal of Thrombosis and Haemostasis, **3**: 2149–2153

IN FOCUS

Pioglitazone protects against thrombosis in a mouse model of obesity and insulin resistance

P. F. BODARY, F. B. VARGAS, S. A. D. KING, K. L. JONGEWARD, K. J. WICKENHEISER and D. T. EITZMAN

Department of Internal Medicine, Division of Cardiovascular Medicine, University of Michigan Medical Center, Ann Arbor, MI, USA

To cite this article: Bodary PF, Vargas FB, King SAD, Jongeward KL, Wickenheiser KJ, Eitzman DT. Pioglitazone protects against thrombosis in a mouse model of obesity and insulin resistance. *J Thromb Haemost* 2005; **3**: 2149–53.

See also Smyth SS, Jennings JL. PPARc agonists: a new strategy for antithrombotic therapy. This issue, pp 2147–8.

Summary. *Background:* As arterial thrombosis accounts for the vast majority of cardiovascular complications in obese, insulin resistant patients, we hypothesized that improving insulin sensitivity may be effective in reducing the thrombotic response following vascular injury. *Objectives:* We investigated the effect of the thiazolidinedione drug, pioglitazone, on the thrombotic response to injury in obese, insulin resistant mice. *Methods:* Insulin-resistant, obesity-prone mice (KK strain) were treated with pioglitazone, placebo, or the sulfonylurea compound, glipizide, for 2.5 weeks and then subjected to photochemical injury of the carotid artery. *Results:* KK mice have a significant increase in adiposity (7 weeks: 25.6%; 15 weeks: 34.4%; $P < 0.0001$) and thrombotic tendency (7 weeks: 21.2 ± 1.9 min; 15 weeks: 13.7 ± 1.7 min; $P < 0.01$) with age. Pioglitazone provided significant protection from thrombosis at both time points, prolonging the time to occlusive thrombosis by 40% and 68%, at 7 and 15 weeks of age, respectively ($P < 0.05$). Similarly, following a diet-challenge to promote diabetes, pioglitazone provided protection from occlusive thrombus formation (Placebo: 11.3 ± 1.0 min; Pioglitazone: 22.3 ± 3.9 min; $P < 0.05$). However, despite a salient effect of glipizide on the hyperglycemia of the mice, there was no effect on the time to occlusive thrombus formation (13.2 ± 0.9 min, $n = 4$) compared with placebo-treated mice. The pioglitazone protection was paralleled by significantly lower soluble P-selectin and platelet P-selectin expression providing evidence of an antiplatelet effect. *Conclusions:* We conclude that pioglitazone treatment provides protection against arterial thrombosis in an obese, insulin resistant, prothrombotic mouse model.

Keywords: coagulation, diabetes mellitus, obesity, platelet, syndrome X.

Correspondence: Peter F. Bodary, 1150 W. Medical Center Dr., 7301 MSRB III, Ann Arbor, MI 48109-0644, USA.
Tel.: +734 615 7916; fax: +734 936 2641; e-mail: pfbodary@umich.edu

Received 5 August 2004, accepted 1 June 2005

Introduction

Obese and diabetic patients are at increased risk of cardiovascular thrombotic complications such as myocardial infarction and stroke [1,2]. The underlying mechanisms responsible for this increased risk are incompletely understood. However, it appears that therapies such as insulin and sulfonylurea compounds, which normalize hyperglycemia without directly influencing the underlying insulin resistance, may not effectively reduce macrovascular complications [3,4].

The thiazolidinedione (TZD) drug class directly improves insulin sensitivity in part by affecting the adipocyte gene expression profile [5]. This therapy may have a unique benefit on cardiovascular outcomes in obese and diabetic populations. Because the complications of cardiovascular disease are primarily due to thrombosis at sites of endothelial damage, we hypothesized that TZD treatment may be useful in preventing the thrombotic response that occurs following vascular injury.

Methods

Experimental animals

Four- to eight-week-old male KK mice (KKHI/J) were purchased from Jackson Laboratory, Bar Harbor, Maine, USA. Two separate groups of mice were studied to examine the effects of age and diet on the thrombotic response to treatment with the TZD-drug, pioglitazone. The first group examined the thrombotic tendency of 7- and 15-week-old mice provided standard rodent chow, with and without pioglitazone. A second group of mice were evaluated at 15 weeks of age following a dietary challenge with a high fat (35.5% w/w) and sucrose (~36% w/w) diet (#1850, Bioserve Corp., Frenchtown, NJ, USA) from 11 to 13 weeks of age prior to the TZD treatment period. All *in vivo* experiments were performed using 8–11 mice per group. All mice were maintained in specific pathogen-free (SPF) facilities. All animal care and experimental procedures complied with the Principles of Laboratory and Animal Care established by the National Society for Medical

Research and were approved by the University of Michigan Committee on Use and Care of Animals.

Drug treatment

Pioglitazone was added to standard rodent chow (Laboratory Rodent Diet #5001, TestDiet, Richmond, IN, USA) as an admixture (0.012%, Takeda Chemical Industries, Osaka, Japan). To examine the TZD-independent effects of improved glycemic control on thrombosis, the dietary challenge experiment was also performed using an admixture of the sulfonylurea drug, glipizide, at a dose of $\sim$1 mg kg^{-1} day^{-1} [6].

Arterial thrombosis

The response to photochemical injury was determined in all mice 2.5 weeks following the initiation of treatment, as previously described [7]. Briefly, a photochemical (Rose Bengal, Fisher Scientific, Pittsburg, PA, USA) was injected into the circulation via a tail vein and a green laser light source was directed to the right carotid artery. The green light activates Rose Bengal leading to formation of reactive oxygen species with subsequent endothelial damage and thrombosis. The dose of the photochemical was reduced by 25% (to 37.5 mg kg^{-1}) for the KK mice because preliminary experiments (with the 50 mg kg^{-1} dose) in this mouse strain resulted in the formation of occlusive thrombi in $<$12 min. Carotid blood flow was monitored using a microcirculation probe (model 0.5 V, transonic systems) interfaced with a T106 flowmeter (Transonic Systems Inc, Ithaca, NY, USA). Vascular occlusion was defined as zero flow for at least 1 min at which time the experiment was ended and a terminal bleed was performed for blood chemistry analyses.

Blood chemistry analyses

Glucose concentration was measured using an automated glucometer (Glucometer Elite XL, Bayer Corporation, Elkhart, IN, USA). Plasma insulin and adiponectin were measured via commercially available enzyme-linked immunosorbent assay (ELISA) kits (#90060; Crystal Chem Inc., Downers Grove, IL, USA and #K1002-1; B-Bridge International, Sunnyvale, CA, USA). Plasma PAI-1 antigen was determined using the Total Murine PAI-1 antigen assay (Molecular Innovations, Southfield, MI, USA).

Whole-body adiposity

A PIXImus2 Mouse Densitometer (GE Medical Systems, Madison, WI, USA) was used to determine the adiposity of mice at the conclusion of each study.

Platelet activation measures

Soluble P-selectin was measured from serum using a commercially available ELISA kit (R&D Systems, Inc, Minneapolis, MN, USA). Platelet expression of P-selectin was determined by flow cytometry (FACScalibur, BD Biosciences, San Jose, CA, USA) on isolated platelets incubated for 10 min at 37 °C using a commercially available FITC-conjugated rat antimouse CD62P monoclonal antibody (Cat #553744; BD Biosciences). The platelet isolation and flow-cytometry procedure was performed as previously described [8]. The platelet P-selectin expression was determined in the resting (i.e. unstimulated) state.

Statistical analysis

For the comparison of 7- and 15-week-old mice, with and without pioglitazone, a two-way ANOVA on Ranks (non-normal distribution) was performed to examine the effects of age and treatment. For the comparison of the diet-challenged mice, a one-way ANOVA on Ranks was used with a Dunn's *post hoc* test to determine differences among placebo, pioglitazone, and glipizide-treated mice. All other comparisons were made using a student's *t*-test. $P < 0.05$ was considered statistically significant. All statistical procedures were performed using SigmaStat for Windows 3.0 (SyStat Software Inc., Point Richmond, CA, USA). Values are expressed as mean $\pm$ SEM.

Results

Effective delivery of pioglitazone

To provide evidence of an effective dosing regimen in the insulin resistant KK mice utilized for these experiments, we determined the fasting plasma insulin and glucose levels following 2 weeks of pioglitazone treatment. As expected, pioglitazone treatment resulted in significantly lower levels of fasting insulin concentration compared with placebo in the 15-week-old mice fed standard chow (Placebo: 1.2 $\pm$ 0.2; Pio: 0.6 $\pm$ 0.1 ng mL^{-1}; $P = 0.028$). In addition, there was a lower mean fasting glucose concentration in the pioglitazone-treated 15-week-old mice compared with placebo, although this did not reach statistical significance (Placebo: 181 $\pm$ 11 mg mL^{-1}; Pio: 154 $\pm$ 10 mg mL^{-1}; $P = 0.09$). The fasting blood glucose levels were elevated in 15-week-old mice compared with 7-week-old mice (7 week placebo: 140 $\pm$ 7.4; 15 week placebo: 181 $\pm$ 11 mg dL^{-1}; $P = 0.009$) and 7-week-old mice had no change with pioglitazone treatment (7 week pioglitazone: 159 $\pm$ 11 mg dL^{-1}). Fasting insulin concentration was significantly improved in the 7-week-old mice receiving pioglitazone (Pre: 2.6 $\pm$ 0.5 ng mL^{-1}; Post: 1.2 $\pm$ 0.4 ng mL^{-1}) while placebo mice were mostly unchanged (Pre: 1.7 $\pm$ 0.1 ng mL^{-1}; Post: 1.8 $\pm$ 0.3 ng mL^{-1}) during the same period.

Effects of age and pioglitazone on thrombosis in KK mice

KK mice are known to have increasing obesity and insulin resistance with age. We observed marked differences in adiposity via DEXA measurement between these two ages

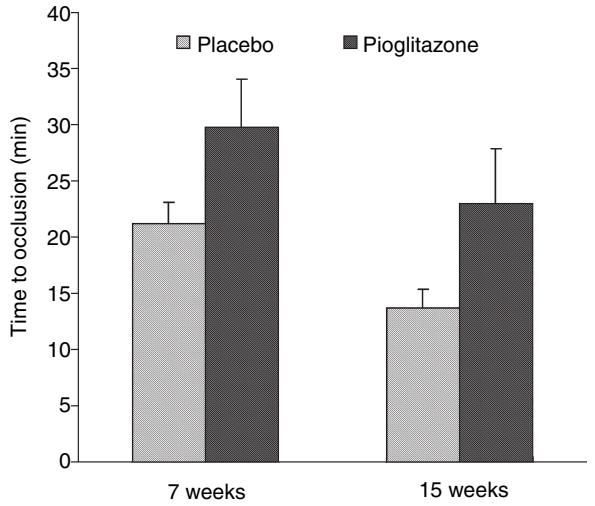

Fig. 1. Effect of pioglitazone or placebo treatment on the time to occlusive thrombus formation for 7- and 15-week-old mice (n = 8-11/group) fed standard rodent chow. Significant main effects for both pioglitazone treatment and age were evident ($P < 0.05$).

(7 weeks: 25.6%; 15 weeks: 34.4%; $P < 0.0001$). Concomitant with this change in age and fatness, we observed a significant increase in the thrombotic tendency whereby 15-week-old mice formed an occlusive thrombus approximately twice as fast as 7-week-old mice (Fig. 1). Interestingly, pioglitazone provided protection to both 7- and 15-week-old mice resulting in 40% and 68% longer times to occlusion for each group, respectively (Fig. 1).

Comparison of pioglitazone and glipizide effects on photochemical-induced thrombosis in diet-challenged KK mice

Previous investigators have demonstrated that dietary fat enhances the diabetes of KK mice. Therefore, to further investigate the effect of pioglitazone on thrombosis in a more clinically relevant state, we provided 2 weeks of high fat chow to the mice prior to the treatment of pioglitazone. This dietary challenge resulted in significantly increased adiposity (challenged: 38.9 ± 1.4% fat; standard chow: 34.4 ± 0.2% fat; $P = 0.001$) and higher fasting insulin levels (challenged: 3.9 ± 1.0 ng mL^{-1}; standard chow: 1.2 ± 0.2 ng mL^{-1}; $P = 0.02$) than standard chow fed mice. In addition, using the diet-challenged mice, we compared the effects of the sulfonylurea compound, glipizide, to that of placebo and pioglitazone-treated mice. The glipizide-treated mice had a significant reduction in fasting glucose compared with placebo mice but no significant effect on fasting insulin levels (Fig. 2). The pioglitazone-treated mice had a significant improvement in fasting insulin but only a trend toward a decrease in fasting glucose (Fig. 2). No statistically significant differences were evident between the glucose levels of the pioglitazone and glipizide-treated mice. Consistent with the effects of pioglitazone on thrombosis observed in 7- and 15-week-old mice on normal chow, the diet-challenged mice provided pioglitazone also had a 65% prolongation in the time to arterial thrombosis compared with placebo mice (Fig. 3). Interestingly, despite the salient effects of the glipizide treatment on fasting glucose concentration, there was no significant benefit on arterial thrombosis compared with placebo-treated mice (Fig. 3).

Pioglitazone effects on measures of platelet activation

To examine the effect of pioglitazone on platelet activation in this mouse model of insulin resistance, we determined the concentration of soluble P-selectin in the serum of placebo and pioglitazone-treated mice following a dietary challenge. Following just 1 week of treatment, the soluble P-selectin concentration was significantly lower in pioglitazone-treated mice compared with the placebo group (Fig. 4A). Because soluble-P-selectin may also result from activated endothelial cells, we examined the effect of pioglitazone treatment on platelet P-selectin expression via flow cytometry of washed platelets isolated from placebo and pioglitazone-treated mice. Using this methodology, we determined that the P-selectin expression of resting platelets was significantly lower in pioglitazone-treated compared with placebo-treated mice, demonstrating that *in vivo* platelet activation was attenuated by TZD treatment (Fig. 4B).

Additional measures

Plasma PAI-1 levels were determined in a separate group of diet-challenged mice but no difference was found between mice with 2 weeks of pioglitazone treatment and the placebo

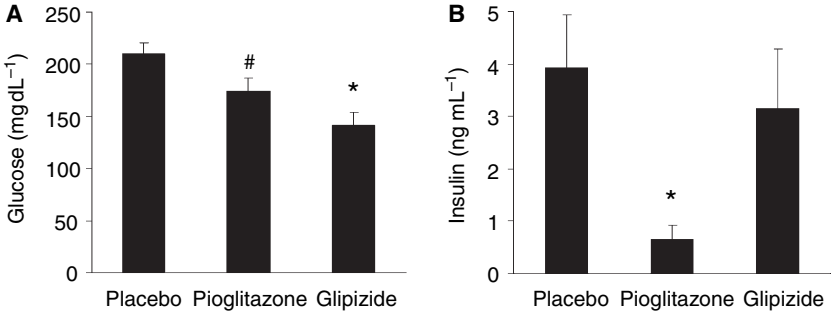

Fig. 2. Plasma was collected following a 5 h fast to determine the effect of placebo, piogltizone, and glipizide treatment on insulin (A) and glucose (B) concentration following 2 weeks of drug treatment. *$P < 0.05$; #$P = 0.09$.

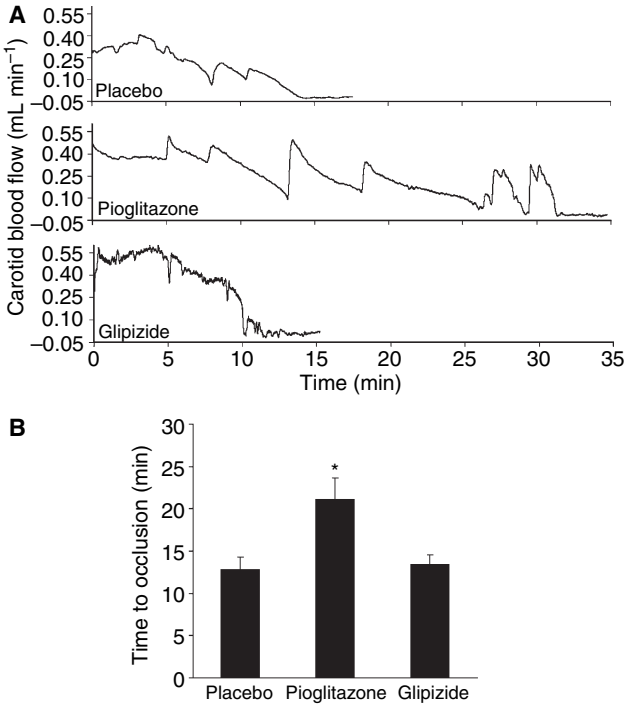

Fig. 3. (A) Representative flow tracings during the photochemical injury thrombosis experiment from diet-challenged, 15-week-old mice treated with placebo, pioglitazone, or glipizide. (B) The mean thrombotic response to photochemical injury from 15-week-old diet-challenged mice following 2 weeks of placebo ($n = 11$), pioglitazone ($n = 9$) or glipizide ($n = 9$) treatment.

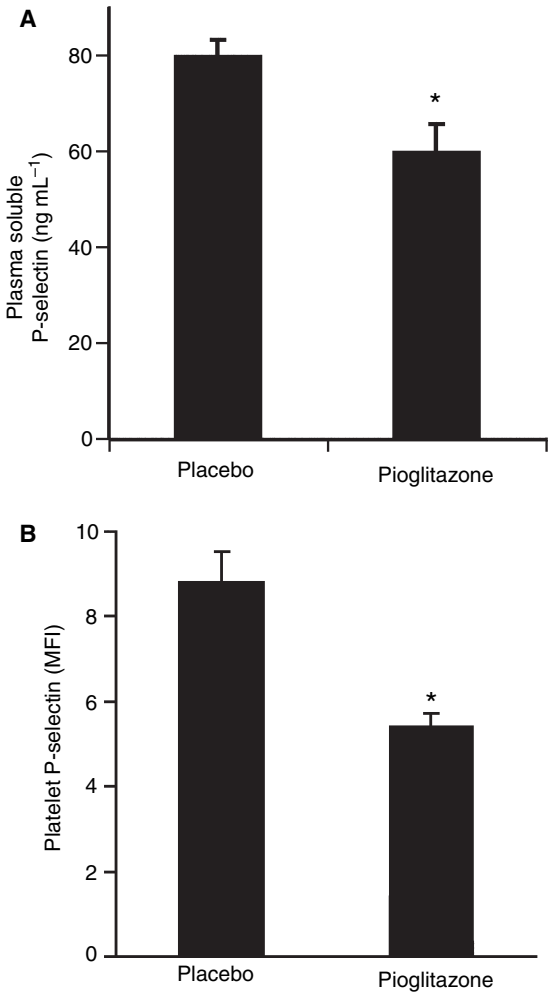

Fig. 4. Effect of placebo or pioglitazone treatment on (A) platelet P-selectin expression ($n = 3$ per group) and (B) soluble P-selectin concentration ($n = 4$ per group). MIF = mean immunofluorescence.

mice (Pioglitazone: 1.0 ± 0.1 ng mL^{-1}; Control: 0.7 ± 0.3 ng mL^{-1}; P = NS). Basal blood flow was assessed in 15-week-old pioglitazone and placebo mice immediately prior to the photochemical injury. Although blood flow in both groups was within the normal range of what we have previously observed in C57BL/6 mice [9], in the current study a significantly higher blood flow was observed in the pioglitazone-treated mice compared with the placebo group (Placebo: 0.39 ± 0.03 mL min^{-1}; Pioglitazone: 0.50 ± 0.03 mL min^{-1}; $P = 0.023$). The baseline blood flow of mice was not associated with the time to occlusive thrombosis ($R^2 = 0.021$).

Discussion

Thrombosis plays a critical role in acute coronary syndromes and stroke [10]. The model of arterial thrombosis used in this study involves endothelial damage mediated by toxic reactive oxygen species, a type of injury that may be common in patients with risk factors for cardiovascular disease [11]. We have previously shown that this photochemical-induced endothelial injury model is sensitive to genetic alterations in fibrinolytic, coagulative, and platelet factors [12–15]. The KK mouse strain used in this study is genetically prone to obesity, insulin resistance and diabetes [16]. We now show that this strain also has an age- and adiposity-associated susceptibility to thrombosis following vascular injury. This is the first study to demonstrate enhanced thrombosis in a diabetic animal model. Thus, this animal model appears well suited to study the effect of therapies used to treat metabolic syndrome and diabetes on arterial thrombosis.

Diabetes is associated with changes in factors that promote vascular disease [1]. Many of the previous therapeutic agents used for type 2 diabetes have not directly affected insulin resistance and/or the underlying causal mechanisms of diabetes. This may explain the neutral or modest effect of maintaining euglycemia with insulin or sulfonylurea treatment on the development of macrovascular complications such as myocardial infarction [3,4]. In contrast, the TZDs represent a new class of drugs that directly improve insulin sensitivity by acting primarily as PPARγ ligands [5]. While they are effective in treating insulin resistance, the ability of these drugs to reduce cardiovascular thrombotic complications is not yet clear. In this study, pioglitazone significantly prolonged the time to occlusive thrombosis following photochemical injury. Interestingly, mice treated with glipizide, which reduces hyperglycemia by direct effects on the pancreas, did not attenuate thrombus formation despite beneficial effects on glycemia. This further

supports the potential antithrombotic benefit of insulin-sensitizing drugs over the insulin-providing compounds of the sulfonylurea class.

Platelet activation is likely an important factor contributing to the prothrombotic state of diabetic patients [17]. One marker of platelet activation which has been identified in diabetic patients is soluble P-selectin. This circulating fragment is believed to closely reflect P-selectin expression of the platelet and has been demonstrated to have direct pro-coagulant effects in animal model experiments [18]. Using this marker to explore the platelet effects of pioglitazone treatment in diet-challenged KK mice, we observed a significant reduction in the concentration of the circulating fragment (soluble P-selectin) as well as P-selectin expression in platelets. Pioglitazone treatment thus leads to a reduction in the platelet activation of insulin resistant mice, which is consistent with results from recent studies in patients [19,20]. These results suggest that the beneficial effect of pioglitazone toward thrombosis may be mediated via platelet inhibition, although we cannot exclude other potential beneficial effects on the vessel wall.

In conclusion, these studies demonstrate a protective role of TZDs in a diabetic model of enhanced thrombosis. Further studies are necessary to confirm the effect in human populations at risk for CVD and to further dissect the underlying mechanisms responsible for the beneficial effect of TZDs on thrombosis.

Acknowledgements

This work was supported by grant funding from NIH grant HL-073150 (DTE), an AHA Established Investigator Award (DTE) and Takeda Pharmaceuticals North America (DTE).

Conflict of interest disclosure

We received partial project support for these studies from Takeda Pharmaceuticals North America.

References

1 Meigs JB, Mittleman MA, Nathan DM, Tofler GH, Singer DE, Murphy-Sheehy PM, Lipinska I, D'Agostino RB, Wilson PW. Hyperinsulinemia, hyperglycemia, and impaired hemostasis: the Framingham Offspring Study. *JAMA* 2000; **283**: 221–8.

2 Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. *JAMA* 1979; **241**: 2035–8.

3 Lebovitz HE. Effects of oral antihyperglycemic agents in modifying macrovascular risk factors in type 2 diabetes. *Diabetes Care* 1999; **22**: C41–4.

4 Boyne MS, Saudek CD. Effect of insulin therapy on macrovascular risk factors in type 2 diabetes. *Diabetes Care* 1999; **22**: C45–53.

5 Spiegelman BM. PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes* 1998; **47**: 507–14.

6 Manohar V, Talpur NA, Echard BW, Lieberman S, Preuss HG. Effects of a water-soluble extract of maitake mushroom on circulating glucose/insulin concentrations in KK mice. *Diabetes Obes Metab* 2002; **4**: 43–8.

7 Bodary PF, Westrick RJ, Wickenheiser KJ, Shen Y, Eitzman DT. Effect of leptin on arterial thrombosis following vascular injury in mice. *JAMA* 2002; **287**: 1706–9.

8 Cambien B, Bergmeier W, Saffaripour S, Mitchell HA, Wagner DD. Antithrombotic activity of TNF-alpha. *J Clin Invest* 2003; **112**: 1589–96.

9 Eitzman DT, Bodary PF, Shen Y, Khairallah CG, Wild SR, Abe A, Shaffer-Hartman J, Shayman JA. Fabry disease in mice is associated with age-dependent susceptibility to vascular thrombosis. *J Am Soc Nephrol* 2003; **14**: 298–302.

10 Fuster V, Stein B, Ambrose JA, Badimon L, Badimon JJ, Chesebro JH. Atherosclerotic plaque rupture and thrombosis. Evolving concepts. *Circulation* 1990; **82**: II47–59.

11 White CR, Chang LY, Crapo J, Ku D, Gianturco SH, Gore J, Freeman BA. Superoxide and peroxynitrite in atherosclerosis. *Proc Natl Acad Sci* 1995; **91**: 1044–8.

12 Eitzman DT, Westrick RJ, Xu Z, Tyson J, Ginsburg D. Hyperlipidemia promotes thrombosis after injury to atherosclerotic vessels in apolipoprotein E-Deficient mice. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1831–4.

13 Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D. Plasminogen activator inhibitor-1 and vitronectin promote vascular thrombosis in mice. *Blood* 2000; **95**: 577–80.

14 He L, Vicente CP, Westrick RJ, Eitzman DT, Tollefsen DM. Heparin cofactor II inhibits arterial thrombosis after endothelial injury. *J Clin Invest* 2002; **109**: 213–9.

15 Westrick RJ, Bodary PF, Xu Z, Shen YC, Broze GJ, Eitzman DT. Deficiency of tissue factor pathway inhibitor promotes atherosclerosis and thrombosis in mice. *Circulation* 2001; **103**: 3044–6.

16 Suto J, Matsuura S, Imamura K, Yamanaka H, Sekikawa K. Genetic analysis of non-insulin-dependent diabetes mellitus in KK and KK-Ay mice. *Eur J Endocrinol* 1998; **139**: 654–61.

17 Ferroni P, Basili S, Falco A, Davi G. Platelet activation in type 2 diabetes mellitus. *J Thromb Haemost* 2004; **2**: 1282–91.

18 Andre P, Hartwell D, Hrachovinova I, Saffaripour S, Wagner DD. Pro-coagulant state resulting from high levels of soluble P-selectin in blood. *Proc Natl Acad Sci U S A* 2000; **97**: 13835–40.

19 Akbiyik F, Ray DM, Gettings KF, Blumberg N, Francis CW, Phipps RP. Human bone marrow megakaryocytes and platelets express PPARgamma, and PPARgamma agonists blunt platelet release of CD40 ligand and thromboxanes. *Blood* 2004; **104**: 1361–8.

20 Sidhu JS, Cowan D, Tooze JA, Kaski JC. Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone reduces circulating platelet activity in patients without diabetes mellitus who have coronary artery disease. *Am Heart J* 2004; **147**: 1032–7.