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Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2016 April 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2015 April ; 35(4): 838–844. doi:10.1161/ATVBAHA.115.305378.

Endothelial PPARγ Protects Against Vascular Thrombosis by Downregulating P-Selectin Expression

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Abstract

Objective—We tested the hypothesis that endothelial proliferator-activated receptor-gamma (PPARγ) protects against vascular thrombosis using a transgenic mouse model expressing a PPARγ mutant (E-V290M) selectively in endothelium.

Approach and Results—The time to occlusive thrombosis of the carotid artery was significantly shortened in E-V290M mice compared with non-transgenic (non-Tg) littermates after either chemical injury with ferric chloride (5.1±0.2 vs. 10.1±3.3 minutes; P = 0.01) or photochemical injury with rose bengal (48±9 vs. 74±9 minutes; P = 0.04). Gene Set Enrichment Analysis demonstrated upregulation of NF-κB target genes, including P-selectin, in aortic endothelial cells from E-V290M mice (P < 0.001). Plasma P-selectin and carotid artery P-selectin mRNA were elevated in E-V290M mice (P < 0.05). P-selectin-dependent leukocyte rolling on mesenteric venules was increased in E-V290M mice compared with non-Tg mice (53±8 vs. 25±7 per minute; P = 0.02). The shortened time to arterial occlusion in E-V290M mice was reversed by administration of P-selectin blocking antibodies or neutrophil-depleting antibodies (P = 0.04 and P = 0.02, respectively) prior to photochemical injury.

Conclusions—Endothelial PPARγ protects against thrombosis through a mechanism that involves downregulation of P-selectin expression and diminished P-selectin-mediated leukocyte-endothelial interactions.

Keywords

PPARγ; Thrombosis; Endothelium; P-Selectin; NF-κB

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Disclosures: S.R.L. is a consultant to Novo Nordisk. The authors declare no competing financial interests.

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Introduction

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a ligand-activated transcription factor that regulates lipid metabolism, adipocyte differentiation, blood pressure, and insulin sensitivity.[1] PPARγ is widely expressed in adipose tissue, liver, muscle, heart, macrophages, and bone, as well as in vascular endothelial and smooth muscle cells.[2] In patients with type 2 diabetes, treatment with therapeutic PPARγ agonists such as thiazolidinediones (TZDs) improves glucose control and lowers blood pressure,[3] and also may protect from progression of atherosclerosis.[4-6] The observation that TZDs exert protective metabolic and vascular effects is consistent with genetic evidence indicating that subjects who carry dominant-negative PPARγ mutations develop severe insulin resistance and hypertension.[7]

Vascular thrombosis is a major complication of cardiovascular disease and is strongly associated with risk factors such as diabetes, metabolic syndrome, atherosclerosis, and hypertension that are known to be modulated by PPARγ.[8] However, the influence of PPARγ on thrombotic susceptibility is not well understood. Some studies performed with cultured endothelial cells have suggested that PPARγ activation may protect against thrombosis by repressing the activation of the transcription factor NF-κB, downregulating the expression of proinflammatory cell adhesion molecules, and enhancing endothelial nitric oxide production.[9-13] In contrast, PPARγ agonists have been found to stimulate increased generation of procoagulant microparticles from monocytes and macrophages,[14] an observation that might help explain the paradoxical increase in risk of myocardial infarction seen in diabetic patients treated with some TZDs.[15] Thus, PPARγ agonists may exert opposing effects on thrombotic susceptibility via actions on different target cells. Clearly, a better understanding of the tissue-specific effects of PPARγ in regulating antithrombotic capacity is needed.[16]

We previously developed transgenic mouse models in which dominant-negative mutations in the ligand-binding domain of human PPARγ (V290M or P467L) are selectively expressed in endothelium under the control of the VE-cadherin promoter.[17, 18] These mutations interfere with basal and agonist-induced PPARγ transcriptional activity and repress PPARγ target genes.[19, 20] We demonstrated that endothelium-specific V290M PPARγ (E-V290M) transgenic mice exhibit increased susceptibility to endothelial vasomotor dysfunction when fed a high fat diet[17] or crossed to apolipoprotein E-deficient mice.[21] In the current study, we used the E-V290M murine model to test the hypothesis that endothelial PPARγ protects against arterial thrombosis *in vivo* and examine the mechanistic role of the endothelial cell adhesion molecule P-selectin.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

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Results

Carotid artery thrombosis is accelerated in E-V290M transgenic mice

To investigate the potential antithrombotic functions of PPARγ specifically in endothelium, we studied transgenic mice expressing a dominant-negative human PPARγ mutant (V290M) targeted to vascular endothelium. Experimental thrombosis of the carotid artery was induced in male E-V290M and non-Tg mice by either transmural chemical injury with ferric chloride (Figure 1A) or luminal injury with the photo-activatable dye, rose bengal (Figure 1B). Compared with non-Tg mice, E-V290M mice exhibited a prothrombotic phenotype with both methods of carotid artery injury. After ferric chloride injury, the time to stable occlusion of the carotid artery was significantly shorter in E-V290M mice than non-Tg mice ($P = 0.01$; Figure 1A). The time to stable occlusion also was shorter in E-V290M mice compared with non-Tg mice after photochemical injury ($P = 0.04$; Figure 1B). Immunohistochemical staining demonstrated the presence of cells expressing the neutrophil antigen Ly-6 and tissue factor within the thrombosed lumen of the carotid artery after photochemical injury (Figure 2). The Ly-6 and tissue factor-positive cells were localized near the intimal layer of the vessel wall, which suggested that activated neutrophils were interacting with the damaged endothelium or subendothelium at the site of injury.

Venous thrombosis is not enhanced in E-V290M mice

Venous thrombosis was induced by ligation of the inferior vena cava (IVC). There were no significant differences in the weight or length of venous thrombi isolated from E-V290M mice compared with non-Tg mice 48 hours after IVC ligation (Supplemental Figure I).

Dominant-negative PPARγ upregulates endothelial NF-κB target genes, including P-selectin

To determine if genes known to be important in the regulation of vascular thrombosis are altered by endothelial PPARγ interference, we analyzed an existing mRNA microarray dataset (available from NCBI-GEO at accession GSE11870) generated from gene expression profiling of endothelial cells derived from E-V290M mice and their non-Tg littermates.[17] We first queried the dataset for genes with established roles in vascular thrombosis (Table 1). Several of these genes exhibited a significant change in expression in endothelial cells of E-V290M mice, with the largest increase observed in the *Selp* gene encoding P-selectin (6.9-fold upregulation; $P < 0.01$). The highly significant upregulation of *Selp*, a known NF-κB target gene,[22] suggested the possibility that the NF-κB pathway was activated in the endothelium of E-V290M mice. To further address this possibility, we queried the dataset using a list of experimentally validated NF-κB target genes (http://bioinfo.lifl.fr/NF-KB/). In addition to statistically significant and robust increases in several individual NF-κB target genes (see Supplemental Table I), the complete set of NF-κB targets, as a group, displayed a significant increase in expression by GSEA ($P < 0.001$, normalized enrichment score 2.14).

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E-V90M mice have elevated levels of P-selectin mRNA and protein

To confirm that the altered expression of the *Selp* gene observed in the microarray dataset analysis was associated with increased expression of P-selectin in E-V90M mice, we measured levels of P-selectin mRNA in the carotid artery by qPCR. We found that P-selectin mRNA was elevated 2.3-fold in E-V290M mice compared with non-Tg mice (P = 0.03; Figure 3A). Similarly, E-V290M mice had significantly elevated levels of circulating soluble P-selectin antigen in plasma compared with non-Tg mice (P = 0.004; Figure 3B). Because plasma soluble P-selectin can originate from platelets as well as endothelial cells,[23] we also measured platelet P-selectin surface expression by flow cytometry. No differences in platelet surface P-selectin were observed between E-V290M and non-Tg mice at baseline or after activation of platelet alpha granule release with thrombin (Figure 4A). Additionally, there were no differences in the level of fibrinogen binding at baseline or after activation with thrombin between E-V290M and non-Tg mice (Figure 4B). These findings suggest that the elevation of plasma soluble P-selectin in E-V290M mice was due to increased expression of P-selectin in endothelial cells rather than release of P-selectin from platelets. These observations also demonstrate that the endothelium-targeted dominant-negative V290M transgene does not have any appreciable effect on platelet activation in E-V290M mice.

E-V290M mice have increased P-selectin-mediated leukocyte-endothelial interactions

To assess the functional activity of endothelial P-selectin, we visualized leukocyte rolling on unstimulated mesenteric veins in real time using phase contrast video microscopy. We observed a 2-fold increase in rolling leukocytes per minute in E-V290M mice (53 ± 8) compared with non-Tg mice (25±7; P = 0.02) (Figure 5 and Supplemental Movies 1 and 2). Leukocyte rolling was almost completely inhibited by pre-treatment with a P-selectin blocking antibody in both E-V290M and non-Tg mice (Figure 5 and Supplemental Movie 3), demonstrating that leukocyte rolling was dependent on P-selectin.

Accelerated carotid artery thrombosis in E-V290M mice is dependent on P-selectin and neutrophils

To determine the mechanistic role of P-selectin-mediated endothelial-leukocyte interactions in the prothrombotic phenotype of E-V290M mice, mice were pre-treated with either the P-selectin blocking antibody or a neutrophil depleting antibody (anti-Ly-6G) prior to inducing carotid artery thrombosis by photochemical injury. Treatment with anti-Ly-6G resulted in a 95% decrease in peripheral blood neutrophil count, from 1.39±0.65 to 0.07±0.04 K/μl (P = 0.04). Compared with E-V290M mice pre-treated with a control IgG, E-V290M mice pre-treated with either the P-selectin blocking antibody or the neutrophil-depleting antibody exhibited prolonged times to thrombotic occlusion (P = 0.04 and P = 0.02, respectively; Figure 6). Antibody pre-treatment did not have any significant effects on the time to occlusion in non-Tg mice (Figure 6). These findings suggest that the accelerated carotid artery thrombosis in E-V290M mice is dependent on both P-selectin and neutrophils.

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Human PPARγ transgene expression in blood cells

We have shown previously that the VE-cadherin promoter confers endothelial-selective expression of dominant-negative human PPARγ in E-V290M mice.[18] Because VE-cadherin is also expressed in hematopoietic stem cells,[24] we measured the expression of human PPARγ V290M mRNA by qPCR in platelets and leukocytes, as well as in lung, which is rich in endothelium (Supplemental Figure II). As expected, strong expression of human PPARγ V290M mRNA was found in lung. Trace expression of human PPARγ V290M mRNA also was detected in peripheral blood leukocytes, at a level that was >15-fold lower than that in lung. No expression of human PPARγ V290M mRNA was detected in platelets.

Discussion

PPARγ is well known to have anti-inflammatory properties mediated by altered expression of pro- and anti-inflammatory genes.[1, 16, 21] However, despite an established association between inflammatory conditions and thrombotic risk,[25] the potential role of PPARγ in protecting from thrombosis remains poorly defined. Administration of high-affinity PPARγ agonists has been reported to protect against thrombosis in a mouse model of insulin resistance[26] and to have anti-platelet effects in vitro.[27, 28] In contrast, some PPARγ agonists have been reported to stimulate increased generation of procoagulant microparticles from monocytes and macrophages.[14] Moreover, the cell-specific influences of PPARγ in vascular endothelium on thrombotic susceptibility remain poorly defined. With this uncertainty in mind, and in consideration of the growing public heath burden of thrombotic complications associated with obesity and insulin resistance,[29] we utilized the E-V290M transgenic mouse model to delineate the effects on thrombosis of endogenous PPARy expressed in endothelium. The major findings from our study are that 1) cell-specific interference with endogenous PPARγ in vascular endothelium increases susceptibility to arterial thrombosis in mice, and 2) the mechanism of accelerated thrombosis in E-V290M mice is mediated by upregulation of P-selectin and increased P-selectin-dependent endothelial-leukocyte interactions.

We chose to use a dominant negative approach, rather than a PPARγ gene knockout approach for a number of reasons. First, dominant negative mutations such as V290M, while rare, are bonafide mutations that cause disease in human patients.[7] Second, the PPARγ null mouse has an embryonic lethal phenotype[30] and there are no null mutations known to exist in humans. Finally, the alternative approach of using an endothelial tissue-specific knockout model[31] has a major limitation. In the unliganded state, PPARγ binds to DNA and actively represses genes by recruiting a co-repressor complex to the gene being silenced[32]. When a ligand is present, the co-repressor complex is replaced by a co-activator complex, leading to gene activation. Deleting PPARγ has a similar effect as ligand activation because there is a small induction of gene expression due to the loss of active repression. This does not occur with the use of dominant negative PPARγ mutants. As expected, we observed strong expression of the PPARγ V290M transgene in endothelium. We also detected trace expression of the transgene by qPCR in peripheral blood mononuclear cells (Supplemental Figure II). It is possible that the low-level expression of the transgene in peripheral blood cells may be due to residual activity of the VE-cadherin promoter in cells of myeloid

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lineage.[24] Alternatively, it may represent the presence of trace amounts of circulating endothelial cells in the mononuclear cell fraction.

PPARγ has been reported to inhibit the activation of NF-κB in endothelial cells,[16] and our gene expression analysis confirmed that known NF-κB target genes are significantly upregulated in aortic endothelial cells of E-V290M mice. In particular, we identified P-selectin as an NF-κB target gene that is highly upregulated in the endothelium of E-V290M mice compared with non-Tg mice. P-selectin (CD62P) is an inducible cell-surface leukocyte adhesion molecule that mediates initial interactions between circulating neutrophils and the activated endothelium.[23] In accordance with the upregulation of P-selectin seen in the microarray dataset analysis, we observed increased levels of P-selectin mRNA in the carotid artery and increased circulating soluble P-selectin in the plasma of E-V290M mice (Figure 3). Importantly, the increased expression of endothelial P-selectin was accompanied by increased leukocyte rolling, indicating that the P-selectin expressed in E-V290M mice was functional in mediating increased leukocyte-endothelial interactions. In addition to mediating leukocyte recruitment to activated endothelium, P-selectin can promote thrombosis by facilitating platelet adhesion and inducing the generation of procoagulant microparticles and neutrophil extracellular traps.[23, 33] We therefore reasoned that P-selectin may be a key mediator of the prothrombotic phenotype of E-V290M mice.

The prothrombotic phenotype of E-V290M mice was apparent when carotid artery thrombosis was induced by either chemical injury with ferric chloride or photochemical injury with rose bengal (Figure 1). For subsequent mechanistic experiments, we chose to focus on the photochemical injury model because it is more endothelium-dependent[34] whereas the ferric chloride injury model is partially mediated by red blood cells.[35] To determine the role of P-selectin-mediated leukocyte-endothelial interactions in the enhanced thrombotic susceptibility of E-V290M mice, we pre-treated mice with a P-selectin blocking antibody that was shown to almost completely eliminate leukocyte rolling (Figure 5). We found that the P-selectin blocking antibody largely reversed the prothrombotic effect of endothelium-specific PPARγ interference (Figure 6). We also observed a similar protective anti-thrombotic effect after depletion of >95% of the circulating neutrophils in E-V290M mice (Figure 6). Together, these findings suggest that endogenous endothelial PPARγ protects from thrombosis through a mechanism that involves downregulation of P-selectin expression, perhaps by antagonizing the transcriptional effects of NF-κB, and diminished P-selectin mediated leukocyte-endothelial interactions.

Both ferric chloride and rose bengal induce vascular injury via oxidative mechanisms, leading to endothelial damage and denudation.[34] The thrombotic response to oxidative injury is thought to be initiated by the adhesion of platelets and leukocytes to the exposed subendothelium. However, recent findings suggest that, at least for the ferric chloride model, the injured endothelium may be retained after ferric chloride exposure and may contribute to thrombosis.[35, 36] Our findings suggest that expression of P-selectin on the retained, injured endothelium contributes to leukocyte adhesion and thrombosis after oxidative injury. We acknowledge that it is possible that additional endothelial PPARγ and/or NF-κB target genes, such as E-selectin, VCAM-1, ICAM-1, and tissue factor also may contribute to the prothrombotic phenotype of E-V290M mice. However, none of these genes emerged from

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the gene expression dataset analysis as significantly upregulated in endothelial cells from E-V290M mice. We note that genes encoding protease-activated receptor 2 (*F2rl1*) and plasminogen activator inhibitor 1 (*Serpine1*) were upregulated 5-fold and 4-fold, respectively, with a borderline level of statistical significance ($P < 0.1$) (Table 1). These may be attractive target genes for future study.

The lack of a venous thrombosis phenotype in E-V290M mice using the IVC stasis model is surprising, because P-selectin is thought to play a mechanistic role in venous thrombosis.[37] We consider these findings to be very interesting, however, because they suggest that non-endothelial sources of P-selectin (e.g. platelet P-selectin) may play a greater role than endothelial P-selectin in driving venous thrombosis, In contrast, endothelial P-selectin may contribute more directly to arterial thrombosis. In support of this idea, it has been suggested that multiple pools of P-selectin promote venous thrombogenesis.[38]

Our findings may have implications for the clinical observation that, despite their generally protective metabolic and cardiovascular effects in diabetic patients, some TZDs have been found to paradoxically increase the risk of thrombotic vascular complications such as myocardial infarction.[15] Our results suggest that activation of PPARγ specifically in vascular endothelium is antithrombotic and protective, raising the possibility that the apparent prothrombotic effects of systemically administered PPARγ agonist may be mediated through effects in other cell types. The protective antithrombotic effect of the P-selectin blocking antibody observed in E-V290M mice suggests that antagonism of P-selectin may be potential therapeutic approach to prevent thrombosis in patients with impaired PPARγ function due to obesity or insulin resistance. Oral P-selectin blocking agents are being developed for several different clinical indications.[39-42]

In summary, this study demonstrates that selective inactivation of PPARγ in vascular endothelium results in a prothrombotic phenotype characterized by upregulation of P-selectin and other NF-κB target genes. Our data further demonstrate that interference with endothelial PPARγ leads to accelerated thrombosis through a mechanism in which increased expression of P-selectin causes enhanced leukocyte recruitment to the vessel wall. These findings suggest that one mechanism for protection against thrombosis by endogenous endothelial PPARγ is by suppressing P-selectin-mediated leukocyte-endothelial interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Severine Groh, Jeff Stevens, and Prem Prakash for technical assistance. The Sysmex automatic hematology analyzer used in this study was provided on an on-loan basis from Sysmex Corporation, Kobe, Japan.

Sources of Funding: The authors gratefully acknowledge the generous research support of the Roy J. Carver Trust (to C.D.S.) and the American Society of Hematology (to S.R.L.). This study was supported in part by research funding from National Institutes of Health (NIH) grants HL063943 and HL062984 to S.R.L., NIH grants HL048058, HL061446, HL062984, HL084207 to C.D.S., NIH grants HL118246 and HL118742 to A.K.C., NIH grant T32 HL007344 to M.A.G., an American Heart Association (AHA) postdoctoral award to H.J., and an AHA predoctoral award to I.O.B.

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Nonstandard Abbreviations and Acronyms

E-V290M	transgenic mouse model expressing the V290M PPARγ mutant selectively in endothelium
FITC	fluorescein isothiocyanate
GSEA	gene set enrichment analysis
IVC	inferior vena cava
PPARγ	proliferator-activated receptor-gamma
non-Tg	non-transgenic
PE	phycoerythrin
TZD	thiazolidinedione

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Significance

Vascular thrombosis is a major complication of cardiovascular disease. Peroxisome proliferator-activated receptor-gamma (PPARγ) is a ligand-activated transcription factor that regulates lipid metabolism, adipocyte differentiation, blood pressure, and insulin sensitivity. The influence of PPARγ on thrombotic susceptibility is not well understood, and PPARγ agonists may exert opposing effects on thrombotic susceptibility via actions on different target cells. Using a transgenic mouse model expressing a dominant-negative PPARγ mutant selectively in endothelium, we tested the hypothesis that PPARγ protects against vascular thrombosis by altering the gene expression profile in endothelial cells. The novel findings of this study are: 1) selective interference with the transcription factor PPARγ in endothelium causes a prothrombotic phenotype in transgenic mice, and 2) endothelial PPARγ protects against thrombosis by downregulating P-selectin-mediated leukocyte-endothelial interactions.

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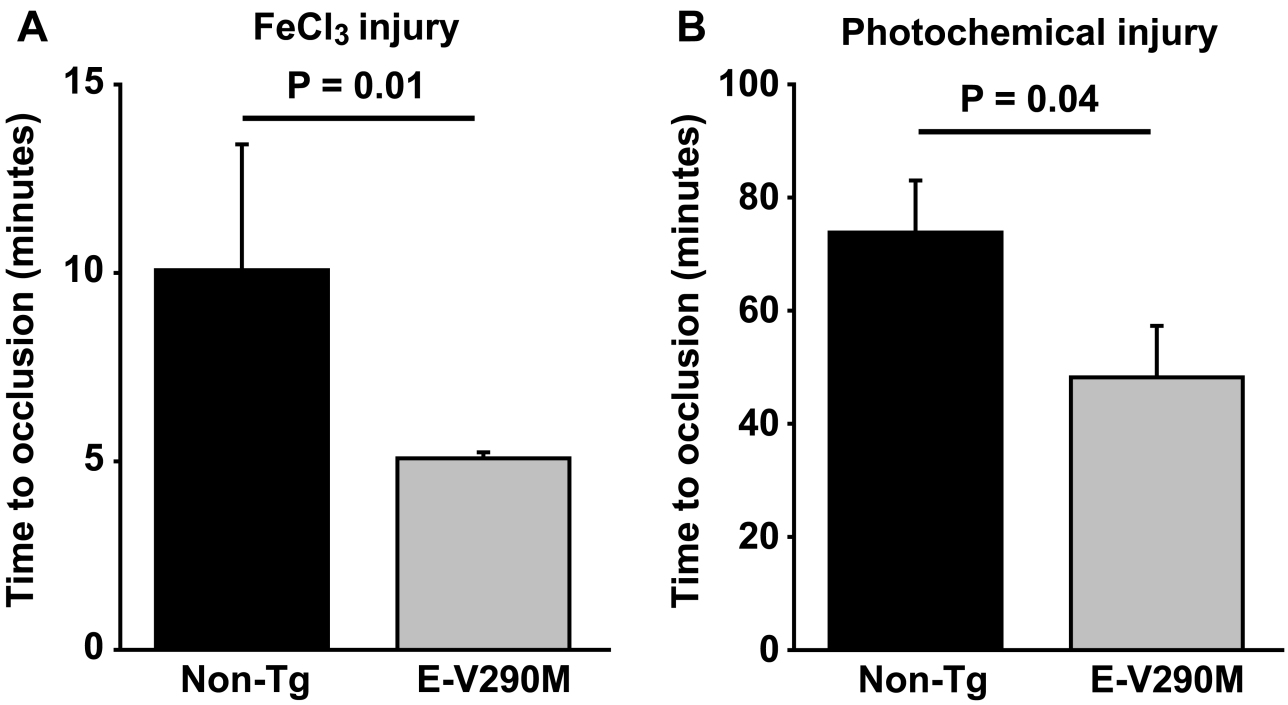

Figure 1.

Carotid artery thrombosis is accelerated in E-V290M transgenic mice. Carotid artery thrombosis was induced by either chemical injury with (A) 7% $FeCl_3$ (N = 5 to 7) or (B) photochemical injury with rose bengal (N = 7 to 8) in male non-Tg or E-V290M mice at 14-16 weeks of age. The time to stable occlusion was measured using a Doppler flow probe. Values are mean ± SE. The P-values were determined using the rank sum test.

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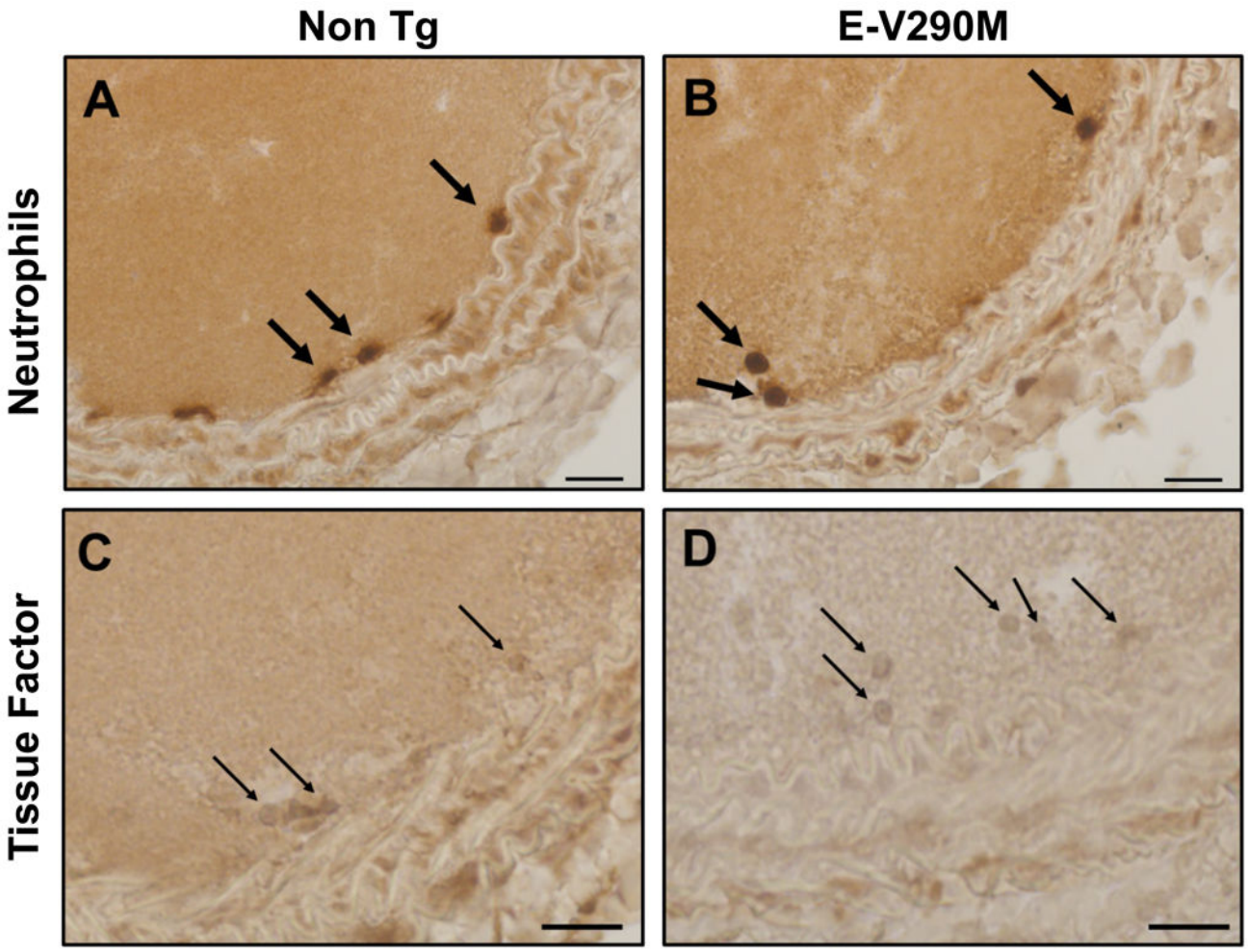

Figure 2.

Immunohistochemical detection of neutrophils and tissue factor in carotid artery thrombi. Carotid artery thrombosis was induced by photochemical injury with rose bengal in male non-Tg and E-V290M mice, and the carotid arteries were harvested and subjected to immunohistochemical staining for neutrophils (Ly-6) or tissue factor (PAA524Mu01). Cells staining positively for neutrophils (thick arrows) and tissue factor (thin arrows) were detected within the thrombus adjacent to the intima. Bar indicates 20 μm.

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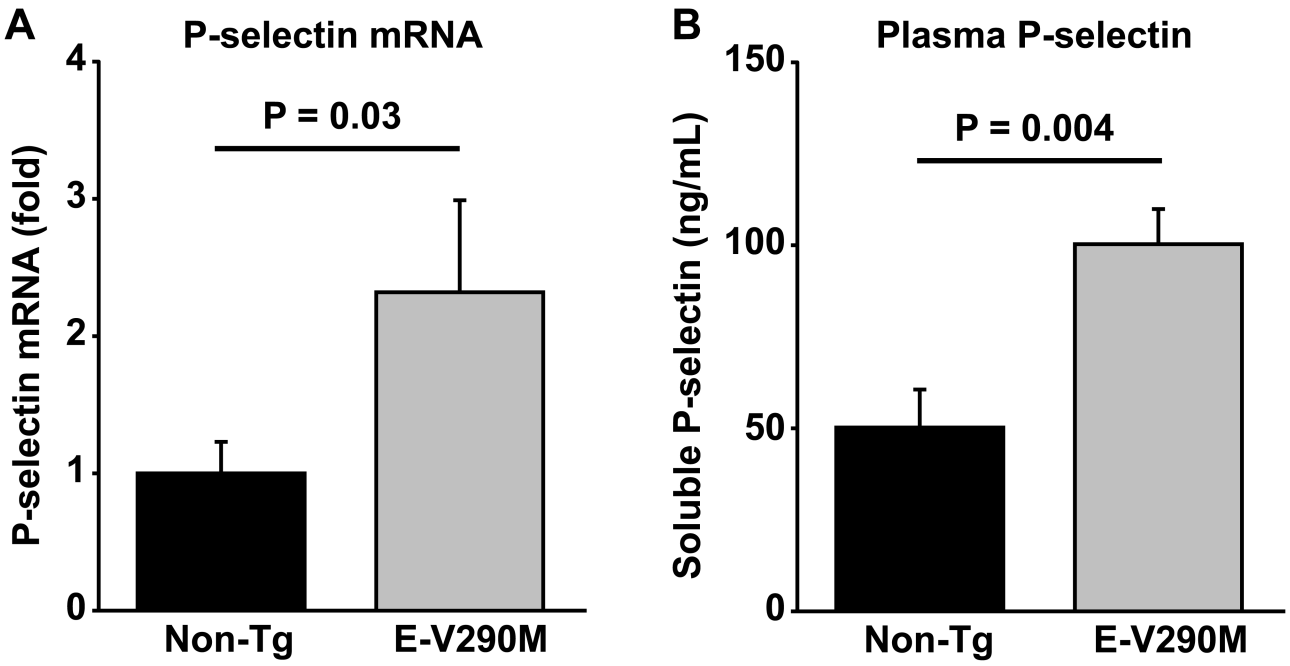

Figure 3.

Elevated levels of P-selectin mRNA and protein in E-V290M mice. (A) Levels of P-selectin mRNA in carotid arteries from E-V290M or non-Tg mice were measured by qPCR (N = 5 to 6). (B) Plasma levels of soluble P-selectin were measured in E-V290M or non-Tg mice (N = 6 to 8). Values are mean ± SE. The P-values were determined using the Student's t-test.

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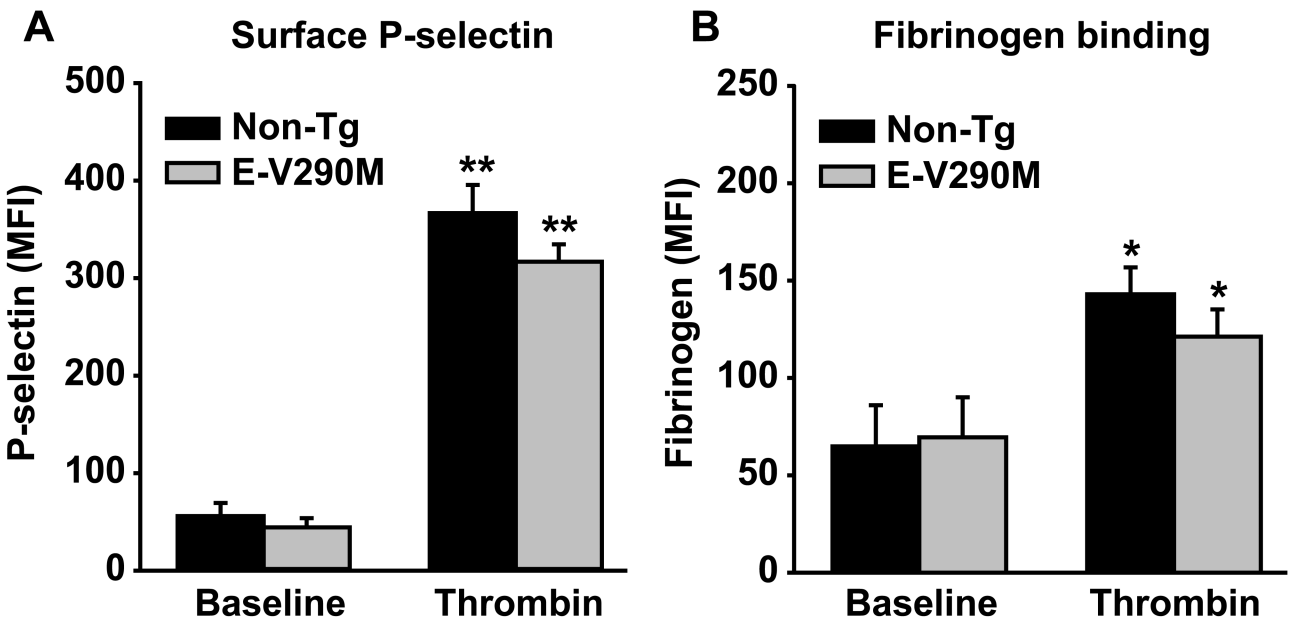

Figure 4.

Platelet activation responses and P-selectin expression do not differ between E-V290M and non-Tg mice. Platelet surface P-selectin (A) and fibrinogen binding (B) were measured by flow cytometry at baseline and after stimulation with 0.05 U/ml thrombin (N = 5 mice in each group). Values are mean ± SE. **P < 0.01; *P < 0.05 vs. baseline by two-way ANOVA. MFI: mean fluorescence intensity.

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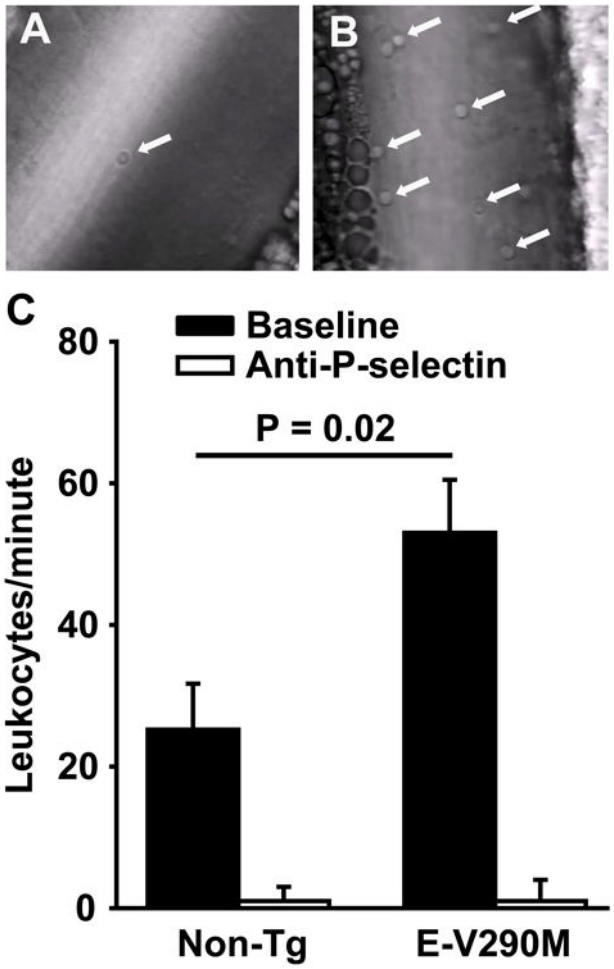

Figure 5.

Increased P-selectin-mediated leukocyte rolling in E-V290M mice. Leukocyte rolling on unstimulated mesenteric veins of male non-Tg (A) or E-V290M (B) mice (4 to 6 weeks of age; N = 6 to 10 per group) was measured in real time using phase contrast video microscopy. Arrows indicate rolling leukocytes. (C) The extent of leukocyte rolling was quantitated by counting the number of cells passing a fixed point per minute (leukocytes/minute). Leukocyte rolling was abolished after pre-treatment with a P-selectin antibody in both E-V290M or non-Tg mice (N = 4 per group). Values are mean ± SE. The P-values were determined using one-way ANOVA. See Supplemental Online Material for video files.

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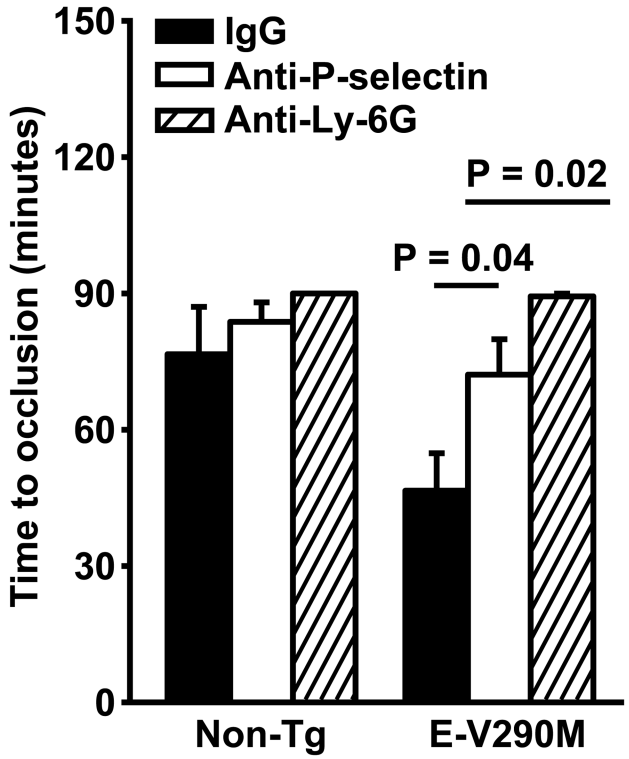

Figure 6.

Accelerated carotid artery thrombosis in E-V290M mice is dependent on both P-selectin and neutrophils. Carotid artery thrombosis was induced by photochemical injury with rose bengal in male non-Tg or E-V290M mice after pre-treatment with either a control IgG, an anti-P-selectin blocking antibody, or a neutrophil-depleting antibody (anti-Ly-6G) (N = 4 to 10 in each group). The time to stable occlusion was measured using a Doppler flow probe. Values are mean ± SE. The P-values were determined using the rank sum test.

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Table 1

Microarray dataset analysis. Changes in expression of thrombosis-related genes in endothelial cells of E-V290M mice compared with non-Tg mice

Gene*	Probe	Fold Change	P-Value	Description
Selp	1420558_at	6.90	0.0061	P-selectin
F2rl1	1448931_at	5.29	0.0856	protease-activated receptor-2
Serpine1	1419149_at	4.15	0.0665	plasminogen activator inhibitor-1
Entpd1	1450939_at	1.72	0.0050	CD39
Thbd	1448529_at	1.29	0.1104	thrombomodulin
Sele	1421712_at	1.25	0.1743	E-selectin
Icam1	1424067_at	1.22	0.1967	Intercellular adhesion molecule-1
F2r	1437308_s_at	1.19	0.3228	protease activate receptor-1
Plat	1415806_at	1.15	0.2174	tissue plasminogen activator
Serpine2	1416666_at	1.15	0.5358	protease nexin-1
Anxa5	1425567_a_at	1.08	0.3687	annexin A5
F3	1417408_at	0.95	0.6423	tissue factor
Vwf	1435386_at	0.92	0.4708	von Willebrand factor
Vcam1	1451314_a_at	0.88	0.3016	vascular cell adhesion molecule-1
Tfpi	1438530_at	0.84	0.2418	tissue factor pathway inhibitor
Adamts1	1450716_at	0.81	0.1452	ADAMTS1
Procr	1420664_s_at	0.77	0.2049	endothelial protein C receptor

*Gene: Official gene symbol from NCBI; Probe: probe set from Affymetrix MOE430 array; Fold change: fold change relative to non-transgenic littermates. For genes with multiple probe sets, the probe set with the lowest p-value was selected. Data are from an existing microarray dataset (available from NCBI-GEO at accession GSE11870).