Published in final edited form as:

Thromb Res. 2013 March ; 131(3): 268–276. doi:10.1016/j.thromres.2012.12.006.

Rosuvastatin reduced deep vein thrombosis in ApoE gene deleted mice with hyperlipidemia through non-lipid lowering effects

K.A. Patterson¹⁻², X. Zhang¹, S.K. Wroblewski¹, A.E. Hawley¹, D. A. Lawrence³, T.W. Wakefield¹, D.D. Myers¹⁻², and J.A. Diaz¹

¹Department of Surgery, Section of Vascular Surgery, Conrad Jobst Vascular Research Laboratories, University of Michigan, Ann Arbor, MI, USA

¹⁻²Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI, USA

³Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

Abstract

Introduction—Statins, particularly rosuvastatin, have recently become relevant in the setting of venous thrombosis. The objective of this study was to study the non-lipid lowering effects of rosuvastatin in venous thrombosis in mice with hyperlipidemia.

Materials and Methods—An inferior vena cava ligation model of venous thrombosis in mice was utilized. Saline or 5mg/kg of rosuvastatin was administered by gavage 48hs previous thrombosis. Blood, the inferior vena cava, thrombus, and liver were harvested 3, 6 hours, and 2 days post-thrombosis. Thrombus weight, inflammatory markers, and plasminogen activator inhibitor-1 expression and plasma levels were measured and neutrophil migration to the IVC was assessed.

Results—Rosuvastatin significantly decreased thrombus weight, plasminogen activator inhibitor-1 expression and plasma levels, expression of molecules related to the interleukin-6 pathway, and neutrophil migration into the vein wall.

Conclusions—This work supports the beneficial effects of rosuvastatin on venous thrombosis in mice with hyperlipidemia due to its non-lipid lowering effects.

Keywords

Statin; Venous Thrombosis; Hyperlipidemia; Rosuvastatin; Interleukin-6

© 2012 Elsevier Ltd. All rights reserved

Corresponding author: Jose Antonio Diaz, M.D. Department of Surgery, Section of Vascular Surgery Conrad Jobst Vascular Research Laboratories University of Michigan, School of Medicine Phone: 734-615-7287, Fax: 734-763-7307 http://sitemaker.umich.edu/jobst/diaz_lab josediaz@med.umich.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement None.

Introduction

Venous thromboembolism (VTE), which comprised deep vein thrombosis (DVT) and pulmonary embolism (PE), is the third most common cardiovascular disease in the United States (1). There are estimated to be more than 900,000 cases in the United States annually, resulting in 300,000 deaths (2, 3). The rate of VTE has not significantly changed in the past 25 years (4). This underscores the importance for developing improved treatments and preventative measures.

Recently, the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) examined a large group of patients with high levels of C-reactive protein (CRP), treated with rosuvastatin or placebo (5). This trial focused on the effects of rosuvastatin on major cardiovascular events, however the patients receiving rosuvastatin were found to have significantly decreased rates of DVT. Statin therapy is usually initiated in patients with hyperlipidemia. Although hyperlipidemia is currently not considered a risk factor for DVT, this concept may change in the future. Most clinical trials investigating hyperlipidemia involve patients on statins, which may be masking the link between hyperlipidemia and DVT. Following this direction, we previously investigated DVT in the context of hyperlipidemia using apolipoprotein E genetically deleted (ApoE^{-/-}) mice. We found that the fibrinolytic system was impaired in ApoE^{-/-} mice due to increased levels of plasminogen activator inhibitor-1 (PAI-1), the main regulator of this system, leading to an increase in venous thrombosis (VT) (6). Whether statins improve fibrinolysis and impact VT remains to be determined.

Statins significantly reduce cardiovascular events and mortality in coronary artery disease patients (7, 8) through lipid lowering effects. They have also been reported to have anti-inflammatory and pro-fibrinolytic properties documented in both *in vitro* and clinical biomarker studies (9–13). In particular, interleukin-6 (IL-6), through signal transducer and activator of transcription 3 (STAT3), and interleukin-1-beta (IL-1 β) were reduced by statins, suggesting anti-inflammatory effects (10). This is important in the context of VT because the relationship between VT and inflammation was hypothesized by Stewart in 1974 (14). Also, the pro-fibrinolytic effects of statins, specifically decreases in PAI-1, have been documented (15). The anti-inflammatory and pro-fibrinolytic effects of statins have not been examined in relationship to VT in a hyperlipidemic state using *in vivo* models. The objective of this study was to evaluate the effects of rosuvastatin (Crestor®) on VT using the inferior vena cava (IVC) stasis animal model in ApoE^{-/-} mice. We hypothesized that rosuvastatin treatment in hyperlipidemic mice would have both anti-inflammatory and pro-fibrinolytic affects in a mouse VT model of IVC ligation.

Materials and Methods

Animals

Male ApoE^{-/-} mice (Stock #2052, Jackson Laboratories) 8–10 weeks of age weighing 20–25 gram were used in this study. All research was approved by the University of Michigan, University Committee on Use and Care of Animals and was completed in compliance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The University of Michigan maintains accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Experimental Design

ApoE^{-/-} mice were used as hyperlipidemic experimental animals. These mice have a 4-fold increase in cholesterol and 2-fold increase in triglycerides on a normal rodent chow compared to wild type (WT, data not shown). Animals were maintained on a normal rodent

chow for the duration of the study. A total of 180 ApoE^{-/-} mice were used in this study. A model of IVC ligation was performed as previously described (16, 17). Time-points were 3 hours, 6 hours, and 2 days post-thrombosis. Mice that did not receive surgery served as non-thrombosed true controls (TC). At the time of euthanasia, blood was collected via cardiocentesis for analysis and the IVC, with thrombus, was harvested for thrombus weight (TW). The thrombus was then removed from the IVC and both were frozen and processed individually for molecular assays. For histological specimens, the sample included the IVC, thrombus, aorta, and surrounding tissues in order to ensure complete visualization of the vein wall and thrombus. Additional sample processing is described below.

In Vivo Treatment with Rosuvastatin or Saline Solution

Animals received rosuvastatin (Crestor® AstraZeneca, Macclesfield, UK), dissolved in saline or an equal volume of saline alone, by oral gavage once daily beginning two days before surgery. Animals with endpoints of 3 and 6 hours post-thrombosis received 2 doses total and were not dosed the day of surgery/harvest. Animals who were harvested at 2 days post-thrombosis received additional doses on the day of and the day after surgery. This dosing schedule was developed based on previous publications (18). The rosuvastatin dose was 5mg/kg (19).

Thrombus Weight (TW)

At the time of harvest, the IVC and the associated thrombus were removed from anesthetized animals. TW (weight of thrombus and vein wall combined) in grams (g) was recorded and used as a reference of thrombosis (16).

Detection of Mouse Soluble P-selectin (sPsel)

Five hundred µl of blood was collected by cardiocentesis at harvest. Mouse plasma samples were evaluated for circulating sPsel levels. EDTA anti-coagulated blood was processed using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's kit for plasma soluble P-selectin (R&D Systems, Minneapolis, MN). All samples were run in duplicate and results normalized to total protein using the standard Pierce BCA assay (Thermo Fisher Scientific, Inc., Rockford, IL).

PAI-1 Activity Assay

Five hundred µl of blood was collected by cardiocentesis at harvest using a syringe with .05 ml of sodium citrate and active and total PAI-1 was determined as previously described (6).

Cholesterol and triglyceride assay

Quantitative circulating cholesterol and triglyceride levels were measured in the serum of ApoE^{-/-} mice using 50 µl of blood collected via cardiocentesis at harvest. Serum was processed according to manufacturer's guidelines for both total cholesterol levels and triglyceride levels (Wako Chemicals USA, Richmond, VA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Ribonucleic acid (RNA) was isolated by homogenizing IVC wall and liver samples in TRIzol reagent (Invitrogen, Carlsbad, CA) per manufacturer's protocol and qRT-PCR was performed as previously described (17). Gene expression of PAI-1, STAT3, nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B), IL-6, chemokine ligand 2 (CCL2), IL-1 β , and CCAAT/enhancer binding protein delta (C/EBP δ), were determined by qRT-PCR using commercially available primers (mouse PAI-1: PPM03093B; mouse STAT3: PPM04643E; mouse NF- κ B: PPM02930E; mouse IL-6: PPM03015A; mouse CCL2: PPM03151F; mouse IL-1B: PPM03109F, mouse C/EBP δ : PPM04676F) and SYBR master

mix (SA Bioscience, Frederick, MD) in a Rotor-Gene 3000 thermocycler (Corbett Life Science, San Francisco, CA). Relative expression levels were normalized to β -actin expression (mouse β -actin: PPM02945B).

Immunohistochemical Staining

Using blank slides prepared from the same paraffin embedded IVC samples as below; neutrophils within the vein wall were analyzed with a neutrophil marker stain NIMP-R14 (Santa Cruz Biotechnology, Santa Cruz, CA) that is a rat monoclonal antibody raised against neutrophils of BALB/c mouse origin. This was completed to visualize any cell count differences in the IVC wall associated with rosuvastatin treatment. Neutrophils were chosen because these cells are associated with acute inflammation. Sections were cut for slides at 4 microns and heated in a 60°F oven for 30 minutes. Samples were deparaffinized and run under water. Antigen retrieval (HIER) was performed in Reveal Solution (citrate based) at 125°F for 30 minutes followed by 95°F for 10 seconds in Decloaker. The samples were cooled in solution on the bench top for 10 minutes, and then rinsed in deionized water and placed in TBS buffer. Slides were placed in Peroxidase 1 for 5 minutes followed by 2 TBS rinses. Samples were then placed in Rodent Block M for 30 minutes and rinsed twice by TBS. Samples were incubated with a 1:200 dilution of rat anti-mouse neutrophil marker NIMP-R14 for 1-hour followed by two TBS rinses. A secondary Rat on Mouse Probe Kit was used for 10 minutes followed by two TBS rinses and then a Tertiary Rat on Mouse HRP Polymer for 30 minutes and two TBS rinses. Color development was performed with DAB substrate for 5 minutes followed by IP Sparkle for 1 minute. Hematoxylin stain was then used for 5 minutes followed by a deionized water rinse. Unless otherwise noted, all kits and reagents were purchased from Biocare Medical (Concord, CA).

IVC Wall Morphometrics

IVC samples were paraffin embedded, processed into slides, stained with hematoxylin and eosin (H&E) and examined under high power oil immersion light microscopy (100 $\times$). A veterinary pathologist, blinded to treatment groups, examined five representative high power fields (cells/5HPFs) around the vein wall and analyzed as previously described(17). Results were added together and the mean $\pm$ standard error was calculated for each vein.

Statistical Analyses

All statistical analyses were completed using GraphPad Prism version 5:01 (GraphPad Software, San Diego, CA). Statistical differences between groups were determined by an unpaired *t* test with Welch's correction. A one-way ANOVA analysis using the Tukey's multiple comparison test was performed between groups for additional comparisons. A *p* value of 0.05 was considered significant. Spearman correlation between TW and soluble P-selectin was also performed. Data was reported as mean $\pm$ standard error of the mean (SEM).

Results

Mice and surgery

All surgeries were performed using the IVC ligation model. The mortality rate for this study was 0.55% (1/180). We found IVC thrombi in all 150 surgical cases (30 animals were true controls that did not undergo surgery). No complications associated with gavage or bleeding complications from surgery were observed.

Rosuvastatin decreased thrombus weight and soluble P-selectin

There were no significant differences in TW between rosuvastatin and saline treated animals at the 3-hour and 6-hour time-points. A statistically significant decrease in TW at the 2-day time-point in the rosuvastatin treated animals, compared to the saline treated animals, was found (0.0304 ± 0.0010 vs. 0.0346 ± 0.0011 grams, $p < 0.05$) (Fig. 1A). Significantly decreased sPsel levels were found at the 3-hour time-point (4.58 ± 0.37 vs. 5.88 ± 0.30 ng/mg, $p < 0.05$), 6-hour time-point (6.92 ± 1.11 vs. 11.51 ± 0.84 ng/mg, $p < 0.05$), and the 2-day time-point (10.60 ± 0.71 vs. 12.97 ± 0.71 ng/mg, $p < 0.05$) in the rosuvastatin treated animals, compared to the saline animals (Fig. 1B). A strong positive correlation was shown between TW and sPsel levels in both the control group (Spearman $r = 0.76$, $p = 0.0001$) and the rosuvastatin group (Spearman $r = 0.78$, $p = 0.0001$) (Fig. 1C–D).

Rosuvastatin ameliorated circulating PAI-1 and PAI-1 gene expression

We found a significant decrease in total PAI-1 levels in rosuvastatin treated animals compared to saline treated animals at the 6-hour time-point (2701.1 ± 453 vs. 13079.6 ± 2818 pg/ml, $p < 0.05$). No significant differences were observed at 3 hours and 2 days post-thrombosis (Fig. 2A). There was also a significant decrease in active PAI-1 levels at the 6-hour time-point in rosuvastatin treated animals, compared to saline treated animals (2568 ± 607 vs. 7100 ± 1567 pg/ml, $p < 0.05$). No significant differences were observed at 3 hours and 2 days post-thrombosis (Fig. 2B).

Significant decreases were found in PAI-1 levels in the liver of rosuvastatin treated animals, compared to saline treated animals at the 6-hour time-point (0.0299 ± 0.0100 vs. 0.2090 ± 0.0578 gene/ β -actin, $p < 0.05$), respectively. There were no significant differences at the 3-hour and 2-day post-thrombosis time-points (Fig. 2 C and D). In addition, there was a trend in decrease PAI-1 in the rosuvastatin group but not statistically significant differences in vein wall at all-time points.

Rosuvastatin did not decrease circulating lipids in ApoE $-/-$ mice

No significant differences were observed in cholesterol and triglycerides levels between mice treated with rosuvastatin compared to saline (Figure 3).

Rosuvastatin altered inflammatory marker gene expression

IL-6—IL-6 gene expression was significantly reduced in the IVC wall among the rosuvastatin treated animals at the 2-day time-point, versus saline controls, (0.01441 ± 0.0 vs. 0.05904 ± 0.0 gene/ β -actin, $p < 0.05$). No significant differences in IL-6 were seen in the IVC wall at the 3-hour and 6-hour post-thrombosis time-points (Fig. 4A). There was a significant decrease in IL-6 messenger RNA (mRNA) in the liver in rosuvastatin treated animals at the 3-hour time-point, versus saline controls (0.00051 ± 0.00011 vs. 0.00198 ± 0.00051 gene/ β -actin, $p < 0.05$). No significant differences in IL-6 were seen in the liver at the 6-hour and 2-day post-thrombosis time-points (Fig. 4B).

IL-1 β —There were no significant differences in IL-1 β gene expression in the IVC wall (Fig. 4A) or liver (Fig. 4B) at any of the time-points.

CCL2—There were no significant differences in CCL2 mRNA in the IVC wall (Fig. 4A) or liver (Fig. 4B) of rosuvastatin treated animals, compared to saline treated animals at any time points.

NF- κ B—There was a decreased in NF- κ B gene expression, but no statistically significant in the rosuvastatin group compared to controls at all-time points in the IVC wall (Fig. 4A). In

the liver, NF- κ B mRNA levels were significantly decreased in rosuvastatin treated animals at the 3-hour time-point (0.01216 ± 0.00199 vs. 0.08677 ± 0.01216 gene/ β -actin, $p < 0.05$) and at the 6-hour time-point (0.01191 ± 0.00090 vs. 0.02730 ± 0.00343 gene/ β -actin, $p < 0.05$). No significant differences were found at the 2-day post-thrombosis time-point (Fig. 4B).

STAT3—The transcription factor STAT3 gene expression in vein wall was decreased but no statistically significant in the rosuvastatin group compared to control group at all-time points (Fig. 4A). The STAT3 mRNA levels in the liver were significantly decreased in the rosuvastatin treated animals, compared to saline treated animals, at both the 3-hour time-point (0.03891 ± 0.00665 vs. 0.1378 ± 0.01344 gene/ β -actin, $p < 0.05$) and 6-hour time-point (0.08951 ± 0.01035 vs. 0.13220 ± 0.01182 gene/ β -actin, $p < 0.05$). There was no significant difference in STAT3 in the liver at the 2-day post-thrombosis time-point (Fig. 4B).

C/EBP δ —A reduced in C/EBP δ mRNA levels, but no statistically significant, was found at all-time points in IVC wall of rosuvastatin treated animals, compared to saline treated animals (Fig. 4A). In the liver, there was a significant decrease in C/EBP δ gene expression in rosuvastatin treated animals at the 3-hour time-point (0.054 ± 0.009 vs. 0.096 ± 0.010 , $p < 0.05$). There were no significant differences at the 6-hour and 2-day post-thrombosis time-points between groups (Fig. 4B).

Neutrophil recruitment was decreased by rosuvastatin

Neutrophil recruitment was assessed using immunohistochemistry. Decreased staining at day 2 post-thrombosis in the rosuvastatin group versus the control group was observed (Fig. 5A). Using neutrophil cell counts, there was a significant decrease at the 2-day time-point in animals treated with rosuvastatin compared to those receiving saline treatment (7 ± 1 vs. 15 ± 3 cells/5HPF, $p < 0.05$). There were no significant differences in neutrophil counts at the 3-hour and 6-hour time-points (Fig. 5B).

Discussion

Hyperlipidemia is not considered a risk factor for VT, but we believe this is due to a masking effect from statin therapy. Two important facts support our current work: a- Statins has been reported to have pro-fibrinolytic properties, mainly decreasing PAI-1 (15), and anti-inflammatory effects, mainly decreasing IL-6, CRP and MCP-1 (10, 12, 20–22) but it was never investigated in VT. b- We previously investigated the link between hyperlipidemia and VT in ApoE $^{-/-}$ mice and found impaired fibrinolysis resulting in significantly larger TW, compared to controls using the ligation model (6). This previous work inspired our current hypothesis, which is that statins will contribute to decreased venous thrombogenesis, in part, due to a correction of the impaired fibrinolysis in the hyperlipidemic mice and also due to an anti-inflammatory effect. Additionally, the JUPITER trial brought to our attention the effect of rosuvastatin on VT (5). The JUPITER trial, in which the patients in were not hyperlipidemic, supports the idea that rosuvastatin has non-lipid lowering effects that could potentially prevent VT. Unfortunately, the mechanisms behind these results were not explored. To our knowledge, the profibrinolytic and anti-inflammatory effects of statins have never been investigated in the context of VT and hyperlipidemia.

Inflammation was linked to VT for the first time in 1974 (14), and one of the main inflammatory cytokines, IL-6, has been demonstrated to play a role in this process (17). Sistemic neutralization of IL-6 during early stages of VT, using the IVC ligation mouse model, modulates CCL2 also known as monocyte chemotactic protein-1 (MCP-1) that is responsible for monocyte recruitment and ultimately modulated thrombus resolution (17).

IL-6 is the principal stimulator of most acute-phase proteins and interestingly, PAI-1 is an acute-phase protein that impairs fibrinolysis (6, 23). A decrease in levels of IL-6 and PAI-1 have been reported in patients on rosuvastatin therapy (24). Based on this information, we hypothesized that rosuvastatin would decrease VT in ApoE^{-/-} mice due to its effects on IL-6 and PAI-1.

In this study we used ApoE^{-/-} on a normal diet and the IVC ligation model to be consistent with previous investigations (6). Rosuvastatin was administered prior to thrombosis because it has been reported that statins prevent, rather than treat, VT (5). We used oral gavage to more closely replicate the human route of administration of statins and a dose of 5 mg/kg as previously reported in mice (19). Five mg/kg was chosen as the initial starting dose since it is the average of the doses (1 – 10mg/kg) used in current statins murine models (9). Allometric scaling confirms that 5 mg/kg is within the clinically used human rosuvastatin dose range of 5 to 40 mg/day.

To address the question of whether rosuvastatin would affect VT, we measured TW. Previous experiments in our laboratory have found that IVC size is consistent for animals within a body weight range of 20–25g, therefore eliminating IVC size as a variable and allowing TW to be compared among animals in this body weight range. We found a significant decrease in TW at 2 days after thrombosis in ApoE^{-/-} mice treated with rosuvastatin, compared to controls. Importantly, statins did not suppress thrombogenesis as shown by the increasing TWs overtime in both controls and rosuvastatin groups (Fig. 1A). This was further supported by the absence of bleeding complications following IVC ligation surgery. These results suggest that treatment with rosuvastatin decreases TW without impairing coagulation, which is a concern of most current therapies.

Soluble P-selectin is an established biomarker for VT as confirmed in both patients (25, 26) and mice that have VT (27, 28). Rosuvastatin significantly decreased soluble P-selectin at all time-points compared to controls. It is known that activated endothelial cells and platelets are the main source of soluble P-selectin in VT initiation (16). Rosuvastatin's effect on soluble P-selectin has not been previously demonstrated and could suggest that rosuvastatin prevents or modulates both endothelial and platelet activation. Importantly, soluble P-selectin increased over time in both the control and rosuvastatin groups, following the same trend as TW. This supports the use of soluble P-selectin as a biomarker of VT in mice. This trend led us to investigate the correlation between TW and soluble P-selectin by groups. We found a strong significant positive correlation between TW and soluble P-selectin in both groups (Spearman $r=0.76$ for control group and 0.78 for the rosuvastatin group, both $p<0.0001$). These results further demonstrated the power of soluble P-selectin as a biomarker for VT and also suggest that soluble P-selectin could be considered as a possible biomarker to “monitor” rosuvastatin's effects on VT, at least in the context of hyperlipidemic mice. Further studies are warranted to translate these findings in the context of human disease.

Previous findings, using the same IVC ligation model in ApoE^{-/-} mice, demonstrated that TW was significantly increased in this mouse strain with hyperlipidemia due to impairment of the fibrinolytic system. Particularly, PAI-1, the main regulator of the fibrinolytic system, was significantly increased in ApoE^{-/-} mice compared to controls (6). In addition, the link between statins and PAI-1 was previously reported (29, 30). In order to address the question of how rosuvastatin modulates the fibrinolytic system in hyperlipidemic mice, we tested active and circulating PAI-1 in plasma and PAI-1 gene expression in the IVC and liver, for local and peripheral rosuvastatin effects. Significant decreases in circulating active and total PAI-1 were found 6 hours after thrombosis in the rosuvastatin group. Also, gene expression of PAI-1 was decreased in the IVC and significantly decreased in liver in the rosuvastatin

group at the same time-point. These results suggest that rosuvastatin decreased, but not suppressed, PAI-1, and ultimately improved the fibrinolytic system in hyperlipidemic mice. Our previous and current results support the fact that rosuvastatin regulates the fibrinolytic system that was acutely impaired when VT occurred in ApoE^{-/-} mice (6). This suggests that the early improvement of the fibrinolytic system observed in the rosuvastatin group may have contributed, at least in part, to the decreased TW at day 2, linking rosuvastatin, fibrinolysis and VT in the context of hyperlipidemia.

An increase in cholesterol enhances daily expression of PAI-1 in the mouse liver, impairing the fibrinolytic system (31). It could be argued that statins decrease cholesterol and circulating PAI-1 through a lipid-lowering effect and not by a direct effect on PAI-1 expression. In this work, lowering cholesterol does not appear to be the primary mechanism affecting PAI-1 expression and levels. Our results showed that the significant reduction in PAI-1 expression and levels in liver and plasma was independent of the rosuvastatin effects on circulating lipids. We tested both cholesterol and triglyceride levels in rosuvastatin and control groups and no significant differences were found in our ApoE^{-/-} mice population in concordance with previous reports (Figure 3) (32). The absence of the lipid lowering effect of rosuvastatin could most likely be explained by the fact that ApoE is a strong acceptor of cellular cholesterol and the lack of ApoE in ApoE^{-/-} mice impedes cholesterol cellular intake (33).

There are reports of anti-inflammatory effects of statins in basic science studies (10, 22) and clinical research (20, 34). Specifically, it was reported that statins reduced IL-6 and MCP-1 gene expression in monocytes from hyperlipidemic patients linking hyperlipidemia and inflammation (21). Numerous publications support the fact that gene-expression is a useful tool to determine statin effects on inflammatory markers (22) and PAI-1 (35, 36). Particularly, Jougasaki M, *et al* performed *in-vitro* studies evaluating the effects of statins on the IL-6 pathway using gene-expression in human vascular endothelial cells (22). In the present work, the only difference between groups was the administration of saline vs. rosuvastatin. Thus, the fact that we observed differences in IL-6 gene-expression and other inflammatory markers along with PAI-1, supports the idea that the gene-expression data was sufficient to detect rosuvastatin effects *in-vivo*.

Importantly, we previously reported that CCL2 was significantly reduced when IL-6 was neutralized in mice, using the same IVC ligation model, demonstrating an association between inflammation and VT (17). However, there is no reported data on rosuvastatin's anti-inflammatory effects in the context of hyperlipidemia and VT. We studied the effect of rosuvastatin on gene expression of transcription factors, inflammatory cytokines involved in the IL-6 signaling pathway. Gene expression of NF- κ B, IL-6 and STAT3 was significantly decreased in the rosuvastatin group 3 hours after thrombosis in the liver, compared to controls and decreased, but not significantly, in the vein wall. IL-1 β and CCL2 were decreased (but not significantly) in both, the liver and vein wall of the rosuvastatin group, compared to controls. IL-6 signal transduction is predominantly mediated by the JAK/STAT signaling pathway and STAT3 is a key downstream transcription factor(37). The JAKs IL-6 receptor-associated tyrosine kinase phosphorylates STAT3, promoting its translocation to the nucleus and regulating the acute phase response and inflammation (38). Upstream in the IL-6 pathway, NF- κ B has been described as a transcription factor that regulates IL-6 and STAT3 expression in head and neck squamous cell carcinoma (39). The fact that NF- κ B, IL-6 and STAT3 were significantly decreased early on in the thrombotic process, possibly suggests an initial anti-inflammatory effect of rosuvastatin in the liver through, at least in part, the IL-6 pathway. In the vein wall, there was a decreased trend in STAT3 but the absence of statistically significant data, at this point, makes it difficult to conclude if the IL-6 pathway is dependent or independent on the JAK/STAT signaling pathway. It was also

reported that IL-6 regulates CCL2 expression in myeloma cells (40) and also in VT, using the IVC ligation model (17). CCL2 was decreased in liver and vein wall in the rosuvastatin group, compared to controls. Of note, the actual liver values for gene expression of all inflammatory markers were much lower compared to vein wall.

IL-6 triggers the association between its receptor and the trans-membrane protein glycoprotein 130 (gp130) present in endothelial cells. This mechanism is known as the IL-6 *trans-signaling* pathway. This IL-6 *trans-signaling* pathway has been, reported to regulate cell trafficking promoting expression of selectins (41, 42), including P-selectin (43). If the anti-inflammatory effects of rosuvastatin are modulated, at least in part, by regulation of the IL-6 *trans-signaling* pathway, leukocyte trafficking should be affected. The main inflammatory cells in our mouse model of VT, at the acute time-point (day 2 post thrombosis), are neutrophils. To address the question of whether the IL-6 regulation by rosuvastatin has an impact on leukocyte trafficking, we evaluated the neutrophils in the vein wall by cell counts and IHC staining. The number of neutrophils was significantly decreased in the rosuvastatin group at day 2, compared to controls. This was demonstrated by both IHC and cell counts (Figure 5A and B) further demonstrating the anti-inflammatory effects of rosuvastatin. An interesting concept for future investigations would include distinguishing cell counts in the different layers of the vein wall.

Finally, it was reported that IL-6 induces PAI-1 mRNA and protein (35, 36) and that this mechanism involves the transcription factor C/EBP δ (36). We found that the liver C/EBP δ gene expression was significantly decreased in the rosuvastatin group 3 hours after thrombosis, compared to controls. As we mentioned previously, PAI-1 expression in the liver was significantly decreased by rosuvastatin 6 hours after thrombosis, showing a delay related to the NF- κ B/IL-6/STAT3 findings. This suggests a downstream regulation of PAI-1 by IL-6 in the context of hyperlipidemia in VT. Our findings are summarized in Figure 6, connecting the anti-inflammatory and pro-fibrinolytic effects of rosuvastatin in VT using hyperlipidemic mice observed during thrombogenesis at acute time points. Further investigation will evaluate the effects of rosuvastatin at chronic time points.

In conclusion, rosuvastatin had an immediate and sustained effect on decreasing soluble P-selectin at all-time points leading to speculate a major and dominating function of rosuvastatin in decreasing thrombosis in this IVC ligation model. Given that rosuvastatin affects the hepatocyte function by inhibiting HMG-CoA reductase, it would appear that the primary events occur in the liver, while the secondary effects of rosuvastatin on the vein wall are a result of the inhibition of liver production of circulating pro-inflammatory and anti-fibrinolytic molecules. In addition, hyperlipidemia could be a risk factor for VT but this is masked by statin therapy. This work supports, for the first time, the fact that rosuvastatin has pleiotropic effects, independent of lipid-lowering effects, that significantly decreased VT in ApoE $^{-/-}$ mice. These effects involved anti-inflammatory properties of rosuvastatin, mainly through regulation of upstream and downstream transcription factors related to IL-6 *trans-signaling* pathway and improvement of an impaired fibrinolytic system in ApoE $^{-/-}$ mice with hyperlipidemia. Our previous (6) and current work supports this conclusion in hyperlipidemic mice. Further investigation in the clinical setting is warranted in order to investigate this concept in VT patients, since the involvement of statins in DVT prevention is an attractive avenue to help decrease side-effects of current DVT therapy.

Acknowledgments

We would like to thank Robert E. Sigler DVM, DACVP for the histopathology evaluation, Mark Warnock BS for performing the PAI-1 assay, and Carrie Schray MA, HT (ASCP), Paula Arrowsmith BS, HT (ASCP), Ingrid Begin MS, VMD, DACLAM, DACVP, and Kathryn Eaton DVM, PhD, DACVP for assistance with the immunohistochemistry.

Sources of Funding Supported by NIH 1P01HL089407-01A1 (Lawrence, PI), Animal Core A, NIH 1 K01 HL080962-01A2 (Myers, PI) and University of Michigan CVC McKay grant (Diaz, PI).

Abbreviations

VT	venous thrombosis
IVC	inferior vena cava
TW	thrombus weight
PAI-1	plasminogen activator inhibitor-1
IL-6	interleukin-6
VTE	venous thromboembolism
DVT	deep vein thrombosis
PE	pulmonary embolism
JUPITER	justification for the use of statins in prevention: an intervention trial evaluating rosuvastatin
CRP	c-reactive protein
ApoE^{-/-}	apolipoprotein E genetically deleted
STAT3	signal transducer and activator of transcription 3
IL1-β	interleukin-1 beta
WT	wild type
TC	true controls
sPsel	soluble p-selectin
qRT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
NF-κB	nuclear factor kappa-light chain enhancer of activated B cells
CCL-2	chemokine ligand 2
C/EBPδ	CCAAT/enhancer binding protein delta
H&E	hematoxylin and eosin
HPFs	high power fields
mRNA	messenger ribonucleic acid
MCP-1	monocyte chemoattractant protein-1
gp130	glycoprotein 130
SS	saline solution
RV	rosuvastatin
SEM	standard error of the mean
H	hours
D	days

References

1. Heit JA. The epidemiology of venous thromboembolism in the community. *Arterioscler Thromb Vasc Biol.* [Research Support, N.I.H., Extramural, Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. Mar; 2008 28(3):370–2.
2. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. *J Thromb Haemost.* [Research Support, Non-U.S. Gov't]. Apr; 2007 5(4):692–9.
3. White RH, Keenan CR. Effects of race and ethnicity on the incidence of venous thromboembolism. *Thromb Res.* [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review]. 2009; 123(Suppl 4):S11–7.
4. Silverstein MD, Heit JA, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ 3rd. Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study. *Arch Intern Med.* [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. Mar 23; 1998 158(6):585–93.
5. Glynn RJ, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, et al. A randomized trial of rosuvastatin in the prevention of venous thromboembolism. *N Engl J Med.* [Multicenter Study Randomized Controlled Trial Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. Apr 30; 2009 360(18):1851–61.
6. Diaz JA, Ballard-Lipka NE, Farris DM, Hawley AE, Wroblewski SK, Myers DD, et al. Impaired fibrinolytic system in ApoE gene-deleted mice with hyperlipidemia augments deep vein thrombosis. *J Vasc Surg.* Mar; 2012 55(3):815–22. [PubMed: 22119245]
7. Gotto AM Jr. Safety and statin therapy: reconsidering the risks and benefits. *Arch Intern Med.* [Comment Editorial]. Mar 24; 2003 163(6):657–9.
8. Gotto AM Jr. Statins, cardiovascular disease, and drug safety. *Am J Cardiol.* [Review]. Apr 17; 2006 97(8A):3C–5C.
9. Monetti M, Canavesi M, Camera M, Parente R, Paoletti R, Tremoli E, et al. Rosuvastatin displays anti-atherothrombotic and anti-inflammatory properties in apoE-deficient mice. *Pharmacol Res.* [Research Support, Non-U.S. Gov't]. May; 2007 55(5):441–9.
10. Arnaud C, Burger F, Steffens S, Veillard NR, Nguyen TH, Trono D, et al. Statins reduce interleukin-6-induced C-reactive protein in human hepatocytes: new evidence for direct antiinflammatory effects of statins. *Arterioscler Thromb Vasc Biol.* [Research Support, Non-U.S. Gov't]. Jun; 2005 25(6):1231–6.
11. Schonbeck U, Libby P. Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation.* [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. Jun 1; 2004 109(21 Suppl 1):II18–26.
12. Jain MK, Ridker PM. Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. *Nat Rev Drug Discov.* [Research Support, N.I.H., Extramural Review]. Dec; 2005 4(12):977–87.
13. Undas A, Celinska-Lowenhoff M, Domagala TB, Iwaniec T, Dropinski J, Lowenhoff T, et al. Early antithrombotic and anti-inflammatory effects of simvastatin versus fenofibrate in patients with hypercholesterolemia. *Thromb Haemostasis.* Jul; 2005 94(1):193–9. [PubMed: 16113803]
14. Stewart GJ, Ritchie WG, Lynch PR. Venous endothelial damage produced by massive sticking and emigration of leukocytes. *Am J Pathol.* Mar; 1974 74(3):507–32. [PubMed: 4814899]
15. Laumen H, Skurk T, Hauner H. The HMG-CoA reductase inhibitor rosuvastatin inhibits plasminogen activator inhibitor-1 expression and secretion in human adipocytes. *Atherosclerosis.* [Research Support, Non-U.S. Gov't]. Feb; 2008 196(2):565–73.
16. Myers D Jr, Farris D, Hawley A, Wroblewski S, Chapman A, Stoolman L, et al. Selectins influence thrombosis in a mouse model of experimental deep venous thrombosis. *J Surg Res.* Dec; 2002 108(2):212–21. [PubMed: 12505044]
17. Wojcik BM, Wroblewski SK, Hawley AE, Wakefield TW, Myers DD Jr, Diaz JA. Interleukin-6: a potential target for post-thrombotic syndrome. *Ann Vasc Surg.* Feb; 2011 25(2):229–39. [PubMed: 21131172]

18. Li JJ, Yang P, Liu J, Jia YJ, Li ZC, Guo YL, et al. Impact of 10 mg rosuvastatin daily or alternate-day on lipid profile and inflammatory markers. *Clin Chim Acta*. [Research Support, Non-U.S. Gov't]. Jan 18; 2012 413(1–2):139–42.
19. Calkin AC, Giunti S, Sheehy KJ, Chew C, Boolell V, Rajaram YS, et al. The HMG-CoA reductase inhibitor rosuvastatin and the angiotensin receptor antagonist candesartan attenuate atherosclerosis in an apolipoprotein E-deficient mouse model of diabetes via effects on advanced glycation, oxidative stress and inflammation. *Diabetologia*. [Research Support, Non-U.S. Gov't]. Sep; 2008 51(9):1731–40.
20. Albert MA, Danielson E, Rifai N, Ridker PM. Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study. *JAMA*. Jul 4; 2001 286(1):64–70. [PubMed: 11434828]
21. Rezaie-Majd A, Maca T, Bucek RA, Valent P, Muller MR, Husslein P, et al. Simvastatin reduces expression of cytokines interleukin-6, interleukin-8, and monocyte chemoattractant protein-1 in circulating monocytes from hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol*. Jul 1; 2002 22(7):1194–9. [PubMed: 12117737]
22. Jougasaki M, Ichiki T, Takenoshita Y, Setoguchi M. Statins suppress interleukin-6-induced monocyte chemo-attractant protein-1 by inhibiting Janus kinase/signal transducers and activators of transcription pathways in human vascular endothelial cells. *Br J Pharmacol*. Mar; 2010 159(6):1294–303. [PubMed: 20136831]
23. Rerolle JP, Hertig A, Nguyen G, Sraer JD, Rondeau EP. Plasminogen activator inhibitor type 1 is a potential target in renal fibrogenesis. *Kidney Int*. Nov; 2000 58(5):1841–50. [PubMed: 11044203]
24. Rodriguez AL, Wojcik BM, Wroblewski SK, Myers DD Jr, Wakefield TW, Diaz JA. Statins, inflammation and deep vein thrombosis: a systematic review. *J Thromb Thrombolysis*. May; 2012 33(4):371–82. [PubMed: 22278047]
25. Rectenwald JE, Myers DD Jr, Hawley AE, Longo C, Henke PK, Guire KE, et al. D-dimer, P-selectin, and microparticles: novel markers to predict deep venous thrombosis. A pilot study. *Thromb Haemost*. Dec; 2005 94(6):1312–7. [PubMed: 16411411]
26. Ramacciotti E, Blackburn S, Hawley AE, Vandy F, Ballard-Lipka N, Stabler C, et al. Evaluation of soluble P-selectin as a marker for the diagnosis of deep venous thrombosis. *Clin Appl Thromb Hemost*. Aug; 2011 17(4):425–31. [PubMed: 21593019]
27. Diaz JA, Hawley AE, Alvarado CM, Berguer AM, Baker NK, Wroblewski SK, et al. Thrombogenesis with continuous blood flow in the inferior vena cava. A novel mouse model. *Thromb Haemost*. Aug; 2010 104(2):366–75. [PubMed: 20589322]
28. Diaz JA, Wroblewski SK, Hawley AE, Lucchesi BR, Wakefield TW, Myers DD Jr. Electrolytic inferior vena cava model (EIM) of venous thrombosis. *J Vis Exp*. 2011; (53):e2737. [PubMed: 21775963]
29. Dunoyer-Geindre S, Fish RJ, Kruithof EK. Regulation of the endothelial plasminogen activator system by fluvastatin. Role of Rho family proteins, actin polymerisation and p38 MAP kinase. *Thromb Haemost*. Mar; 2011 105(3):461–72. [PubMed: 21174002]
30. Rosenson RS, Tangney CC. Antiatherothrombotic properties of statins: implications for cardiovascular event reduction. *JAMA*. May 27; 1998 279(20):1643–50. [PubMed: 9613915]
31. Kudo T, Nakayama E, Suzuki S, Akiyama M, Shibata S. Cholesterol diet enhances daily rhythm of *Pai-1* mRNA in the mouse liver. *Am J Physiol Endocrinol Metab*. Oct; 2004 287(4):E644–51. [PubMed: 15361354]
32. Bea F, Blessing E, Bennett B, Levitz M, Wallace EP, Rosenfeld ME. Simvastatin promotes atherosclerotic plaque stability in apoE-deficient mice independently of lipid lowering. *Arterioscler Thromb Vasc Biol*. Nov 1; 2002 22(11):1832–7. [PubMed: 12426212]
33. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM, et al. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler Thromb Vasc Biol*. Aug; 2007 27(8):1706–21. [PubMed: 17541027]
34. Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, et al. C-reactive protein levels and outcomes after statin therapy. *N Engl J Med*. Jan 6; 2005 352(1):20–8. [PubMed: 15635109]

35. Dong J, Fujii S, Goto D, Furumoto T, Kaneko T, Zaman TA, et al. Increased expression of plasminogen activator inhibitor-1 by mediators of the acute phase response: a potential progenitor of vasculopathy in hypertensives. *Hypertens Res.* Sep; 2003 26(9):723–9. [PubMed: 14620928]
36. Dong J, Fujii S, Li H, Nakabayashi H, Sakai M, Nishi S, et al. Interleukin-6 and mevastatin regulate plasminogen activator inhibitor-1 through CCAAT/enhancer-binding protein-delta. *Arterioscler Thromb Vasc Biol.* May; 2005 25(5):1078–84. [PubMed: 15718495]
37. Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene.* May 15; 2000 19(21):2468–73. [PubMed: 10851045]
38. Yu Z, Zhang W, Kone BC. Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor kappaB. *Biochem J.* Oct 1; 2002 367(Pt 1):97–105. [PubMed: 12057007]
39. Squarize CH, Castilho RM, Sriuranpong V, Pinto DS Jr. Gutkind JS. Molecular cross-talk between the NFkappaB and STAT3 signaling pathways in head and neck squamous cell carcinoma. *Neoplasia.* Sep; 2006 8(9):733–46. [PubMed: 16984731]
40. Arendt BK, Velazquez-Dones A, Tschumper RC, Howell KG, Ansell SM, Witzig TE, et al. Interleukin 6 induces monocyte chemoattractant protein-1 expression in myeloma cells. *Leukemia.* Oct; 2002 16(10):2142–7. [PubMed: 12357369]
41. Vardam TD, Zhou L, Appenheimer MM, Chen Q, Wang WC, Baumann H, et al. Regulation of a lymphocyte-endothelial-IL-6 trans-signaling axis by fever-range thermal stress: hot spot of immune surveillance. *Cytokine.* Jul; 2007 39(1):84–96. [PubMed: 17903700]
42. Chen Q, Fisher DT, Clancy KA, Gauguier JM, Wang WC, Unger E, et al. Fever-range thermal stress promotes lymphocyte trafficking across high endothelial venules via an interleukin 6 trans-signaling mechanism. *Nat Immunol.* Dec; 2006 7(12):1299–308. [PubMed: 17086187]
43. Fisher DT, Chen Q, Skitzki JJ, Muhitch JB, Zhou L, Appenheimer MM, et al. IL-6 trans-signaling licenses mouse and human tumor microvascular gateways for trafficking of cytotoxic T cells. *J Clin Invest.* Oct; 2011 121(10):3846–59. [PubMed: 21926464]

Effects of rosuvastatin on IVC/thrombus weight, soluble P-selectin and correlation between IVC/thrombus weight and soluble P-selectin

Figure 1. Effects of rosuvastatin in IVC/Thrombus Weight-Soluble P Selectin-Correlation
A: Weights, in grams, of thrombus from mice treated with saline solution (SS; solid bars) or rosuvastatin (Rv; hatched bars). Bars represent $\pm$ SEM of tissue harvested from indicated number of mice. **B:** Plasma Soluble P-Selectin normalized to total protein from mice treated with SS (solid bars) or Rv (hatched bars). Bars represent $\pm$ SEM. **C:** Thrombus weight and soluble P-selectin correlation in control group and **D:** Thrombus weight and soluble P-selectin correlation in rosuvastatin group. Asterisk indicates significant difference between rosuvastatin treatment and controls at matching time-points. SS=saline solution, Rv=rosuvastatin, TC=true control, H=hours, D=days, post-thrombosis; n=5 for TC; n=10 for each time-point group.

Figure 2. Total and Active PAI-1 in Plasma and PAI-1 Relative Gene Expression in Vein Wall and Liver

A: Effect of rosuvastatin on total PAI-1 levels in plasma and **B:** on active PAI-1 in plasma **C:** **PAI-1** gene expression determined by quantitative real-time polymerase chain reaction (qRT-PCR) in vein wall. **D:** PAI-1 gene expression determined by qRT-PCR in the liver. Asterisks indicate significant difference between rosuvastatin treated and saline treated animals at matching time-points (n=5 for true control, n=10 for each time-point group).

Rosuvastatin does not decrease circulating lipids

Figure 3. Rosuvastatin does not decrease circulating lipids in ApoE^{-/-} mice

A: Quantitative determination of free cholesterol in serum in mice treated with saline (solid bar) or rosuvastatin (hatched bar). **B:** Quantitative determination of free triglycerides in serum in mice treated with saline (solid bar) or rosuvastatin (hatched bar).

Effects of rosuvastatin on transcription factors, cytokines and chemokine gene expression

Figure 4. Effects of Rosuvastatin on Transcription Factors, Cytokines and Chemokine Relative Gene Expression in Vein Wall and Liver

A: Effect of rosuvastatin in vein wall gene expression of IL-6, IL-1 β , CCL2 (top panel), and NF- κ B, STAT3, and C/EBP6 (lower panel) determined by quantitative real-time polymerase chain reaction (qRT-PCR) in mice treated with saline (solid bars) or rosuvastatin (hatched bars). **B:** Gene expression of IL-6, IL-1 β , CCL2 (top panel), and NF- κ B, STAT3, and C/EBP6 (lower panel) in liver of mice treated with saline (solid bars) or rosuvastatin (hatched bars), determined by qRT-PCR; n=5 for TC; n=10 for each time-point group.

Vein wall neutrophil assessment

Figure 5. Neutrophil assessment

A. Immunohistochemistry: Representative tissue sections (pictures at 200×) stained with a rat anti-mouse neutrophil marker NIMP-R14 demonstrating the degree of neutrophil recruitment to the vein wall and thrombus in mice treated with saline or rosuvastatin harvested at day-2 post-thrombosis. **B. Morphometrics:** Number of neutrophils present in vein wall of mice treated with saline (solid bars) or rosuvastatin (hatched bars). Counts were determined using H&E stained sections. Bars represent $\pm$ SEM of 5 HPF per-slide. Asterisk indicates a significant difference between rosuvastatin treated and saline treated animals at coordinating time-points SS=saline solution, Rv=rosuvastatin, TC=true control, H=hours, D=days, post-thrombosis; n=5 for TC; n=10 for each time-point group.

Figure 6.
Proposed Anti-inflammatory and Pro-fibrinolytic Mechanisms of Rosuvastatin Effects in ApoE^{-/-} mice with hyperlipidemia.