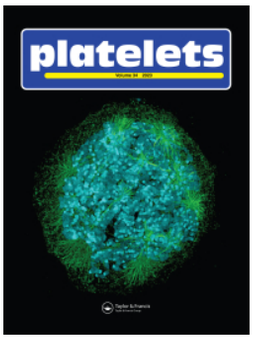

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RESEARCH ARTICLE

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Atorvastatin-mediated inhibition of prenylation of Rab27b and Rap1a in platelets attenuates their prothrombotic capacity and modulates clot structure

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Abstract

Statins inhibit the mevalonate pathway by impairing protein prenylation via depletion of lipid geranylgeranyl diphosphate (GGPP). Rab27b and Rap1a are small GTPase proteins involved in dense granule secretion, platelet activation, and regulation. We analyzed the impact of statins on prenylation of Rab27b and Rap1a in platelets and the downstream effects on fibrin clot properties. Whole blood thromboelastography revealed that atorvastatin (ATV) delayed clot formation time ($P < .005$) and attenuated clot firmness ($P < .005$). ATV pre-treatment inhibited platelet aggregation and clot retraction. Binding of fibrinogen and P-selectin exposure on stimulated platelets was significantly lower following pre-treatment with ATV ($P < .05$). Confocal microscopy revealed that ATV significantly altered the structure of platelet-rich plasma clots, consistent with the reduced fibrinogen binding. ATV enhanced lysis of Chandler model thrombi 1.4-fold versus control ($P < .05$). Western blotting revealed that ATV induced a dose-dependent accumulation of unprenylated Rab27b and Rap1a in the platelet membrane. ATV dose-dependently inhibited ADP release from activated platelets. Exogenous GGPP rescued the prenylation of Rab27b and Rap1a, and partially restored the ADP release defect, suggesting these changes arise from reduced prenylation of Rab27b. These data demonstrate that statins attenuate platelet aggregation, degranulation, and binding of fibrinogen thereby having a significant impact on clot contraction and structure.

Plain Language Summary

What is the context?

- Statins such as Atorvastatin (ATV) are 3-hydroxy, 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which block the cholesterol biosynthetic pathway to lower total serum levels and LDL-cholesterol.
- The cholesterol pathway also provides a supply of isoprenoids (farnesyl and geranylgeranyl) for the prenylation of signaling molecules, which include the families of Ras and Rho small GTPases.
- Prenyl groups provide a membrane anchor that is essential for the correct membrane localization and function of these proteins.
- Statins deplete cells of lipid geranylgeranyl diphosphate (GGPP) thereby inhibiting progression of the mevalonate pathway and prenylation of proteins.
- Rab27b and Rap1 are small GTPase proteins in platelets that are involved in the secretion of platelet granules and integrin activation.

Keywords
Fibrin, platelets, Rab27b, Rap1a, statins, thrombosis

History
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What is new?

- In this study, we found that ATV impairs prenylation of Rab27b and Rap1a and attenuates platelet function.
- These effects were partially rescued by GGPP, indicating the involvement of the mevalonate pathway.
- Platelet aggregation and degranulation was significantly attenuated by ATV.
- The impact of statins on platelet function altered clot formation, structure and contraction generating a clot that was more susceptible to degradation.

What is the impact?

- This study demonstrates a novel mechanism whereby statins alter platelet responses and ultimately clot structure and stability.

Introduction

Platelets are finely tuned to modulate the response to mechanical injury or vascular breach. A crucial characteristic of platelet function is packaging and uptake of cargo that is subsequently released upon platelet activation. Membrane trafficking is also imperative to granule biogenesis and maturation in megakaryocytes and platelets.[1] Small guanosine triphosphatases (GTPases)[2,3] act as molecular switches cycling between an inactive GDP-bound' form to an active "GTP-bound" form to regulate effector proteins which are crucial for trafficking events. GTPases localize to the cytosolic face of subcellular vesicles[3] and modulate platelet function at several levels including regulation of platelet activation, integrin exposure[4,5] and granule secretion.[6,7] Rap1 GTPase belongs to the Ras family[8] and exists as two isoforms Rap1a and Rap1b, which are encoded by separate genes but share approximately 95% sequence identity. Rap1a and Rap1b function in activation of the integrins αIIbβ3 and β1 and consequently impact on fibrinogen binding, aggregation, and cytoskeletal responses, including spreading.[5,9] Rap1b is the predominant isoform Ras family member in platelets[10] and plays additional roles over Rap1a in terms of α-granule secretion.[4]

Platelets harbor about 40 Rab GTPases, which range in level of copy number.[11] Rab27a and Rab27b are two isoforms[12] which function in trafficking of platelet dense granules.[13,14] Rab27b-/- mice reveal a 50% reduction in the number of dense granules per platelet and impaired exocytosis of dense but not alpha granule secretion.[13] These mice also exhibit impaired aggregation and prolonged bleeding times.[13] Rab GTPases are dependent on the post-translational addition of a lipid tag, known as prenylation, for their correct localization. A mouse strain, gunmetal (*Rggtagm*), bearing a mutation in Rab geranylgeranyl transferase (RGGT), the enzyme that carries out this modification, accumulates unprenylated Rab GTPases in platelets and exhibits prolonged bleeding times.[15]

The 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors (statins) are extensively prescribed as a first-line agent for lowering serum concentrations of LDL via inhibition of the mevalonate pathway required for cholesterol synthesis.[16–18] Inhibition of this pathway also inhibits the downstream pathways resulting in impaired prenylation of proteins by depleting cells of lipid including farnesylpyrophosphate (FPP) and its derivative geranylgeranyl-PP (GGPP).[17,19] Both FPP and GGPP are lipid substrates that are essential for protein prenylation further downstream of the mevalonate pathway. Depleting cells of FPP and GGPP by statins can attenuate protein prenylation.[19]

Inhibition of prenylation may offer an additional beneficial effect of statins, by interfering with the function of small GTPases. The aim of this study is to investigate whether Atorvastatin (ATV), one of the more potent and widely used statins, can directly inhibit prenylation of Rab and Rap in platelets and thereby interfere with clot formation, contraction, and stability.

Materials and methods

Blood collection and preparation of plasma

All blood samples were obtained from healthy donors after approval from the University of Aberdeen College Ethics Review Board, in accordance with the Declaration of Helsinki and after obtaining written consent. Peripheral blood was collected into sodium citrate 3.2% or acid citrate dextrose (ACD) solution A vacuettes (Greiner Bio-one LTD, Stonehouse, UK). Platelet-rich plasma (PRP) was prepared by centrifugation at 170 × *g* for 10 min at room temperature. The remaining sample was further centrifuged at 1860 × *g* for 30 min at 4°C to obtain platelet-poor plasma (PPP). Pooled normal plasma (PNP) was prepared from blood from at least 20 healthy donors by centrifuging at 1860 × *g* for 30 min at 4°C.

Preparation of washed platelets

Washed human platelets were isolated from outdated human apheresis platelets, kindly provided by the Scottish National Blood Transfusion Service, Aberdeen, as previously described[20] or isolated from whole blood freshly collected in ACD tubes.[21] Cell counts were adjusted to a final concentration of 2.5×10^8 platelets/ml in resuspension buffer, unless otherwise as stated.

Thromboelastography

Thromboelastography (ROTEM® Analysis) was performed, according to manufacturer's instructions, in whole blood pre-treated with ATV (0–40 μM; Sigma-Aldrich, UK) for 5 min or 24 h at 37°C. EXTEM and FIBTEM tests were performed which are both initiated with tissue factor but cytochalasin D (cytoD) is additionally present in FIBTEM to eliminate the platelet contribution to the clot and focus only on the fibrin component.

Turbidity assays

Plasma clots were formed with 30% PRP or PPP as previously described[22] following pretreatment overnight with ATV (0–40 μM). Clotting was initiated with (20 μM) thrombin receptor activator peptide 6 (TRAP-6; Sigma-Aldrich-UK) and $CaCl_2$ (10 mM) and monitored at 405 nm for 30 min at 37°C a FLX-800 plate reader (Biotek Instruments). After this, the pre-formed clots were overlaid with tPA (5 nM; Genentech) to induce clot lysis and absorbance read for a further 6 h at 37°C.

Thrombodynamic analysis

Thrombodynamic analysis was performed using the Hemacore T2F thrombodynamic analyzer according to the manufacturer's instructions with the following modifications. PRP was treated

overnight with ATV (0–40 μM) at 37°C before addition of tPA (5 nM). Clotting was initiated by insertion of a tissue factor (TF)-coated comb. Readings were taken every 6 sec for 1 h at 37°C. Full lysis position was calculated using Karmin software (Hemacore).

Chandler model thrombi

Whole blood was incubated with increasing concentrations of ATV (0–40 μM) for 24 h at 37°C before forming model thrombi in a Chandler system as previously described.[23] Thrombi were then removed, washed in 0.9% (w/v) saline, blotted in filter paper, and lysed by bathing in tPA (15 nM) at 37°C. The released fluorescence was read at 525 nm.

Clot retraction

PRP was pre-treated with increasing concentrations of ATV (0–40 μM) or with inhibitors including CytoD (10 μM; Sigma-Aldrich, UK) or Tirofiban (1 μg/mL; Sigma-Aldrich, UK) for 24 h at 37°C. PRP (33.3%) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) was clotted with thrombin (0.125 U/ml) and $CaCl_2$ (2 mM) and incubated for 2 h at 37°C. Percentage of clot retraction was determined as both the clot weight and by measuring the tube weight before and after removal of the clot.[24]

ADP assay

Washed platelets (2×10^8 platelets/ml) were incubated with increasing concentrations of ATV (0–40 μM) for 24 h at 37°C. Platelet releasate was prepared by activating with 0.125 U/ml thrombin for 30 min at 37°C before centrifuging at 13 000 × g for 5 min. The supernatant was collected and quantified using the luciferin-luciferase luminescence kit (Sigma-Aldrich) which quantifies ATP within a given samples from which the ADP concentration can be derived from a standard curve.

Flow cytometry

Washed platelets (2×10^8 platelets/ml) in Hepes buffer (pH 7.45) containing $CaCl_2$ (2 mM) were incubated for 24 h with increasing concentration of ATV (0–40 μM) at 37°C. Samples were stimulated with thrombin (0.125 U/ml) for 45 min as previously described[22,25] in the presence of fluorescently labeled antibodies to either PAC-1 (1/20) (Becton Dickinson Immunocytometry System) or P-Selectin (1/20; BioLegend, San Diego). In some experiments (DL488)-Fibrinogen (37 μg/mL) or AlexaFluor (AF647)-Annexin V (1/20; BD Biosciences) was added after 40 min of stimulation. Binding of PAC-1, P-Selectin or fibrinogen was measured using an LSR II flow cytometer (Becton Dickinson). A minimum of 10 000 events were collected. Data analysis was performed using FlowJo V. X.0.6 software (Tree Star Inc.) and results are expressed as fold change in MFI ± SEM.

Fluorescence confocal imaging

PRP was incubated for 24 h at 37°C with increasing concentration of ATV (0–40 μM). Clots were prepared as described[25] with incorporation of 0.25 μM labeled fibrinogen-Alex fluor 647 (AF647; Thermo Fisher Scientific) and 0.5 μg/ml $DiOC_6$ to visualize platelets. Initially, slides were coated with collagen (20 μg/ml) to tether the platelets to the surface for the live imaging. Then PRP was activated with TRAP-6 (20 μM) to observe the effects of ATV on α-granule protein secretion and binding. Fluorescently labeled antibodies to either P-selectin (1/20), fibrinogen (37 μg/mL) PAC-1 (1/20), CD41 (1/20) or plasminogen activator inhibitor-1 (PAI-1, 5.8 μg/ml) were included during stimulation. After 30 min incubation, Alexa Fluor conjugated annexin V (AF488-annexin V; 1/20, Life Technologies) was added in the presence of $CaCl_2$ (2 mM) for a further 5 min. Images were obtained on Zeiss 710 laser scanning confocal microscope with a × 631.40 oil immersion objective using Zeiss Zen 2012 software. Analysis was performed on Bitplane's Imaris × 64 software or Zen 2012 software.

Platelet aggregation

Platelet aggregation was performed in a PAP-8e Platelet Aggregation Profiler according to the manufacturer's instructions with the following modifications. Washed platelets (2×10^8 platelets/ml) were incubated with increasing concentrations of ATV (0–40 μM) for 5 min, 6 h or 24 h at 37°C. Aggregation was initiated at 37°C using thrombin (0.125 U/ml; Sigma-Aldrich) and monitored in the aggregometer for 5 min. Samples were analyzed in duplicate and the result was expressed as a percentage of maximum aggregation (MA %).

Western blot analysis of protein prenylation

Platelets were seeded into 12-well tissue culture plate (Greiner) to give 3.75×10^8 platelets per well and treated with ATV (0–40 μM) ± geranylgeranyl pyrophosphate (GGPP) (10 μM, (Sigma-Aldrich) or 20% DMSO as a vehicle control, for 24 h at 37°C with gentle agitation. Platelets were harvested by gentle pipetting and collected by centrifuging at 1500 × *g* before lysing in Triton X-114 buffer (20 mM Tris, 150 mM NaCl, pH 7.5, 1% Triton X-114) as described.[26]

Platelet lysates (30 μg) were separated under reducing conditions and transferred to nitrocellulose. Blots were then hybridized using polyclonal antibodies to Rab27b (kindly provided by Dr. Tanya Tolmachova, Imperial Collage London)[27,28] diluted 1/400 in SEA-BLOCK (1:1 with TBST: 10 mM Tris; 140 mM Nacl pH 7.4, 0.01% Tween 20), Rap1a (sc1482; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/1000 in SEA-BLOCK (1:1 with TBST) or β-actin (A5060; Sigma-Aldrich-UK) diluted 1/2000 SEA-BLOCK (1:1 with TBST). Donkey-anti-rabbit 800 or donkey-anti-Goat 680 (1/15,000; Invitrogen) and protein bands visualized using LI-COR Odyssey imager (LI-COR, USA). Relative protein concentration were measured in relation to the intensity of band using ImageJ 1.47 v software. Results were expressed as a fold change relative to the control.

Data analysis

Data analysis was performed in GraphPad Prism® 5.04. Statistical analysis determined by one-way analysis of variance with Dunnett's multiple comparison post-hoc test. P values of $< .05$ were considered significant. Rates of lysis (FU/min−1) for Chandler model thrombi were determined by slope best fit to a centered second-order polynomial quadratic in GraphPad Prism 5.04, and used to calculate fold differences in lysis.

Results

Clot formation and structure is modulated by atorvastatin

We initially evaluated the effect of statins on coagulation and viscoelastic properties using thromboelastography. Pre-treatment of whole blood with ATV for 5 min did not alter EXTEM and

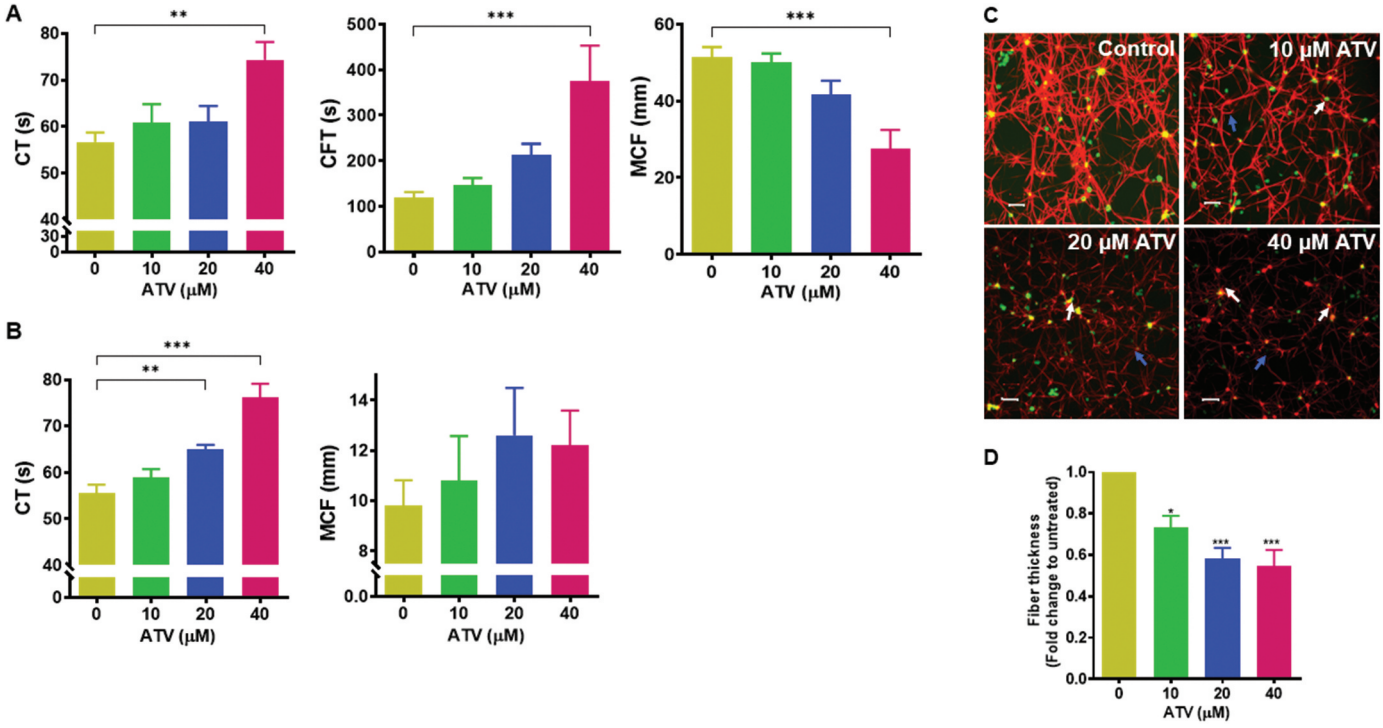

Figure 1. Clot formation and structure is modulated by atorvastatin. Coagulation time (CT), maximum clot firmness (MCF) and CFT were examined using thromboelastography with (a) Extem and (b) Fibtem tests in whole blood pre-treated overnight with ATV (0, 10, 20, 40 μM). (c) PRP was treated overnight with ATV (0, 10, 20 and 40 μM) before clotting was induced by 0.125 U/ml thrombin and 10 mM $CaCl_2$ in the presence of Alexa 647-labeled fibrinogen (red), and DIOC6-labeled platelets (green). Arrows point to the reduction in platelet aggregates (white) and thinner fibrin fibers (blue). Images were recorded on a LSM710 Zeiss Confocal microscope using a × 631.4 oil immersion objective was used together with Zeiss Zen 2012 software. In total, 25 images (Z-stacks) were taken from three different fields for each sample. Images are representative of $n = 8$. Scale bars represent 10 μm. (d) Quantification of fibrin fibers using Angio Tool software 0.6. *P < .05, **P < .01, ***P < .001 compared to 0 μM ATV ($n \geq 3$).

FIBTEM coagulation parameters (data not shown). In contrast, incubation of ATV (40 μM) with whole blood for 24 h, delayed the clot time (CT) in the EXTEM test (74.3 ± 3.8 s, $P < .01$) compared to the control (56.7 ± 2.1 s) (Figure 1a). Clot formation time (CFT) was significantly delayed with 40 μM ATV (375.12 ± 78 vs. 119.10 ± 12.4 s, $P < .001$) and maximum clot firmness (MCF) reduced (27.5 ± 4.9 vs. 51.5 ± 2.6 mm, $P < .001$) compared to the control. Changes in the MCF value are usually indicative of changes in platelet function and/or fibrinogen concentration.

To test whether the reduced clot firmness arises from platelet dysfunction, we performed a FIBTEM test. Again, there was a significant prolongation of the CT in the FIBTEM analysis after treatment of whole blood for 24 h with ATV (65 ± 0.9 s, $P < .05$ and 76.2 ± 2.9 s, $P < .001$ at 20 μM and 40 μM ATV respectively) compared to the control (55.6 ± 1.7 s) (Figure 1b). No significant changes in the MCF in the FIBTEM analysis were observed. These data indicate that the changes in MCF values with ATV observed in the EXTEM test arise due to platelet dysfunction.

The direct impact of ATV on fibrin clot structure revealed significant differences following pre-treatment of PRP with ATV (Figure 1c). Quantification of fibrin fibers revealed a significant reduction in fiber diameter and fiber length in ATV-treated PRP versus control (Figure 1d).

Atorvastatin enhances fibrinolysis

Pre-treatment of PRP for 24 h with ATV significantly impaired time to maximum absorbance compared to untreated samples (21.73 ± 1.6 min vs. 16.46 ± 0.86 min, $P < 0.01$) (Figure 2a). Maximal absorbance was also significantly reduced in the presence of ATV (40 μM) compared to the control (0.50 ± 0.03 vs. 0.57 ± 0.05, $P < .001$). These data indicate that ATV can delay fibrin clot formation consistent with the altered clot structure. Following clot formation tPA was added to the surface to induce clot lysis (Figure 2b). Preincubation of PRP with ATV produced clots that were significantly more susceptible to tPA-mediated lysis than the control (Figure 2b; $P < .05$).

The effect of ATV on tPA-mediated lysis may arise due to fibrinogen dysfunction rather than platelet-mediated effects. We therefore tested the effect of 24 h incubation of ATV in the absence of platelets using PPP before analyzing by plasma clot lysis assay. No effect of ATV was observed on clot formation in PPP clots (Figure S1a) and there was no discernable effect on clot lysis after overlaying with tPA (Figure S1b). This contrasts the significant attenuation of clot formation and enhanced lysis observed in PRP clots containing ATV thereby suggesting an impact on platelet function.

Thrombodynamics of clot formation were analyzed to garner data on the rate of clot growth and the rate of spontaneous clot lysis. Consistent with the clot lysis assay, maximum clot formation time with ATV was significantly longer (Figure 2c). ATV delayed clot formation initiated at the TF-comb, spontaneous clot growth and clot lysis (Figure 2d). Indeed, in the presence of ATV, there was clear evidence of clot formation and lysis occurring simultaneously, whereas in the control clot, it took longer before onset of lysis was visualized (21.75 ± 3.2 min vs. 39 ± 3.3 min, $P < .01$). Similarly, lysis of whole blood thrombi was 1.4-fold faster with ATV compared to the control ($P < .05$; Figure 2e). Interestingly, preincubation of ATV with PRP significantly inhibited thrombin-induced clot retraction in a dose-dependent manner (Figure 2f) to a similar extent as the tirofiban and cytoD controls.

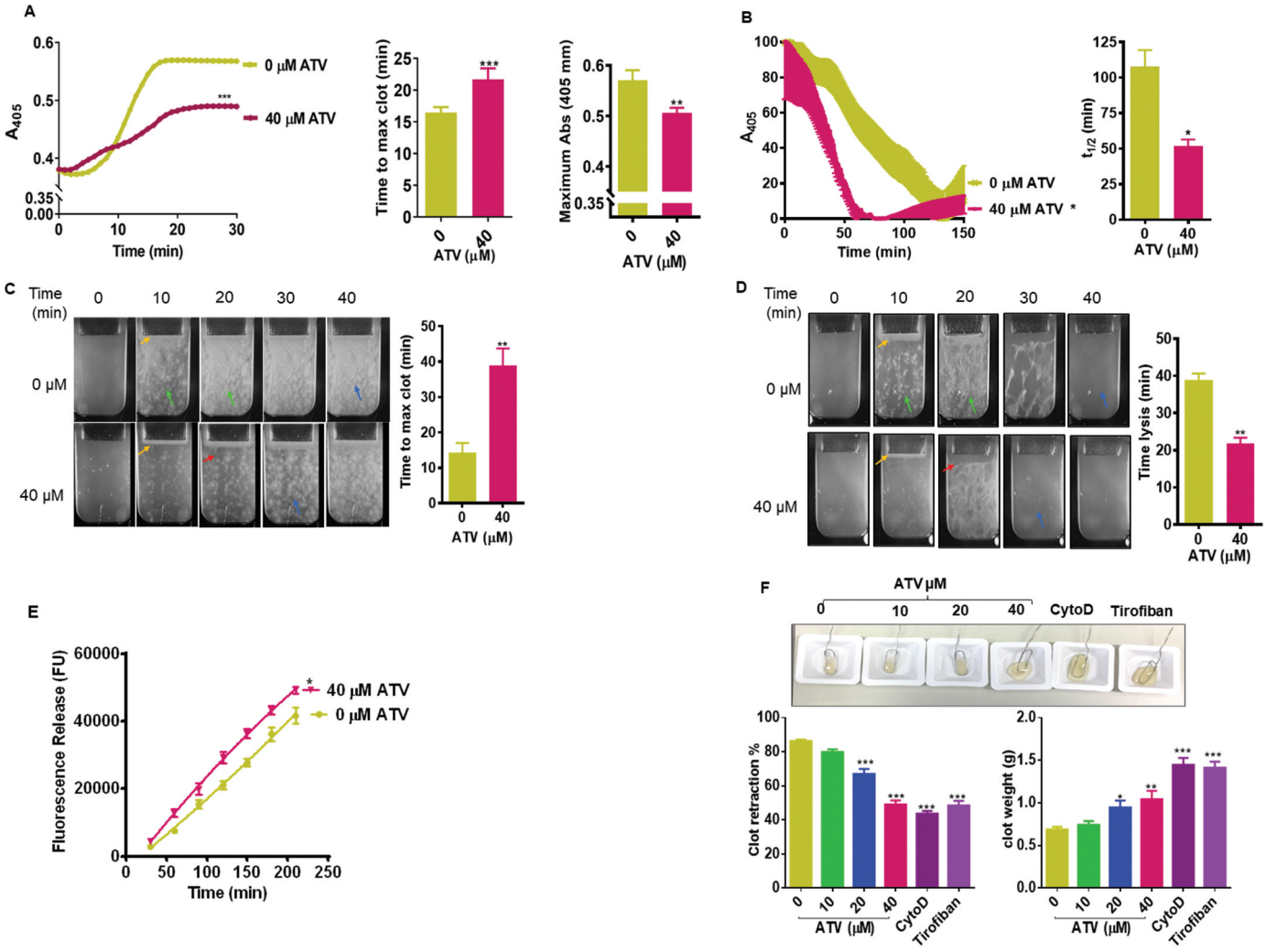

Figure 2. Atorvastatin enhances fibrinolysis. Fibrin clots were formed with 30% PRP treated overnight ±40 μM ATV and clotted with TRAP-6 (20 μM) and $CaCl_2$ (10 mM). Time to maximum absorbance (a) and subsequent 50% clot lysis times following addition of 5 nM tPA (b) Representative images of clot growth (c) and Tpa-mediated lysis (d) in PRP after overnight treatment with ATV. Arrows indicate initiation of clot formation (yellow), spontaneous clot growth (green), start of lysis (red) and full lysis (blue). Whole blood was incubated ±40 μM ATV after which thrombi were formed by recalcification with $CaCl_2$ (10.9 mM) in the presence of FITC-labeled fibrinogen and subsequently lysed with 15 nM tPA (E). (F) Clot retraction was analyzed by addition of 0.125 U/ml thrombin and 2 mM $CaCl_2$ to PRP for 2 h at 37°C following pre-treatment with 0–40 μM ATV, cytoD (10 μM) or tirofiban (1 μg/mL). Data are expressed as the mean ± SEM ($n \geq 3$), from different independent donors. (*P < .05) (**P < .01) (***P < .001) vs control.

ATV modulates platelet function and fibrinogen binding to integrin αIIbβ3

The influence of ATV on granule secretion in thrombin-stimulated washed platelets was investigated by quantifying ADP, which was significantly attenuated in a concentration-dependent manner (Figure 3a). Flow cytometric analysis of thrombin-stimulated washed platelets revealed that P-selectin expression was down-regulated by ATV (Figure 3b). Binding of fibrinogen and PAC-1, to monitor αIIbβ3 activation, was also significantly attenuated in a concentration-dependent manner following pre-incubation with ATV compared to untreated platelets (Figure 3c,d).

Previously, we have observed retention of α-granule proteins localized to the platelet "cap" of dual-stimulated platelets.[22,25] Confocal microscopy did not reveal changes in the number of phosphatidylserine-positive platelets between untreated and those pre-treated with ATV. However, similar to flow cytometry, there was a significant reduction in staining for P-selectin, PAC-1, fibrinogen and CD41 stain intensity (Figure 3e-h) following treatment of platelets with ATV. Additionally, ATV reduced PAI-1 exposure on the platelet membrane (Figure S2).

ATV significantly diminished platelet aggregation in response to 0.125 U/ml thrombin after 5 min pre-incubation (Figure 4a). This indicates that high concentrations of ATV induce a rapid inhibitory effect on platelet aggregation, suggesting that statins may mediate their effect via interfering with platelet receptors. Overnight incubation of platelets reduced their general responsiveness to agonist stimulation, however, incubation with ATV abolished all aggregatory responses (Figure 4b).

We examined the influence of a therapeutic range of ATV (0.5, 1 or 10 μM)[27] on platelet aggregation. There was no effect on aggregation following a 5 min incubation with ATV at concentrations within the therapeutic range (Figure 4c). Incubation of platelets with ATV at therapeutic doses for 6 h revealed a significant reduction in MA which was further pronounced after 24 h.

ATV attenuates Rab27b and Rap1a prenylation in platelets

To assess whether statins play a role in regulating the inhibitory effect of platelet function by reducing Rab27b and Rap1a prenylation, the accumulation of un-prenylated Rab27b and Rap1a in the

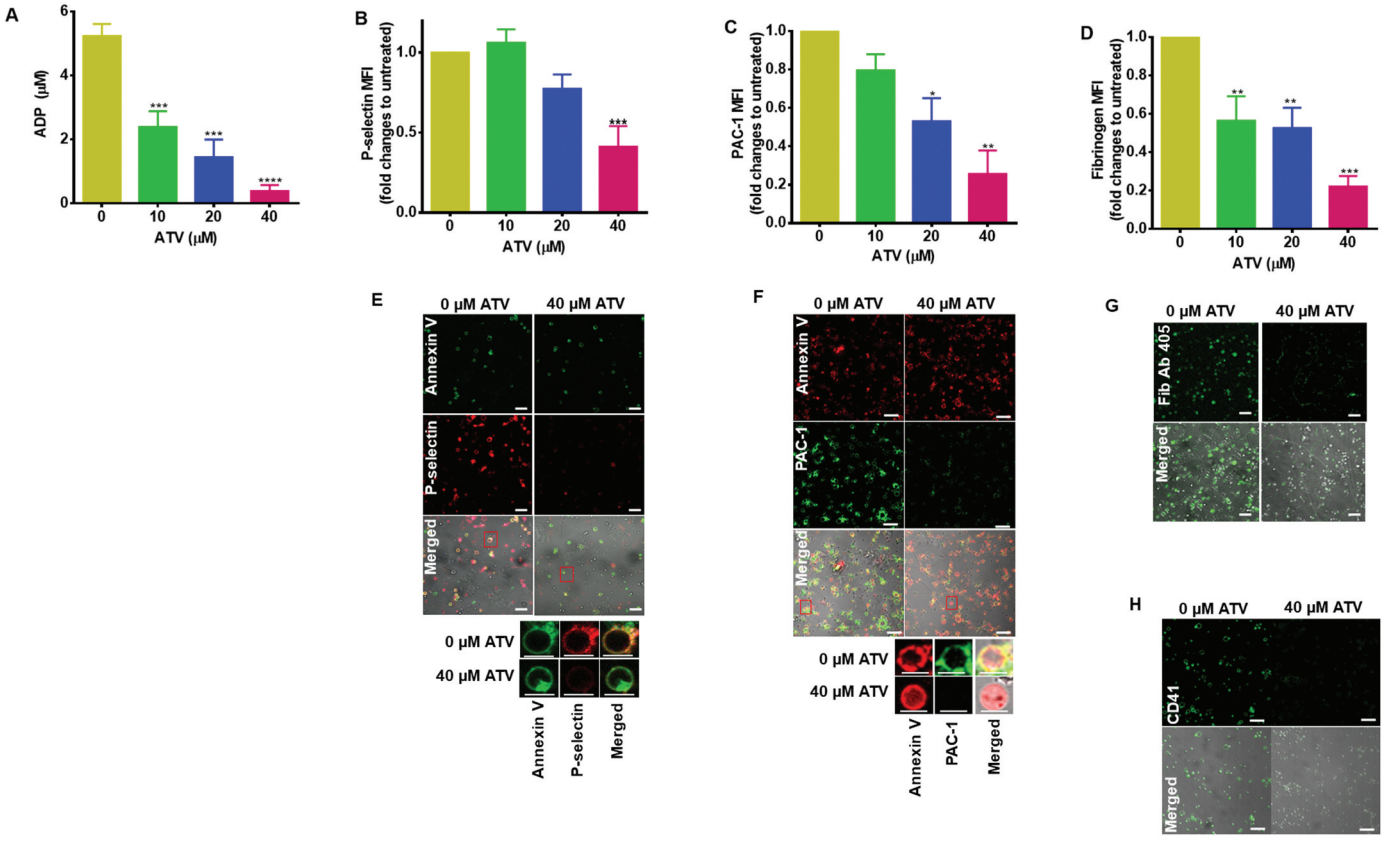

Figure 3. ATV modulates platelet function and fibrinogen binding to integrin αIIbβ3 and regulates P-selectin, fibrinogen, PAC-1 and CD41 exposure in stimulated platelets. Washed platelets (2×10^8 platelets/mL) were treated with 0, 10, 20 or 40 μM ATV for 24 h, before being stimulated with 0.125 U/ml thrombin for 30 min at 37°C and quantifying ADP release (a) Washed human platelets were treated with 0–40 μM ATV for 24 h and incubated with P-selectin (CD62P) Ab (b) or PAC-1 antibody to assess conformational changes and activation of $\alpha_{II}b\beta_3$ integrin (c) to assess binding to the platelet surface via $\alpha_{II}b\beta_3$ integrin or DL488-labeled fibrinogen (d) Platelets were stimulated with thrombin (0.125 U/ml) for 45 min at 37°C before analyzing by flow cytometry. Data are expressed as MFI normalized to fold changes to the control (untreated sample) *** $P < .001$, 40 μM ATV vs. 0 μM ATV. **$P < .01$ 10 and 20 μM ATV vs. 0 μM ATV. Data from $n \geq 3$ independent experiments with separate blood donors are represented as mean ± SEM. PRP was treated with ATV (0 or 40 μM) for 24 h before being added to slides coated with 20 μg/ml collagen to tether the platelets to the surface for the live imaging. PRP was activated with 20 μM TRAP-6 in the presence of FITC-488 Annexin-V to detect phosphatidylserine (green) and PE/cy5 CD62P (p-selectin) (red). Images represent Full field view (E). PRP was activated with 20 μM TRAP-6 in the presence of a labeled FITC-anti PAC-1 antibody (green; F), anti-fibrinogen antibody (Fib-405; green; g) and stained using AF-647 Annexin-V to detect phosphatidylserine (red) or FITC-CD41 (green; h). Scale bars represent 10 μm and single platelets taken from the full field (red box) represent 5 μm. Representative images of n ≥ 3 separate experiments with different donors.

aqueous fractions were measured. There was clear dose-dependent accumulation of unprenylated forms of Rab27b and Rap1a in the aqueous fractions of platelets pre-treated with ATV, and no accumulation in untreated platelets (Figure 5a,b). To confirm that the effect of ATV on prenylation arises due to inhibition of the mevalonate pathway, we performed a rescue experiment with GGPP. Replenishing GGPP attenuated accumulation of unprenylated Rab27b (Figure 6a) and Rap1a (Figure 6b) in the aqueous fractions.

Similarly, an almost 60% recovery of ADP secretion from activated platelets pre-incubated with 40 μM ATV was observed with GGPP (Figure 6c). These observations confirm that the inhibitory effect of statins on platelet function is at least partially mediated by blocking prenylation of Rab 27b.

Discussion

Statins elicit a wide range of pleiotropic effects in cardiovascular disease that extend beyond their lipid lowering function; these include improvement in endothelial function, reducing LDL oxidation and oxidative stress, decreasing vascular inflammation and antithrombotic actions[28–31] Various direct actions of these drugs on platelet function have been previously described.[32–37] These include an effect on protein kinase C α (PKCα), mediated via PPAR receptor activation, independent of their cholesterol lowering action.[33] Simvastatin inhibits platelet aggregation by enhancing platelet NOS and guanylyl cyclase activity resulting in increased NO and cyclic GMP formation.[34] Herein, we analyzed the direct impact of ATV on the dynamics of clot formation, stability and integrity and, specifically, the impact of ATV on prenylation of small GTPases that are important for normal platelet function. ATV inhibits prenylation of Rab27b and Rap1a in platelets and down-regulates dense granule release of ADP. The addition of GGPP overcomes the inhibitory effect of ATV on Rab27b and Rap1a prenylation, as well as their effect on ADP release, implying involvement of the mevalonate pathway. Furthermore, statins attenuate fibrinogen-binding to activated platelets, clot retraction and modulate fibrin binding to platelet aggregates, suggesting a potential alternative cardiovascular protective mechanism for these drugs *in vivo*.

Pre-incubation of PRP with ATV gave rise to clots comprised of thinner fibrin fibers with a more porous network, with increased susceptibility to tPA-mediated lysis. Similarly, a study performed in 30 individuals with LDL below 3.4 nM demonstrated that simvastatin treatment for 3 months significantly enhanced clot permeability and accelerated clot lysis.[38] Furthermore, simvastatin treatment

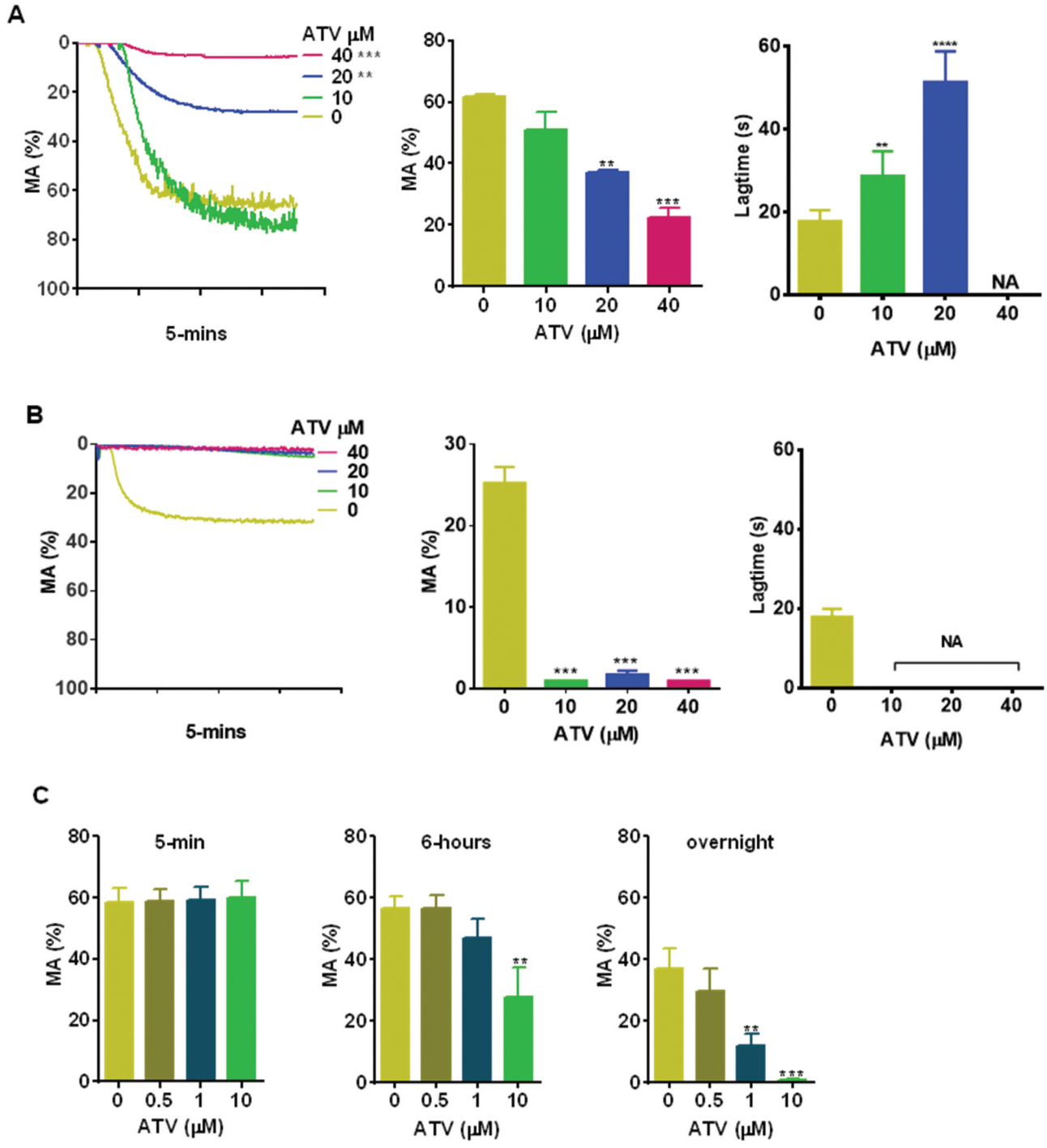

Figure 4. ATV attenuate platelets aggregation. Washed platelets (2×10^8 platelets/ml) were treated with 0–40 μM ATV for 5 min (a), overnight (b) or treated with 0, 0.5, 1 and 10 μM ATV for 5 min; 6 h; and overnight incubation (c), then pre-warmed for 2 min, before being stimulated with 0.125 U/ml thrombin. Aggregation was monitored for 5 min under constant stirring (1000 rpm) at 37°C using light aggregometry. Results were expressed as a percentage of maximum aggregation (MA %) and Lag time required for the platelets to start aggregating (NA: no aggregation). Representative aggregation traces generated by PAP-8e computer software. Data from $n \geq 3$ independent experiments from different donors are represented as mean ± SEM. (**P < .01) (***P < .001) vs control.

of patients with chronic obstructive pulmonary disease for 90 days significantly reduced clot lysis rate and enhanced fibrin permeability.[39] Moreover, myocardial infarction patients, randomly administrated with either ATV or simvastatin for 30 days, demonstrated faster fibrinolysis time and increased permeability post-treatment.[40] A similar observation was observed in a smaller cohort of patients with type 1 diabetes and dyslipidemia who received ATV for 60 days.[41]

Platelet α-granules contain a plethora of hemostatic proteins,[42] and impaired granular release could ultimately influence thrombus stability. We found that ATV attenuated exposure of α-granule proteins including P-selectin, PAI-1, and the active integrin αIIbβ3 on the activated platelet membrane. P-selectin is a vital adhesive molecule that bridges platelets and leukocytes.[43] Studies in P-selectin-deficient mice have previously revealed the crucial role of this adhesive protein in leukocyte accumulation in atherosclerotic lesions.[44] Our previous studies showed retention of PAI-1 on activated platelets that was dependent on αIIbβ3 and fibrin.[25] Interestingly, a significant reduction in the plasma level of

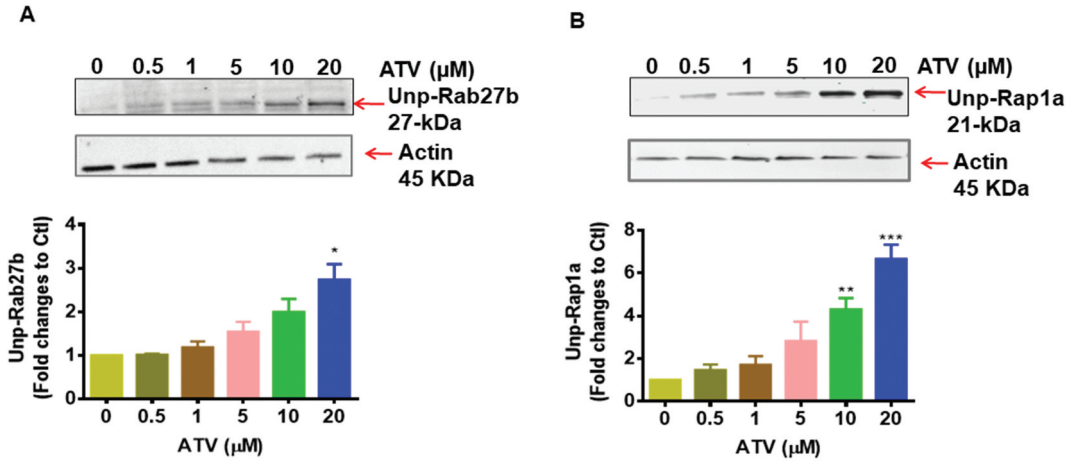

Figure 5. Attenuates Rab27b and Rap1a prenylation in platelets. Platelets (2.5×10^8/ml) were incubated with 0, 0.5, 1, 5, 10 or 20 μM ATV at 37°C for 24 h. Platelets were harvested and lysed before being separated into their cytosolic and membrane components by Triton X-114 and centrifugation. Lysates (30 μg/lane) of aqueous, which only contains the unprenylated protein, loaded into this blot. The blots were probed for an antibody that specifically recognizes Rab27b (a) or Rap1a (b). An antibody against β-actin was used as a loading control. Representative of $n > 8$. Densitometry analysis of the unp- Rab27b or Rap1a in platelets, normalized to actin and expressed as fold changes compared to the untreated. Pooled results of $n = 8$. (*P < .05 vs. control).

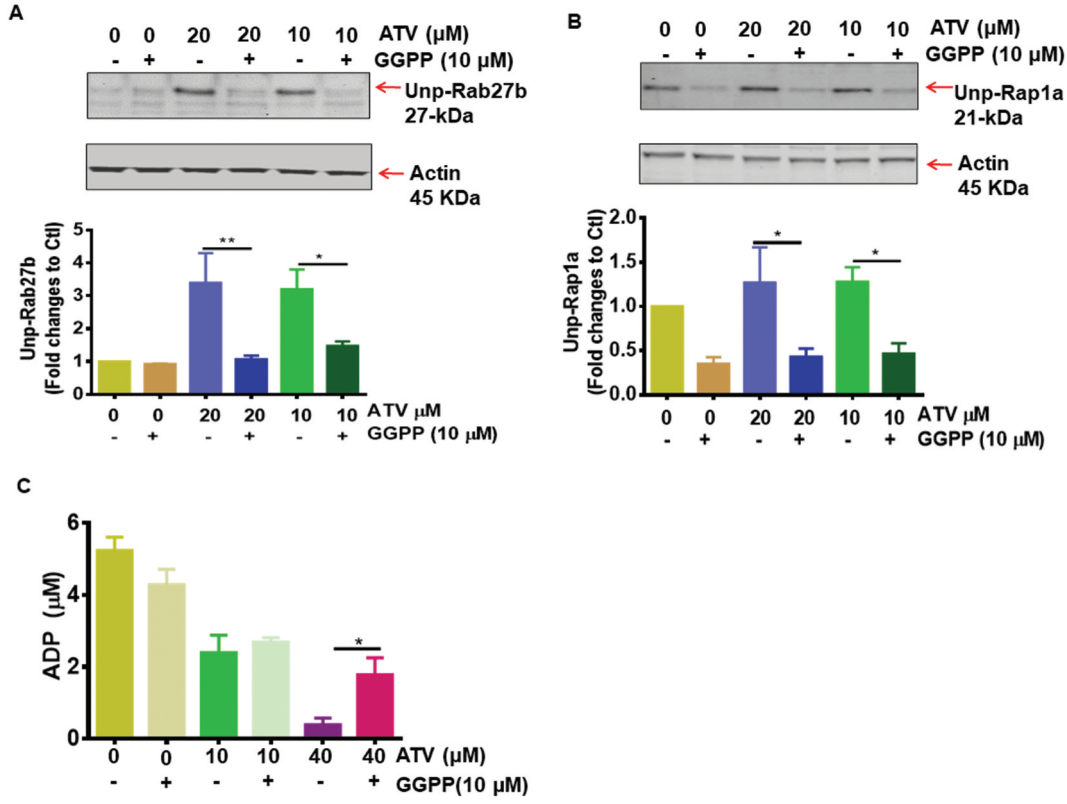

Figure 6. Exogenous GGPP abolish the effect of ATV in Rab27b and Rap1a prenylation and ADP release in platelets. Platelets (2.5×10^8 platelets/ml) were incubated with 0, 10, or 20 μM ATV at 37°C for 24 h ±10 μM GGPP. Platelets were harvested and lysed before being separated into their cytosolic and membrane components by Triton X-114 and centrifugation. Lysates (30 μg/lane) of aqueous, which contains only unprenylated protein were analyzed. The blots were probed for an antibody that specifically recognizes Rab27b (a) or Rap1a (b). An antibody against β-actin was used as a loading control. Data represent $n > 8$ and densitometry analysis of the unp- Rab27b or Rap1a in platelets was normalized to actin and expressed as fold change compared to untreated. Washed platelets (2×10^8 platelets/ml) were treated with 0, 10 or 40 μM ATV for 24 h in the presence or absence of 10 μM GGPP, before being stimulated with 0.125 U/ml thrombin for 30 min at 37°C (c). ADP concentration was quantified by ADP luciferase assay. Results were expressed as a percentage of maximum aggregation (MA %). Data from $n = 5$ independent experiments with different donors are represented as mean ± SEM. (*P < .05, **P < .01 vs. control).

PAI-1 has been observed following treatment with ATV for 1 year.[45] A reduction in active integrin αIIbβ3 and fibrinogen on activated platelets could attenuate PAI-1 retention on the activated membrane thereby increasing susceptibility of thrombi to lysis. Indeed, we observed faster lysis of thrombi treated with ATV, which may arise due to changes in PAI-1

secretion and binding on platelets.[25] ATV also attenuated the copy number of αIIbβ3 integrin on activated platelets. Dense granule release was attenuated at low concentrations of ATV (10 μM) in comparison to the observed effect on P-selectin exposure, which required in excess of 40 μM ATV. Interestingly, in patients with coronary artery disease, ATV reduced platelet P-selectin exposure in response to ADP-stimulation.[46] As ADP secretion would be impacted at this high a concentration of ATV this could be a potentially explanatory mechanism for this observation. Collectively these findings indicate that statins modulate platelet intercellular mechanisms and granular release.[47,48]

Platelet activation induces a αIIbβ3 receptor conformational change from the low-affinity to the high-affinity state[49] which in turn activates outside-in signaling. This process stimulates clot retraction via interaction with the intracellular cytoskeleton.[50] In this study, we demonstrate that ATV attenuates αIIbβ3 integrin expression, activation, and clot retraction, potentially via modulation of intracellular signaling. Other platelet receptors are known to bind fibrin, including αvβ3[51] and GPVI,[52] and the impact of statins on these interactions should be a focus of future studies.

Statins are reported to have a protective effect in patients with high risk of venous thromboembolism (VTE) whereas aspirin was ineffective.[53] The protective mechanism of statins in VTE is unknown and may arise due to the action of this drug at various levels including; reduction in platelet aggregation; decreased prothrombin, factor V and FXIII activation; attenuated fibrin formation; or by decreasing PAI-1 levels.[54,55] Our current findings reveal that ATV reduces platelet aggregation and activation, as well as altering fibrin clot formation structure and susceptibility to lysis which may help define the protective mechanism of statins against VTE.

It has been shown that statins, including simvastatin,[56] fluvastatin,[57] and cerivastatin[58] decrease tissue factor expression in cultured human monocytes and macrophages. The effect could be overcome by mevalonate, the precursor of FPP and GGPP, but not cholesterol, indicating this effect is mediated via intracellular GGPP biosynthesis.[57] Herein, we have shown that statins inhibit prenylation of Rab27b and Rap1A in platelets, which are known to regulate many aspects of platelet function, secretion, and activation.[4,13] Inclusion of GGPP overcame ATV inhibition of platelet function and restored ADP secretion. These data thereby suggest that the inhibitory effect of statins on platelets is partially mediated through inhibition of prenylation of small GTPases. Herein, we have shown that statins exhibit anti-thrombotic properties on platelet function which, could relate to altercations in prenylation of small GTPases such as Rab27b and Rap1, as observed *in vitro* studies. Additional *in vivo* studies are necessary to assess the effect of statins on protein prenylation of Rab27b and additional small GTPases proteins present in platelets, such as Rac1 and Rap1b.

In conclusion, statins directly inhibit Rab27b and Rap1a prenylation in platelets and down-regulate dense granule release by inhibiting the mevalonate pathway. The pleotropic actions of statins on platelet function have a downstream impact on clot formation, integrity, and stability. These findings provide additional utility in the use of statins in prevention of various thrombotic conditions.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

M.M.J. performed the research, analyzed the data and wrote the manuscript; C.S.W analyzed the data and edited the manuscript. F.P.C. and N.J.M designed and supervised the research, analyzed the data and wrote the manuscript.

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