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Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Stimulates Platelet-Dependent Arterial Thrombosis

Yan Liu,[1,2] Liqun Wang,[2] Meiping Ren,[2] Ni Chen,[2] Jing He,[2] Wei Xiong,[2] Rong Li,[2] Yi Li,[2] Pei Luo,[1] Jianbo Wu,[1,2,*]

[1]Faculty of Chinese Medicine and State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau, People's Republic of China
[2]Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease of Sichuan Province, Drug Discovery Research Center of Southwest Medical University, Luzhou, Sichuan, People's Republic of China

***Corresponding author:** Jianbo Wu, PhD, Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease of Sichuan Province, Drug Discovery Research Center of Southwest Medical University, 319 Zhongshan Street, Luzhou, Sichuan 646000, People's Republic of China; Tel: 86-0830-3161702; Fax: 86-0830-3161702; Email: jbwucn1996@yahoo.com

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Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays a critical role in lipid metabolism and is a drug target for treating hyperlipidemia and reducing cardiovascular risk. However, little is known about non-lipid pathways by which PCSK9 might contribute to ischemic cardiovascular events. Our goal was to test the hypothesis that PCSK9 regulates arterial thrombosis and platelet function. The effects of genetic deficiency of PCSK9 and recombinant PCSK9 protein on carotid artery and cremasteric arteriolar thrombosis, platelet aggregation, and platelet agonists signaling pathways were studied in mice. Platelet-dependent arterial thrombosis was significantly delayed in *pcsk9*$^{-/-}$ mice compared to wild-type (WT) controls and intravenous infusion of PCSK9 accelerated thrombosis. PCSK9 bound to platelets in a concentration-dependent, saturable manner. Compared to WT controls, platelets from *pcsk9*$^{-/-}$ mice exhibited reduced ADP-induced aggregation, which was corrected by addition of PCSK9 protein. Platelets from PCSK9-deficient mice also contained less $P2Y_{12}$, the ADP receptor, and exhibited a defect in ADP-induced activation of Akt serine-threonine kinase. PCSK9 binds to platelets, stimulates ADP-induced platelet aggregation, regulates expression of $P2Y_{12}$, and controls platelet signaling downstream of $P2Y_{12}$. These findings suggest that PCSK9 promotes ischemic cardiovascular events not only by driving atherosclerosis through effects on plasma lipids, but also through effects on platelet function and arterial thrombosis.

Keywords: PCSK; Blood platelets; Phosphotransferases Receptors; purinergic P2 Receptors

Introduction

The low density lipoprotein receptor (LDLR), which is expressed by hepatocytes, plays a critical role in lipid metabolism by removing LDL cholesterol from circulating plasma [1]. After LDL cholesterol binds to LDLR, the complex is internalized and LDLR is either degraded in endosomes or recycled back to the membrane surface, where it can again bind and internalize LDL cholesterol. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a plasma protein that binds to LDLR and preferentially targets it for endosomal degradation after the receptor is internalized [2,3]. Drugs that inhibit binding of PCSK9 to LDLR have been developed to treat hyperlipidemia and reduce atherosclerotic cardiovascular disease [4,5].

PCSK9 has the potential to regulate biological pathways other than LDL cholesterol metabolism by binding and promoting intracellular degradation of other LDL receptor family members, such as LDL receptor-related protein 1 (LRP1), which has many functions, including clearance of factors that regulate hemostasis, thrombosis, and inflammation [3,6,7]. In this study, we tested the hypothesis that PCSK9 regulates arterial thrombosis. Our results identify a functional role of PCSK9 in regulating platelet-dependent arterial thrombosis and suggest that the increased risk of major adverse cardiovascular events associated with enhanced PCSK9 expression may be mediated not only by effects on lipid metabolism and atherogenesis, but also on platelet function and arterial thrombosis.

Materials and Methods

Reagents

DNA oligonucleotides for PCR genotyping of *pcsk9*$^{-/-}$ mice (olMR5170,olMR5171, olMR5172) and *ldlr*$^{-/-}$ mice (19799, 19800; oIMR7770) were purchased from Invitrogen Biotechnology Co., Ltd (Shanghai, China). Recombinant human PCSK9 was from ACRO Biosystems (Bethesda, MD, USA). Adenosine diphosphate (ADP) was from Helena Laboratories (Beaumont, TX, USA). PCSK9 [Biotinylated]-LDLR Binding Assay Kit was from BPS Bioscience Inc. (San Diego, CA, USA). Alexa-Fluor-488 labeled anti-CD41 antibody was from Biolegend (La Jolla, CA, USA). Rabbit anti-P2Y12, P2Y1, CD42a, pVASP^{Ser157}, GPVI, and PAR1 antibodies were from Abcam (Cambridge, UK). Anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody was from Abcam (Shanghai, China). Antibodies against phosphorylated Akt and non-phosphorylated Akt were from Santa Cruz Biotechnology (Dallas, TX, USA).

Animals

C57BL/6J mice were obtained from Chongqing Tengxin Bioscience Inc. (Chongqing, China). *Pcsk9*$^{-/-}$ mice (strain: B6.129S6-PCSK9tm1jdh/J; Stock number: 005993) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained on a 12:12-h light-dark cycle. All protocols for animal use were approved by the Animal Care Committee of Southwest Medical University in accordance with Institutional Animal Care and Use Committee guidelines, and conformed to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All mice were fed normal chow for 4 weeks after weaning, after which some mice continued on normal chow until undergoing thrombosis experiments, while others were changed to a western diet high in fat (45% kcal% fat), cholesterol, and sucrose (D12451; Research Diet, New Brunswick, NJ), which they continued for 14 weeks until being studied in thrombosis experiments. Plasma levels of total cholesterol (C), LDL-C, HDL-C, and triglycerides were measured using an AU680 analyzer (Beckman Coulter, Indianapolis, IN).

Carotid artery thrombosis model

Carotid artery thrombosis was induced by ferric chloride ($FeCl_3$), as described previously [8,9]. Male mice were anesthetized by intraperitoneal (IP) injection of sodium pentobarbital (50 mg/kg) and euthanized by rapid cervical dislocation. The mid-portion of left carotid common artery was exposed and covered for 3 min with a 1×2 mm rectangular piece of filter paper soaked in 10% $FeCl_3$ solution. After removing the filter paper, the carotid artery was rinsed with phosphate-buffered saline (PBS) and blood flow was monitored continuously with a vascular flow probe (Transonic Systems, Ithaca, NY, USA) from the completion of vascular injury until the onset of stable occlusion (defined as no detectable flow for 10 min) or for 60 min if occlusion did not occur. To determine the effect of PCSK9 on arterial thrombosis, mice were injected by tail vein with either sterile water or recombinant human PCSK9 (30 μg) in 150 μL sterile water 5 min before $FeCl_3$ injury.

Laser-induced vascular injury

After inducing anesthesia with IP sodium pentobarbital (50 mg/kg), the cremasteric muscle was exteriorized and arterioles (diameter 30-50 μm) were visualized with a Zeiss Examiner A1 microscope. Alexa-488-labeled anti-CD41 antibody (0.1 μg/g body weight) was administered via the tail vein. Five minutes later vessel wall injury was induced with a Micropoint Laser System

(Andor Technology, wavelength 435 nm), as described previously [10,11]. Fluorescence data were captured digitally via a single fluorescence channel (488/520 nm) before and continuously after laser injury. The kinetics of thrombus formation was determined by plotting median fluorescence intensity *vs*. time for all thrombi in each experimental group, with 3-4 thrombi studied per mouse and at least 3 mice in each experimental group.

In vitro platelet aggregation

Blood (approx. 0.8 mL) was collected from the inferior vena cava of anesthetized mice into a syringe containing ACD anticoagulant (tri-sodium citrate 51 mM, citric acid 22 mM, D-glucose 74 mM). After centrifugation (100g) for 10 min at 22°C, platelet-rich plasma (PRP) was removed and the remaining blood was centrifuged at 2700g for 10 min at 22°C to obtain platelet-poor plasma (PPP). Platelets in PRP were counted with a hemocytometer and the concentration was adjusted to 3×10^8/mL using PPP. Platelet aggregation was analyzed with a turbidimetric aggregation-monitoring device (Helena Laboratories, Beaumont, TX, USA). PRP (225 µL) was incubated with recombinant PCSK9 (30 µg/mL) or vehicle control for 5 minutes at 37°C, after which aggregation was induced by addition of ADP (10 µM) under constant stirring conditions (600 rpm).

Measurement of plasma PCSK9

Blood was collected into citrate anticoagulant and plasma was prepared by centrifugation. *Plasma levels of* PCSK9 were measured by a commercial enzyme-linked immunoadsorbent assay (Abcom, cat. ab215538). Data are expressed as pg/ml. The intra-assay and inter assay coefficients of variance were 2.4% and 8.4%, respectively.

Platelet PCSK9 binding assay

Binding of PCSK9 to platelets was studied by incubating murine platelets ($2x10^6$) in Tyrode's buffer for 4 hours at 37°C in 96 well microplates, after which increasing amounts (0-60 µg/mL) of biotinylated PCSK9 (BPS Bioscience, Inc., San Diego, CA) were added to wells. After a 2 hour incubation at room temperature, supernatants were gently removed from each well and platelets were washed 3 times (100 µL/wash) with PCSK9 assay buffer (PCSK9 [Biotinylated]-LDLR Binding Assay Kit, BPS Bioscience). Using reagents provided in the kit, wells were blocked, after which streptavidin-HRP and HRP chemiluminescent substrate were added sequentially, with intervening washes, after which wells were read in a Molecular Devices chemiluminescence plate reader to measure binding of PCSK9 to platelets. Control experiment in which biotinylated PCSK9 (60 µg/mL) was added to wells lacking platelets and processed identically as described above yielded no detectable chemiluminescent signal.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from platelets and livers by the use of TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. cDNA was synthesized with a Prime Script RT reagent kit (TaKaRa, Dalian, China), subjected to qRT-PCR with an ABI PRISM 7700 cycler (Applied Biosystems, Foster City, CA). Fold changes in gene expression were determined using the $2-\Delta\Delta CT$ method, as described previously [12]. For amplification of murine PCSK9, the sequences 5'-CAGGGAGCACATTGCATCC-3' and 5'- TGCAAAATCAAGGAGCATGGG-3' were used as forward and reverse primers, respectively. For amplification of GAPDH, the sequences 5'-TTCACCACCATGGAGAAGG-3' and 5'-CTCGTGGTTCACACCCATC-3' were used.

Western blotting

Platelets lysates were prepared as described [13] and subjected to SDS-PAGE (using either 7.5% homogeneous or 4-20% gradient polyacrylamide gels) and Western transfer. To study platelet $P2Y_{12}$ expression, blocked membranes were incubated with anti-$P2Y_{12}$, anti-P2Y1, anti-CD42a, and anti-PAR1 antibodies, respectively. After washing, blots were incubated with horseradish-peroxidase (HRP)-conjugated goat IgG raised against rabbit IgG (Cell Signaling Technology) and developed with ECL substrate (Pierce). Thereafter, blots were stripped and re-probed with anti-GAPDH antibody, using similar techniques. To study platelet Akt activation, blocked membranes were incubated with rabbit IgG raised against phosphorylated Akt. Secondary antibody and blot development were as described for $P2Y_{12}$. Thereafter, blots were stripped and re-probed for total Akt expression with anti-Akt antibody, using similar techniques. NIH ImageJ software was used to quantify band densities.

Statistical analyses

Data are presented as mean ± standard error of the mean. Experimental groups were compared by the two-tailed Student's t-test or one-way analysis of variance (ANOVA). Tukey's multiple comparison test was used to determine statistical significance, employing the LSD method for equal variance and the Tamhane's T2 method for unequal variance.

Results

PCSK9 Promotes Carotid Artery and Cremasteric Arteriole Thrombosis

The murine carotid artery $FeCl_3$ model was used to determine the effect of PCSK9 on thrombosis. Recombinant human PCSK9, administered via tail vein injection, significantly accelerated formation of an occlusive thrombus compared to vehicle control in a normal chow fed WT mice (Figure 1A,B). Conversely, mice with complete genetic deficiency of PCSK9 exhibited delayed thrombus formation compared to WT controls, with the defect being correctable by IV administration of recombinant PCSK9 (Figure 1A,B). The $FeCl_3$ model generates platelet-rich thrombi [8]. There was no significant difference in the blood platelet count between WT mice ($1362 \pm 107 \times 10^9$/L; n=9) and *pcsk9*$^{-/-}$ mice ($1171 \pm 71 \times 10^9$/L; n=8; p=0.17). To further explore the effects of PCSK9 on platelet accretion after vascular injury, and to examine the effects of PCSK9 on microvascular thrombosis, the cremasteric arteriole laser injury model was employed, using fluorescent anti-CD41 antibody to specifically examine platelet-dependent thrombosis. Platelet deposition at sites of vascular injury was significantly reduced in *pcsk9*$^{-/-}$ mice compared to WT controls (Figure 1C-E). Both WT mice and *pcsk9*$^{-/-}$ mice were fed a western diet for 14 weeks, after which carotid artery and cremasteric arteriole thrombosis were studied. Interestingly, DKO (i.e. *ldlr*$^{-/-}$, *pcsk9*$^{-/-}$) mice fed a western diet exhibited significantly attenuated platelet-dependent thrombosis compared to *ldlr*$^{-/-}$ mice receiving the same diet (Figure 2). Next, we measured the mRNA levels of PCSK9 in platelets by quantitative RT-PCR. Compared with mice fed the normal chow diet, the WT-WD mice demonstrated increased PCSK9 mRNA expression (Supplementary Figure 1A). Furthermore, platelets exhibited a significantly higher relative PCSK9 mRNA expression in WT mice fed WD compared with that fed CD. We also measured the plasma PCSK9 level by ELISA from WT in normal chow feeding. The results showed that there is a significantly higher PCSK9 plasma level from WT mice fed WD compared with that fed CD (2388±102 Vs.118±4.12 ng/mL; p<0.05). Plasma lipid profiles of each experimental group are shown in Table 1. These results indicate that PCSK9 regulates the thrombotic response to vascular injury, as well as plasma cholesterol concentration.

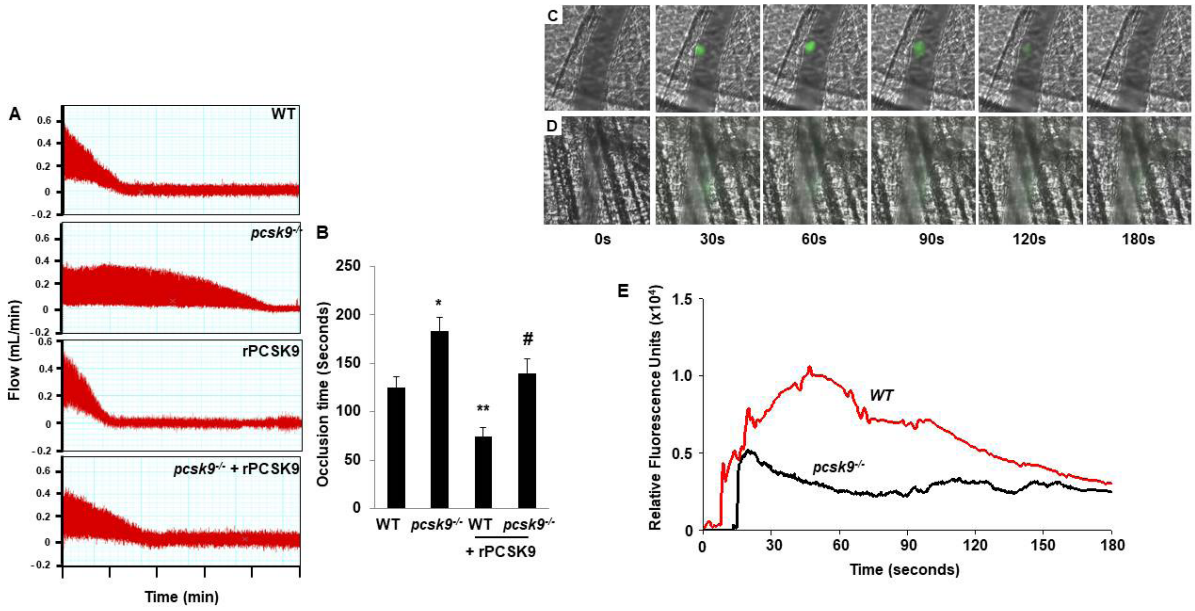


Figure 1. PCSK9 regulates thrombus formation in mice fed normal chow. (A) Recombinant PCSK9 (30 μg) or vehicle control was administered to WT mice via the tail vein 5 min before injuring carotid arteries with topical $FeCl_3$. Carotid blood flow tracings are shown. (**B**) Mean carotid artery occlusion times after $FeCl_3$ injury (n=11/group); *P<0.05 vs vehicle control group; **P>0.05 vs vehicle control group; #P<0.05 vs vehicle control group. WT, n=5; *pcsk9*$^{-/-}$, n=13; *pcsk9*$^{-/-}$ + rPCSK9, n=7. The data were shown as mean ± SEM. *P<0.05 vs WT; #P<0.05 vs *pcsk9*$^{-/-}$ mice that did not receive rPCSK9. WT, wild-type. **(C)** Time-lapse images showing formation of platelet thrombus (green) in a cremasteric arteriole of a WT mouse after laser injury. Alexa Fluor 488-conjugated anti-CD41 antibody was used to label platelets. Scale bar (shown in 0s images) = 10 μm. **(D)** Corresponding images of a laser-injured cremasteric arteriole of a *pcsk9*$^{-/-}$ mouse. **(E)** Median integrated platelet fluorescence intensity (thrombus formation) in cremasteric arterioles after laser injury (n=3 mice/group). s, seconds; WT, wild-type.

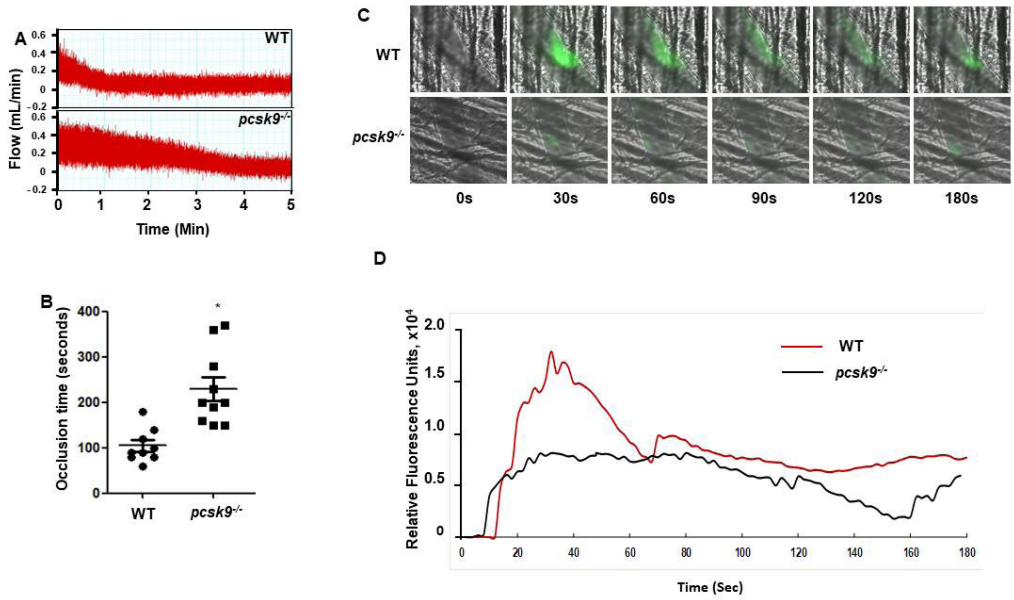


Figure 2. PCSK9 deficiency delays thrombosis in mice fed a western diet. (A) Representative blood flow tracings after $FeCl_3$ injury of carotid arteries of mice with indicated genotypes. **(B)** Aggregate carotid artery occlusion time data; WT, n=9; *pcsk9*$^{-/-}$, n=10. Mean of each group is indicated by a horizontal bar; The data were shown as mean ± SEM. *P<0.05 vs. WT mice. **(C)** Representative time-lapse photography images of platelet thrombus formation (green) in cremasteric arterioles of mice from each group. Scale bar = 10 μm. **(D)** Median integrated platelet fluorescence intensity (thrombus formation) in cremasteric arterioles after laser injury (n=3 mice/group). WT, wild-type.

Diet	Genotype	Total-C	TG	LDL-C	HDL-C
Chow	WT	1.48±0.07	0.42±0.13	0.30±0.01	1.10±0.07
	pcsk9$^{-/-}$	0.75±0.09*	1.15±0.22*	0.13±0.01*	0.56±0.08*
Western	WT	2.76±0.21	0.30±0.06	0.72±0.10	1.40±0.07
	pcsk9$^{-/-}$	2.26±0.13$^{\#}$	0.19±0.04*	0.39±0.02*	1.48±0.11

Table 1. Plasma Lipid Profiles

All units are mM. Values are mean±SEM. * p<0.01 *vs*. WT mice receiving same diet; $^{\#}$p>0.05 *vs*. WT fed western diet; n=3-4 mice per group. Total-C, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; WT, wild-type.

PCSK9 Binds to Platelets and Enhances Aggregation

To determine if PCSK9 binds to platelets, biotinylated PCSK9 was incubated with washed murine platelets for 2 hours and bound PCSK9 was measured by chemiluminescence. PCSK9 bound to platelets in a concentration-dependent, saturable manner, with half-maximal binding at a concentration of approximately 7 μg/mL (Figure 3). To determine if PCSK9 deficiency alters platelet reactivity, PRP was prepared from WT mice and PCSK9-deficient mice and ADP-induced aggregation was studied by light aggregometry. Aggregation of platelets from PCSK9-deficient mice fed a chow diet was significantly attenuated compared to that observed with platelets from WT mice, and addition of recombinant PCSK9 to platelets from PCSK9-deficient mice restored normal platelet aggregation (Figure 4A,B). In animals fed western diet, PCSK9 deficiency also inhibited platelet aggregation (Figure 4C,D). Thus, platelet PCSK9 was sufficient to enhance the aggregation response state. Together, these results suggested that PCSK9 binds platelets and enhances platelet reactivity.

$P2Y_{12}$ Receptor Expression and Downstream Signaling Are Reduced in Platelets from PCSK9-Deficient Mice

ADP induces platelet aggregation by binding the $P2Y_{12}$ receptor, which activates integrin $\alpha_{IIb}\beta_{IIIa}$ and leads to platelet degranulation, thromboxane production, and aggregation [14]. To study effects of PCSK9 on platelet $P2Y_{12}$, lysates were prepared from washed platelets obtained from WT mice and PCSK9-deficient mice and subjected to SDS-PAGE and Western blotting. $P2Y_{12}$ expression was significantly reduced in platelets from *pcsk9*$^{-/-}$ mice compared to those from WT controls (Figure 5A). However, the expression of $P2Y_1$ (Figure 5B), PAR1 (Figure 5C), and CD42a (Figure 5D) did not change significantly, suggesting that PCSK9 specifically regulates platelet $P2Y_{12}$ expression. To determine if decreased $P2Y_{12}$ expression in platelets from PCSK9-deficient mice is associated with a defect in downstream intracellular signaling, we incubated washed platelets in the presence or absence of ADP, after which Akt activation was studied by Western blot analysis of platelet lysates. Akt phosphorylation, which is induced by activation of $P2Y_{12}$ receptor by ADP [15], was significantly less in ADP-stimulated platelets from PCSK9-deficient mice compared to WT controls, whereas Akt-phosphorylation in unstimulated platelets did not differ significantly between PCSK9-deficient mice and WT mice (Figure 5E).

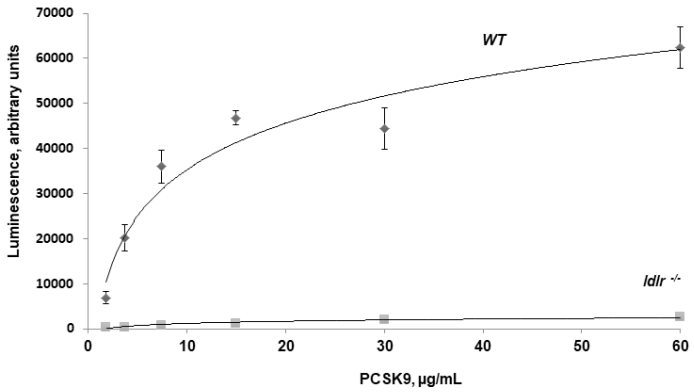


Figure 3. PCSK9 binds to platelets. Washed platelets prepared from wild-type and *ldlr*$^{-/-}$ mice fed normal chow were incubated with increasing concentrations of biotinylated PCSK9 for 2 hours. After extensive washing, platelet-bound PCSK9 was measured. Results shown are representative of triplicate experiments.

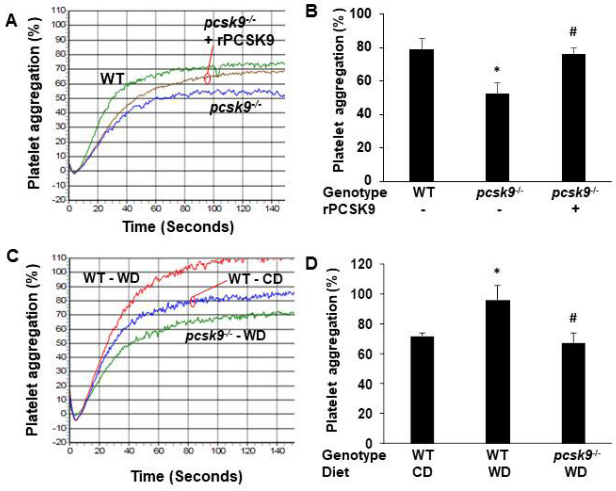


Figure 4. PCSK9 enhances platelet aggregation. (A) ADP-induced platelet aggregation in PRP from mice of the indicated genotypes, all receiving CD, was studied. rPCSK9 (30 μg/mL) was added to PRP from a *pcsk9*$^{-/-}$ mouse 5 min prior to addition of ADP, as indicated. Light transmission aggregometry tracings are shown. **(B)** Bar graph showing mean maximal % aggregation (n=3-5 mice/group); The data were shown as mean ± SEM. *p<0.05 *vs*. WT; $^{\#}$p<0.05 *vs*. *pcsk9*$^{-/-}$ without added rPCSK9. **(C)** ADP-induced platelet aggregation in PRP from mice of the indicated genotypes, receiving CD or WD, as indicated, was studied. **(D)** Bar graph showing mean maximal % aggregation (n=3/group); The data were shown as mean ± SEM. *p<0.05 *vs*. WT receiving CD; $^{\#}$p<0.05 *vs*. WT receiving WD. ADP, adenosine diphosphate; CD, chow diet; PRP, platelet-rich plasma; WD, western diet; WT, wild-type. The bar graph shows the aggregation results expressed as maximal amplitude of aggregation (n=3-5 mice/group).

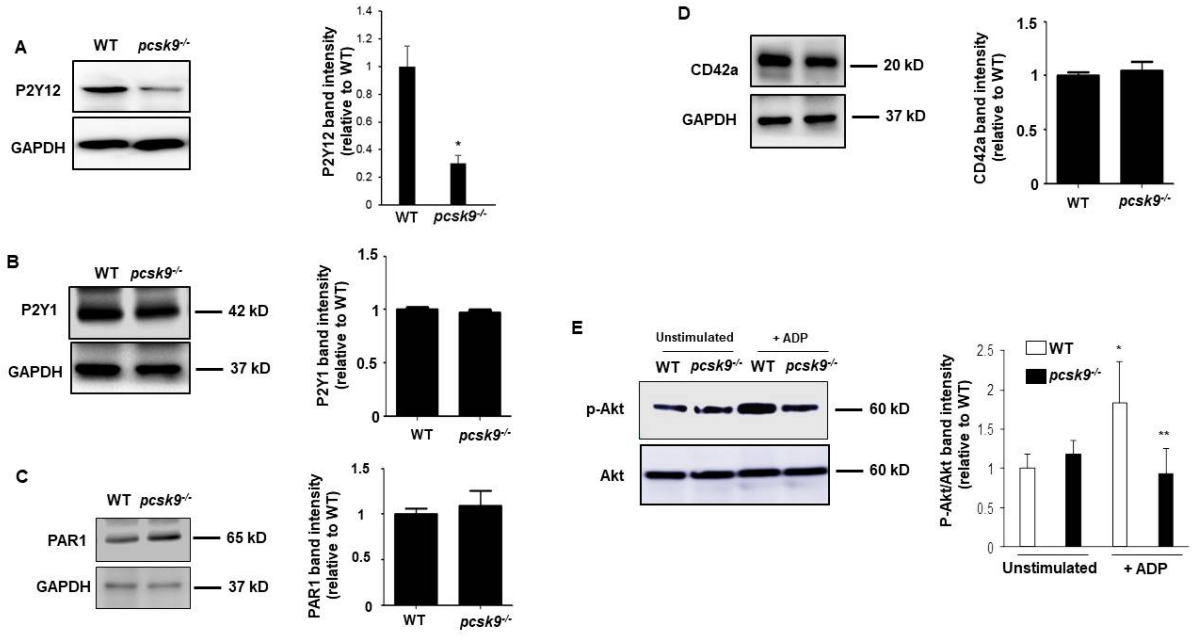
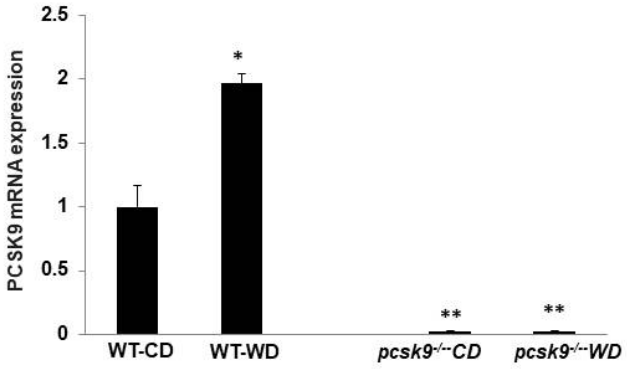


Figure 5. ADP signaling is attenuated in platelets from PCSK9-deficient mice. (A-D) Platelet lysates from WT mice and PCSK9-deficient mice fed normal chow were subjected to SDS-PAGE and Western blotting with anti-$P2Y_{12}$, anti-$P2Y_1$, anti-PAR1, and anti-CD42a antibodies, respectively. Densitometry analyses of 3 independent experiments; *P<0.05 *vs*. WT. **(E)** Washed platelets from WT and PCSK9-deficient mice fed normal chow diet were incubated 5 min in the presence or absence of ADP (10 μM), after which platelet lysates were prepared and subjected to Western blotting using antibodies against phosphorylated (p) and non-phosphorylated Akt. Densitometry analyses of 3 independent experiments. The ratio of the densities of the p-Akt and non-Akt bands was calculated for each experiment. Data shown are normalized to those of unstimulated platelets from WT mice, which were assigned a value of 1.0. The data were shown as mean ± SEM. *P<0.05 *vs*. unstimulated platelets from WT mice; **P<0.05 *vs*. ADP-stimulated platelets from WT mice. ADP, adenosine diphosphate; p-Akt, phospho-Akt; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WT, wild-type.



Supplemental Figure 1. PCSK9 mRNA in platelets and livers. PCSK9 mRNA was assessed by quantitative RT-PCR in platelets of the indicated genotypes, receiving CD or WD, as indicated, was studied. Data (mean ± SEM) are for 3 experiments and are expressed as fold-control. **P*<0.05 vs. WT-ND. ***P*<0.05 vs. WT-WD.

Discussion

In this study we examined the effects of PCSK9 on arterial thrombosis and platelet aggregation in mice. Our main conclusions are the following: 1) complete PCSK9-deficiency produces delayed thrombosis in carotid arteries and cremasteric arterioles of mice, whereas administration of recombinant PCSK9 accelerates the thrombotic response to vascular injury; 2) PCSK9 binds to platelets, 3) PCSK9 deficiency significantly attenuates ADP-induced platelet aggregation, which is corrected by addition of recombinant PCSK9, and 4) platelets from PCSK9-deficient mice exhibit reduced expression of $P2Y_{12}$, the ADP receptor, and decreased downstream Akt-mediated intracellular signaling. As a whole, our findings suggest that PCSK9 plays a significant and previously uncharacterized role in regulating platelet-dependent arterial thrombosis.

PCSK9 regulates plasma cholesterol concentration, with genetic deletion of PCSK9 producing a decrease in plasma cholesterol concentration [16], as we showed in our studies. Elevations in plasma cholesterol have been shown to enhance platelet reactivity and accelerate thrombosis [17, 18]. While it is possible that reductions in plasma cholesterol in PCSK9-deficient mice may have contributed to their thrombotic defect, several lines of evidence argue strongly that the effects of PCSK9 on thrombosis observed in our experiments are not mediated simply by downstream effects on plasma lipoprotein concentrations. First, infusion of recombinant PCSK9 acutely altered arterial thrombosis, producing an effect within several minutes of administration, and PCSK9 enhanced ADP-induced aggregation of washed platelets. Second, the pro-thrombotic effect of PCSK9 was observed in mice with normal LDLR expression consuming a chow diet, which is associated with low plasma lipoprotein concentrations, regardless of PCSK9 genotype, suggesting that plasma lipoprotein concentrations in themselves did not produce a major effect on the thrombotic response to arterial injury under the experimental conditions we employed. Additional experiments, such as evaluation of PCSK9 inhibitors, will be necessary to further clarify the potential importance in future studies.

Our results suggest that the effects of PCSK9 on thrombosis are mediated, at least in part, by alterations in platelet function. We showed that ADP-induced platelet aggregation is down-regulated in PCSK9-deficient mice and increased by recombinant PCSK9. Furthermore, we demonstrated that platelets from PCSK9-deficient mice have significantly reduced expression of $P2Y_{12}$, the ADP receptor, compared to WT controls. We also showed that platelets from PCSK9-deficient mice exhibit reduced phosphorylation of Akt in response to ADP stimulation. Given that Akt phosphorylation is induced by $P2Y_{12}$ activation [15], our results suggest that the reduced expression of $P2Y_{12}$ observed in platelets from PCSK9-deficient mice produces a functional defect in platelet activation. Our experiments also revealed that PCSK9 binds platelets. The concentration of PCSK9 needed to achieve half-maximal platelet binding under static conditions, approximately 7 μg/mL, is significantly higher than the median plasma levels reported in humans, which is approximately 0.5 μg/mL [19]. However, plasma concentrations of PCSK9 vary widely in humans, with some individuals having values approaching those employed in our study [19]. It is possible that the biotin used to label PCSK9 in our experiments may have inhibited the binding interaction of PCSK9 with platelets. Flow conditions or other factors present in plasma, which were

absent in our binding experiments, could possibly also affect PCSK9-platelet interactions. Our experiments do not determine the molecular mechanism(s) by which PCSK9 binds to platelets and regulates $P2Y_{12}$ expression. However, it is interesting to note that $P2Y_{12}$ undergoes internalization after binding ADP and can recycle back to the platelet surface to be reactivated [20,21]. PCSK9 is not known to increase the plasma membrane concentration of any receptor, so it seems unlikely that it would enhance plasma membrane expression of $P2Y_{12}$, even if were to bind to it. However, $P2Y_{12}$ is known to exhibit cross-talk behavior with other platelet receptors [15, 22]. It is tempting to hypothesize that a factor present on the surface of platelets could down-regulate $P2Y_{12}$ expression and/or signaling, while also binding PCSK9 and thereby be directed towards degradation pathways after internalization. Under such a scenario, deficiency of PCSK9 would up-regulate surface expression of the putative negative regulator of $P2Y_{12}$, leading to down-regulation of $P2Y_{12}$ expression and ADP-induced platelet aggregation, with addition of recombinant PCSK9 producing an opposite effect. Further experiments are warranted to define the molecular mechanisms by which PCSK9 regulates platelet activation and arterial thrombosis.

Wang et al. recently reported that PCSK9-deficient mice exhibit diminished venous thrombosis after inferior vena cava ligation compared to wild-type animals [23]. This group also demonstrated that venous thrombi in PCSK9-deficient mice contained fewer leukocytes and formed less neutrophil extracellular traps. Recently, plasma levels of PCSK9 were shown to correlate positively with platelet reactivity in patients with acute coronary syndromes [24]. Furthermore, Camera et al. showed very recently that recombinant PCSK9 reduces the lag-time in epinephrine-induced platelet aggregation [25,26]. This group also observed a trend towards reduced $FeCl_3$-induced thrombosis in PCSK9-deficient mice *vs.* WT controls, though the differences between groups did not appear to achieve statistical significance. Compared to these intriguing studies, our work demonstrates that PCSK9 definitively regulates arterial thrombosis, both in conductance arteries and the microcirculation, acts acutely to promote the thrombotic response, binds to platelets, and enhances ADP-induced platelet activation through effects on $P2Y_{12}$ receptor expression and downstream intracellular signaling.

While our study establishes a cause-and-effect relationship between PCSK9 and arterial thrombosis and provides important mechanistic insights, there are limitations in the ability of any murine study to reproduce human pathology. Furthermore, our study has not identified the molecular binding interaction(s) by which PCSK9 accelerates arterial thrombosis. We also have not explored non-platelet mechanisms by which PCSK9 might regulate arterial thrombosis, such as by direct effects on vascular wall cells. Additional studies are warranted to resolve these important issues.

In summary, our experiments elucidate a previously uncharacterized role of PCSK9 in regulating arterial, platelet-dependent thrombosis. Specifically, we have shown that PCSK9 enhances the thrombotic response to arterial injury and ADP-dependent platelet activation through effects on platelet $P2Y_{12}$ expression and downstream signaling. Elevated plasma concentration of PCSK9 is associated with major arterial ischemic events, including myocardial infarction and stroke [2], and pharmacologic inhibition PCSK9 reduces these events [4]. Our study significantly broadens the current understanding of the pathological mechanisms by which PCSK9 contributes to cardiovascular diseases – i.e. not only by promoting atherosclerosis through effects on plasma lipoprotein concentrations, but also through effects on platelet function and arterial thrombosis. Additional studies are warranted to more precisely define the molecular and cellular pathways by which PCSK9 regulates the thrombotic response to arterial injury and assess the anti-thrombotic effects of PCSK9 inhibiting drugs.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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