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Deficiency of LRP8 in mice is associated with altered platelet function and prolonged time for *in vivo* thrombosis

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Abstract

Introduction—Our group has previously reported genetic studies associating polymorphisms in the low density lipoprotein receptor related protein 8 (*LRP8*) gene with myocardial infarction. The aim of this study was to define the role of platelet surface LRP8 in thrombosis.

Materials and Methods—Flow cytometry, aggregometry, intravital microscopy and tail bleeding assays were used to examine platelet function and hemostasis in *LRP8*-deficient mice and littermate controls.

Results—We demonstrated that activation of platelets from both $LRP8^{+/-}$ and $LRP8^{-/-}$ mice was reduced *in vitro* in response to either ADP or thrombin. *In vivo*, *LRP8*-hemizygous and $LRP8^{-/-}$ mice demonstrated 200% and 68% increased time for carotid occlusion in response to $FeCl_3$ injury, respectively. Moreover, lipidated apoE3, a ligand for LRP8, inhibited platelet activation in a dose-dependent fashion. This inhibition was markedly attenuated in $LRP8^{-/-}$ but not $LRP8^{+/-}$ mice and did not result from membrane cholesterol efflux or a nitric oxide dependent pathway. Tail bleeding times were unaffected in both genotypes.

Conclusions—Our results suggest that LRP8 is capable of altering thrombosis without affecting normal hemostasis through mechanisms both dependent on and independent of apoE. This suggests a means whereby clot formation could be affected in humans with LRP8 gene variants.

Keywords

ApoER2; hemostasis; LRP8; platelet; thrombosis

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Introduction

Multiple risk factors have been elucidated for myocardial infarction (MI), but the most significant and least understood of the epidemiologic factors associated with MI is family history [1]. Coronary artery disease (CAD) and MI are recognized to have polygenic modes of inheritance and complex interaction with environmental factors. Our group has previously reported the results of a genetic linkage study for MI susceptibility genes in which we detected a novel MI susceptibility locus on chromosome 1p34-36 [2]. Sixteen candidate genes in this region were subsequently evaluated; however, the only single nucleotide polymorphisms (SNPs) significantly associated with MI were in or near the low density lipoprotein receptor related protein 8 (LRP8, also termed apoE receptor 2 or apoER2) gene [3]. Moreover, the positive associations between SNPs or rare haplotypes of *LRP8* and MI has been replicated in three other large case-control populations [3].

LRP8 is a member of the low density lipoprotein receptor (LDLR) family [4]; however, *LRP8* contains an additional exon that encodes a novel 59 amino acid segment in the cytoplasmic tail, including three potential copies of the minimal consensus sequence for the Src homology 3 (SH3)-binding motif [5]. Interactions between adapter molecules and the cytoplasmic tail of LRP8 have been shown to have an important role in signal transduction in the brain [6]; and LRP8 has been shown to localize to caveolae in CHO cell monolayers [7], further supporting a role in cellular signaling. LRP8-deficient mice have defects in sperm maturation [8], and mice deficient in both LRP8 and the VLDL receptor have defects in cortical and cerebellar neuronal layering, similar to that observed in mice deficient in Reelin or mouse Disabled 1 [6,9]. Known apolipoprotein/lipoprotein ligands for LRP8 include apoE and LDL [5,10]; however, LRP8 is not thought to play a role in the endocytosis and degradation of lipoproteins [11,12].

Although three splice variants of LRP8 have been identified on human platelets [5,13], the function of this protein on platelets remains incompletely characterized. Lipidated apoE has been shown to inhibit human platelet reactivity by stimulating intracellular nitric oxide (NO) synthase, eventually resulting in elevated levels of anti-aggregatory cGMP [14]. It has been suggested that this effect is mediated through LRP8 [5]. The purpose of this study was to characterize the function of LRP8 in platelets and provide data that could explain the association between polymorphisms in *LRP8* and myocardial infarction.

Materials and Methods

Mice

B6;129S6-*Lrp8*^{tm1Her}/J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine), and heterozygotes were mated to generate *LRP8*$^{+/+}$, *LRP8*$^{+/-}$ and *LRP8*$^{-/-}$ littermates. Mice were maintained on a mixed C57BL/6 and 129S6 background, and male animals were used for experimentation. All experiments were performed in accordance with protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Isolation and purification of platelets

While anesthetized with ketamine (170 mg/kg)/xylazine (5 mg/kg), 800 μL of blood was drawn from the inferior vena cavae into a syringe containing 100 μL of acid citrate dextrose (85 mM trisodium citrate dehydrate, 65 mM citric acid monohydrate and 111 mM glucose, pH 4.6) and 100 μg prostaglandin E_1 (Sigma-Aldrich, St. Louis, MO). Blood was then diluted 1:1 in Tyrode's Buffer (137 mM sodium chloride, 12 mM sodium bicarbonate and 2.5 mM potassium chloride, pH 7.2). Platelet rich plasma (PRP) was collected by two sequential centrifugations at 138× *g*, and platelets were sedimented at 863× *g* and resuspended in 250 μL of Tyrode's

Buffer containing 0.1% glucose and 0.35% BSA before gel-filtration through a sepharose 2B (Sigma-Aldrich) column. All centrifugations were performed at room temperature (24°C). Final concentrations of 1 mM $MgCl_2$ and 2 mM $CaCl_2$ were added to the platelet rich fraction.

Preparation of apoE3/DMPC complexes

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) large unilamellar vesicles (LUVs) were prepared by extrusion, as previously described [15]. Human recombinant apoE3 (Invitrogen, Carlsbad, CA) was then added at a lipid to protein ratio of 3.75:1 (w/w) and incubated at room temperature overnight to form apoE3/DMPC complexes [16]. DMPC only controls were prepared similarly, using PBS instead of apoE, and therefore had equivalent DMPC content.

Flow cytometry

Flow cytometry was performed on 1×10^6 fresh, gel-filtered platelets using 5 μL phycoerythrin (PE)-conjugated JON/A or P-selectin antibody (Emfret, Eibelstadt, Germany) in a 20 μL final volume. The JON/A antibody selectively binds to activated mouse integrin αIIbβ3. Platelets were pre-incubated for 5 minutes with PBS, 10-30 μg/mL of apoE3/DMPC complexes or DMPCs alone. Activation was then initiated through the addition of either 1 μM ADP (Chrono-log, Havertown, PA) or 6.9 mU/mL of human thrombin (Sigma-Aldrich). 300 nM murine fibrinogen (Sigma-Aldrich) was added to all ADP reactions. Incubation occurred in the dark at room temperature for 30 minutes following the addition of ADP and for 15 minutes following the addition of thrombin. Agonist concentrations and time courses were selected from a standard curve such that approximately 50-60% of maximal activation was achieved. Reactions were quenched by addition of 400 μL of PBS and quantitated immediately using a Guava EasyCyte flow cytometer (Guava Technologies, Hayward, CA).

For studies of the role of nitric oxide, platelets were pretreated with 1.2 mM *N*-nitro-*L*-arginine methyl ester (L-NAME) (Sigma-Aldrich) for 30 minutes prior to treatment with 20 μg/mL of apoE3/DMPC complexes and 6.9 mU/mL of thrombin, as described, and activation was quantitated using the JON/A antibody.

Aggregometry

Aggregometry was performed using approximately 1×10^8 gel-filtered platelets/ml in a final reaction volume of 250 μL. Aggregation was stimulated using 0.5 μM ADP or 50 mU/mL of thrombin. 300 nM fibrinogen was added to platelet suspensions before stimulation with ADP. Tyrode's buffer containing 0.1% glucose and 0.35% BSA was used to establish baseline turbidimetric readings. All assays were performed using a Chrono-log whole blood aggregometer and data was recorded using AGROLINK software.

Intravital microscopy

Intravital microscopy was performed on similarly-sized 8 week old mice. Mice were anesthetized, and the right jugular vein and left carotid artery were exposed through a midline cervical incision. The carotid artery was suspended on a piece of black plastic in order to provide contrast for the microscope, and the vessel was stripped of adventitia. 100 μL Rhodamine 6G (0.5 mg/mL, Sigma-Aldrich) was injected directly into the right jugular vein in order to fluorescently label platelets. A 1 × 2 mm piece of filter paper was saturated with a 12.5% $FeCl_3$ solution and was then applied to the left carotid artery for 1 min. The filter paper was removed, and the vessel was rinsed with saline. Clot formation was observed in real-time under a Leica DMLFS fluorescent microscope (Leica, Issaquah, WA) using a Leica water immersion objective at 20× magnification and an attached Gibraltar Platform (EXFO, Quebec, Canada). The time to complete occlusion of flow was determined through visual inspection

using real time video image capture with a QImaging Retigo Exi 12-bit mono digital camera (Surrey, Canada) and Streampix version 3.17.2 software (Norpix, Montreal, Canada). The assay end points were defined as 1) cessation of blood flow for more than 30 seconds or 2) 45 minutes without carotid occlusion (in which case the time was recorded as 45 minutes).

Cholesterol efflux assays

Gel-filtered platelets resuspended in 250 μL of Tyrode's buffer containing 0.1% glucose and 0.35% BSA were incubated with 1 μCi of ^{3}H-cholesterol (Amersham Biosciences, Piscataway, NJ) for 1 hr at room temperature. The platelets were then pelleted, resuspended in fresh buffer, and washed again by filtration through a sepharose 2B column. Incubations with 30 μg/mL of apoE3/DMPC complexes or equivalent DMPC alone were performed in a final volume of 40 μL for a duration of 25 minutes at room temperature. 10 mM methyl-β-cyclodextrin was used as a positive control. Subsequently, sepharose 2B beads suspended in PBS were added to the reaction as carriers and platelets were sedimented at 863 *g*. Supernatants were collected, pellets were lysed in 100 μL of lysis solution (1% SDS (w/w), 10 mM sodium phosphate and 5 mM DTT), and radioactivity was measured in a scintillation counter. Percent efflux was calculated by dividing the DPM in the supernatant over the total DPM in the reaction.

Tail bleeding assays

Tails of anesthetized, 7 week old mice were equilibrated for 5 min in 37 °C PBS. 5 mm of the distal tail was then amputated and the tail was returned to the PBS. The time to cessation of bleeding was recorded as the bleeding time. The assay endpoint for bleeding times was 10 minutes (in which case the time was recorded as 10 minutes).

Statistical Analysis

All data is shown as mean ± standard deviation, unless otherwise stated. Kolmogorov-Smirnov tests were used to determine if data was normally distributed. Comparisons of three groups were performed by ANOVA, unless the data was not normally distributed, in which case the non-parametric Kruskal-Wallis test was used. All statistics were performed using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

Results

Activation of LRP8-deficient platelets is reduced *in vitro* in response to ADP and thrombin

We examined activation of gel-filtered platelets in the absence of apoE using both flow cytometry and turbidimetric aggregometry. Activation of αIIbβ3 integrin, measured by flow cytometry, was significantly reduced in both $LRP8^{+/-}$ and $LRP8^{-/-}$ mice in response to both ADP (Figure 1A) and thrombin (Figure 1B). 1 μM ADP activated 21.2 ± 3.3% of platelets isolated from $LRP8^{+/+}$ mice (n=6) but only activated 12.1 ± 4.2% and 15.9 ± 2.0% of platelets from $LRP8^{+/-}$ (n=6) and $LRP8^{-/-}$ mice (n=6), respectively ($p<0.01$ and $p<0.05$ for $LRP8^{+/-}$ and $LRP8^{-/-}$ platelets compared to wild-type controls). Likewise, 6.9 mU/mL thrombin activated a mean of 66.0 ± 5.9% of platelets isolated from $LRP8^{+/+}$ mice (n=8), whereas it only activated 55.3 ± 5.3% and 53.1 ± 11.4% of platelets from $LRP8^{+/-}$ (n=7) and $LRP8^{-/-}$ mice (n=6), respectively ($p<0.05$ for both $LRP8^{+/-}$ and $LRP8^{-/-}$ platelets when compared to $LRP8^{+/+}$ platelets). In contrast, thrombin mediated P-selectin expression in the LRP8 mutant mice was not statistically different across genotypes (data not shown).

Results generated with turbidimetric aggregometry were consistent with the activated αIIbβ3 expression differences seen using flow cytometry. Both the rate and extent of platelet aggregation, as evidenced by the initial slope of the curve and the maximum percent aggregation achieved were significantly different in response to both ADP and thrombin (Table

1). The initial slope and maximum aggregation were decreased in *LRP8*$^{+/-}$ and *LRP8*$^{-/-}$ mice. Representative aggregation curves are shown for both ADP (Figure 2A) and thrombin (Figure 2B).

Clot formation and vessel occlusion are altered *in vivo*

Both intravital microscopy following injury with ferric chloride and tail bleeding assays were performed to evaluate thrombosis *in vivo* in *LRP8*-deficient mice. Carotid occlusion times in response to ferric chloride injury, as measured by intravital microscopy, were significantly different between the *LRP8*$^{+/-}$ and *LRP8*$^{+/+}$ mice (Figure 3A). The median times to occlusion were 9.5 minutes, 29.0 minutes, and 16.0 minutes in *LRP8*$^{+/+}$, *LRP8*$^{+/-}$, and *LRP8*$^{-/-}$ mice, respectively. Thus, the occlusion times for the hemizygous *LRP8*$^{+/-}$ and the *LRP8*$^{-/-}$ mice were approximately 200% and 68% greater, respectively, than those for the wild-type mice. A non-parametric ANOVA comparing occlusion times between genotypes yielded an overall *p*-value of 0.044, and a Dunn's post-test demonstrated a significant difference between *LRP8*$^{+/+}$ and *LRP8*$^{+/-}$ mice with a *p*-value <0.05. Representative still images of early clot formation for each genotype are shown (Figure 3B). Qualitatively, the *LRP8*$^{+/-}$ mice formed smaller thrombi throughout the time course than either *LRP8*$^{+/+}$ or *LRP8*$^{-/-}$ mice. Compared to the *LRP8*$^{+/+}$ mice, the *LRP8*$^{-/-}$ mice had smaller thrombi at 2 minutes post injury, but typically the thrombus size was only slightly reduced at 4 minutes and later time points.

Despite the differences observed for the occlusion time, the median bleeding times were 80, 103 and 101 seconds for the *LRP8*$^{+/+}$ (n=29), *LRP8*$^{+/-}$ (n=53) and *LRP8*$^{-/-}$ (n=34) mice, respectively, and these differences were not statistically significant by a non-parametric ANOVA (p=0.35, Figure 4).

apoE3 inhibits platelet activation through LRP8

In order to confirm that apoE signals through LRP8 to inhibit platelet activation, we used flow cytometry to measure activated αIIbβ3 integrin in response to thrombin activation, following pretreatment with lipidated human apoE3. We observed a dose-dependent reduction of activation when gel-filtered platelets were pre-treated with apoE3/DMPC complexes. *LRP8*$^{-/-}$ mice demonstrated 50% less inhibition than the wild-type controls in the presence of the highest dose of apoE3/DMPC (Figure 5A). A two-way ANOVA demonstrated interaction between apoE3/DMPC dose and genotype at a significance level of p=0.0118. Bonferroni post-tests confirmed a difference between *LRP8*$^{+/+}$ and *LRP8*$^{-/-}$ platelets at p<0.01 for doses of apoE3 ranging from 10-20 μg/mL and at p<0.001 for 30 μg/mL of apoE3. Inhibition of *LRP8*$^{+/-}$ platelet activation by apoE3/DMPC was comparable to that of *LRP8*$^{+/+}$ platelets and significantly different from *LRP8*$^{-/-}$ platelets (p <0.05 for doses of apoE3 between 10-20 μg/mL and p <0.001 for 30 μg/mL of apoE3). DMPC liposomes in the absence of apoE only mildly inhibited activation of *LRP8*$^{+/+}$ platelets (Figure 5A).

To ensure that the inhibitory effect of apoE was not due extraction of cholesterol from the platelet membrane, we measured cholesterol efflux in response to apoE3/DMPC complexes, DMPC liposomes, and methyl-β-cyclodextrin (Figure 5B). Cholesterol efflux to apoE3/DMPC complexes was approximately 3% over background levels, and was similar to efflux to DMPC liposomes. Thus, the degree of cholesterol efflux did not correlate with the inhibition of platelet activation. There was no *LRP8* genotype effect on cholesterol efflux to any of these acceptors (Figure 5B).

Nitric oxide does not mediate effect of apoE3

The nitric oxide inhibitor L-NAME was used to determine if NO played an important role in apoE3 mediated inhibition of murine platelet activation by thrombin (Figure 5C). The addition of L-NAME did not significantly affect thrombin mediated activation of *LRP8*$^{+/+}$ platelets.

Furthermore, L-NAME did not reverse the inhibition of platelet activation caused by lipidated apoE3.

Discussion

Our group has previously associated polymorphisms in the *LRP8* gene with increased risk for MI [2,3]. Since LRP8 has been identified on platelets [5], we sought to determine if it has a modulatory role in platelet activation and thrombus formation by using *LRP8*-deficient mice, for which we could find no prior reports regarding platelet activation or thrombosis.

Although LRP8 has not been extensively studied in human platelets, it has been proposed that both apoE and LDL are ligands for platelet LRP8 [5,10,14]. Data suggest that apoE attenuates the response of platelets to agonists [5,14], while LDL activates p38 mitogen-activated protein kinase (MAPK) phosphorylation in platelets [10], both presumably through LRP8. In our activation studies, platelet preparations were washed, filtered and diluted in order to separate them from any lipoproteins present in the plasma; although we observed no effect of human LDL on platelet activation as measured by flow cytometry (data not shown). In the absence of lipoproteins, we observed a reduction in the rate and extent of activation for both $LRP8^{+/-}$ and $LRP8^{-/-}$ platelets in response to thrombin and ADP compared to wild-type platelets (Figures 1-2 and Table 1). One potential explanation for this finding is that platelets secrete, or expose on their surfaces, a ligand for LRP8, resulting in autocrine regulation that augments platelet activation. Of the many adhesion proteins, mitogens and protease inhibitors released from the α-granules [17], several are known to interact with other members of the LRP family [18-21], but further investigation would be required to determine if any of these molecules are ligands for LRP8. Alternatively, reduced presence or absence of LRP8 on the platelet surface may alter platelet activation in a ligand independent fashion. Attempts at quantifying levels of LRP8 on platelet membranes (and in brain extracts as a positive control) were unsuccessful, as several commercially available antibodies were unable to detect LRP8 by Western blot or flow cytometry.

Examination of thrombosis *in vivo* in LRP8-deficient mice confirmed the importance of LRP8 in modulating thrombus formation. This data clearly demonstrates deviations from the wild-type thrombosis phenotype in $LRP8^{+/-}$ mice (Figure 3A) and shows qualitative differences early in thrombosis in both $LRP8^{+/-}$ and $LRP8^{-/-}$ mice (Figure 3B), affirming the functional importance of the observed *in vitro* reduction in platelet activation.

The inhibition of activation of gel-filtered platelets that we observed in response to apoE (Figure 5A) was comparable to the effect previously observed with human platelets [14]. We used apoE phospholipid complexes, which mimic the form of apoE secreted by macrophages [22], as lipidation of apoE has previously been demonstrated to be required for binding to LRP8 [5, 14, 16]. As expected, this effect was significantly attenuated in $LRP8^{-/-}$ platelets, confirming that apoE does indeed signal through LRP8 to inhibit platelet activation by agonists. Yet, some level of inhibition was still evident even in $LRP8^{-/-}$ platelets, suggesting that apoE may also signal platelets through non-LRP8 pathways. This is consistent with previous human platelet studies in which receptor-associated protein (RAP), a specific inhibitor of LRP family members, only reversed about 60% of the inhibition of activation caused by apoE [5]. The remainder of the apoE effect cannot be accounted for by cholesterol sequestration from the platelet membrane, which is known to diminish response to agonists [23-25], as the highest dose of DMPC liposomes caused only minor inhibition of platelet activation, but led to the same amount of cholesterol efflux as observed for lipidated apoE (Figure 5B).

Nevertheless, a few differences were noted between human and murine platelets with regard to apoE mediated inhibition. Riddell, *et al* have previously reported that thrombin mediated

activation of human platelets was only inhibited using very high doses of apoE/DMPC (500 μg/mL) or incubation times reaching 30 minutes [14]. In contrast to that study, we found that physiological doses of apoE3/DMPC complexes and a relatively short pre-incubation period were sufficient to inhibit thrombin induced aggregation in murine platelets (Figure 5A). This difference could be accounted for by differences in the concentration of thrombin used or by innate differences between human and murine platelets. Thrombin activates human platelets through activation of the PAR-4 and PAR-1 receptors, with signaling through the PAR-1 receptor predominating at low doses of thrombin, but in murine platelets thrombin signals entirely through PAR-4, with PAR-3 facilitating cleavage of PAR-4 at low doses [26]. Moreover, our results suggest that NO does not play a role in mediating inhibition of platelet activation by lipidated apoE in murine platelets (Figure 5C). This differed from the data reported using human platelets, in which an L-arginine:NO signal transduction pathway for apoE signaling was found [14].

The smaller increase in occlusion time seen in the *LRP8*$^{-/-}$ mice (compared to the large increase seen in the *LRP8*$^{+/-}$ mice, Figure 3A) can potentially be explained by the effect of apoE on platelet activation. Both *LRP8*$^{+/-}$ and *LRP8*$^{-/-}$ platelets exhibit similarly reduced activation in response to agonists (Figures 1 and 2), but *LRP8*$^{-/-}$ platelets alone show evidence of an attenuated response to regulation by apoE (Figure 5A). It is only the hemizygous *LRP8*$^{+/-}$ mice that have two factors inhibiting platelet aggregation: 1) the basal reduction to agonist activation and 2) intact inhibition by apoE, leading to longer occlusion time than in either the wild-type or knockout mice. Therefore, we hypothesize that *LRP8*$^{-/-}$ mice undergo carotid artery thrombosis faster than *LRP8*$^{+/-}$ mice because platelet activation is no longer under negative regulation by apoE, thereby counterbalancing the reduced aggregation observed in response to platelet agonists. Interestingly, we observed no significant effect on bleeding time between genotypes (Figure 4), suggesting that alteration of LRP8 is able to modulate thrombosis without altering this response to wounding.

It is apparent from this work that polymorphisms in *LRP8* have the potential to modify platelet function and thrombus formation, and thereby contribute to the development of early-onset MI. It is currently unknown whether several implicated polymorphisms in *LRP8* are associated with altered expression levels or protein coding changes that could alter LRP8 function, but either would theoretically have the potential to contribute pathologically to an MI. Increased expression or a gain of function of this receptor could augment agonist-induced platelet activation (as we observed for *LRP8*$^{+/+}$ vs. *LRP8*$^{+/-}$ and *LRP8*$^{-/-}$ mice). One important human variant about which slightly more is known, the R952Q variant of LRP8, has recently been demonstrated to increase p38 MAPK phosphorylation *in vitro* and increase platelet aggregation by 14% in patients not on anti-platelet therapy when compared to the common variant not associated with disease [3]. This has clear potential to contribute to myocardial infarction. ApoE signaling through LRP8 may be a key regulator of platelet activation, and since apoE is present in blood and secreted locally in lesions by macrophages [27], it has the potential to be an important regulator of thrombosis during a plaque rupture.

In conclusion, our use of a mouse knockout model demonstrated that LRP8 can modulate platelet activation *in vitro* and thrombosis *in vivo*. These findings lend support to the hypothesis that polymorphisms in *LRP8* may play a role in susceptibility to MI, and they provide a rational mechanism for this association. At the same time, our data show that partial LRP8 deficiency is capable of altering thrombosis in the context of intravascular injury without altering bleeding time, indicating that a partial inhibitor of LRP8 could have potential clinical applications.

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Abbreviations

LRP8
: Lipoprotein receptor related protein 8

ApoER2
: Apolipoprotein E receptor 2

CAD
: coronary artery disease

MI
: myocardial infarction

SNPs
: single nucleotide polymorphisms

LDL
: low density lipoprotein

Q1-Q3
: interquartile range

PE
: phycoerythrin

DMPC
: 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine

L-NAME

N-nitro-*L*-arginine methyl ester

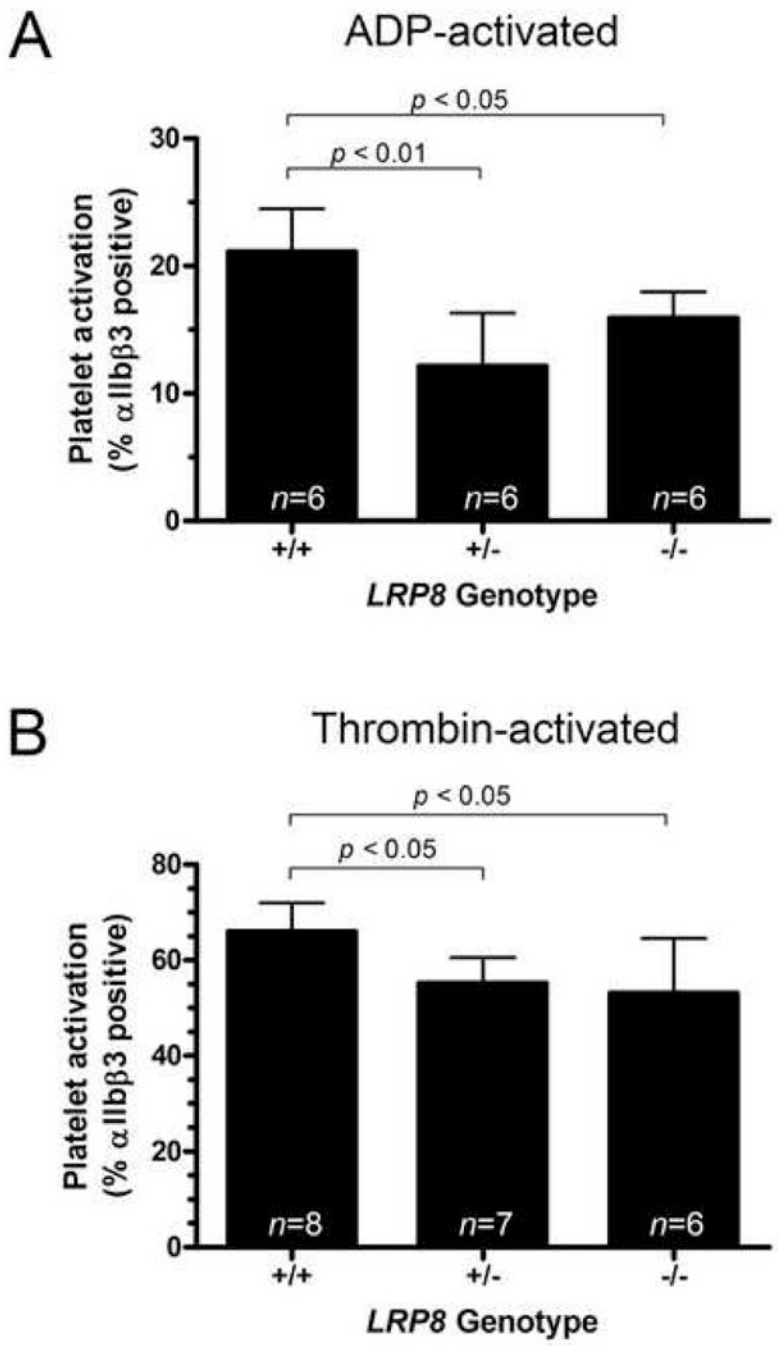

Figure 1. Reduced expression of activated αIIbβ3 integrin on the surface of *LRP8*$^{+/-}$ and *LRP8*$^{-/-}$ platelets in response to ADP and thrombin by flow cytometry

Gel filtered platelets from mice of each of the *LRP8* genotypes were activated with either 1 μM ADP (A) or 6.9 mU/mL thrombin (B) at room temperature.

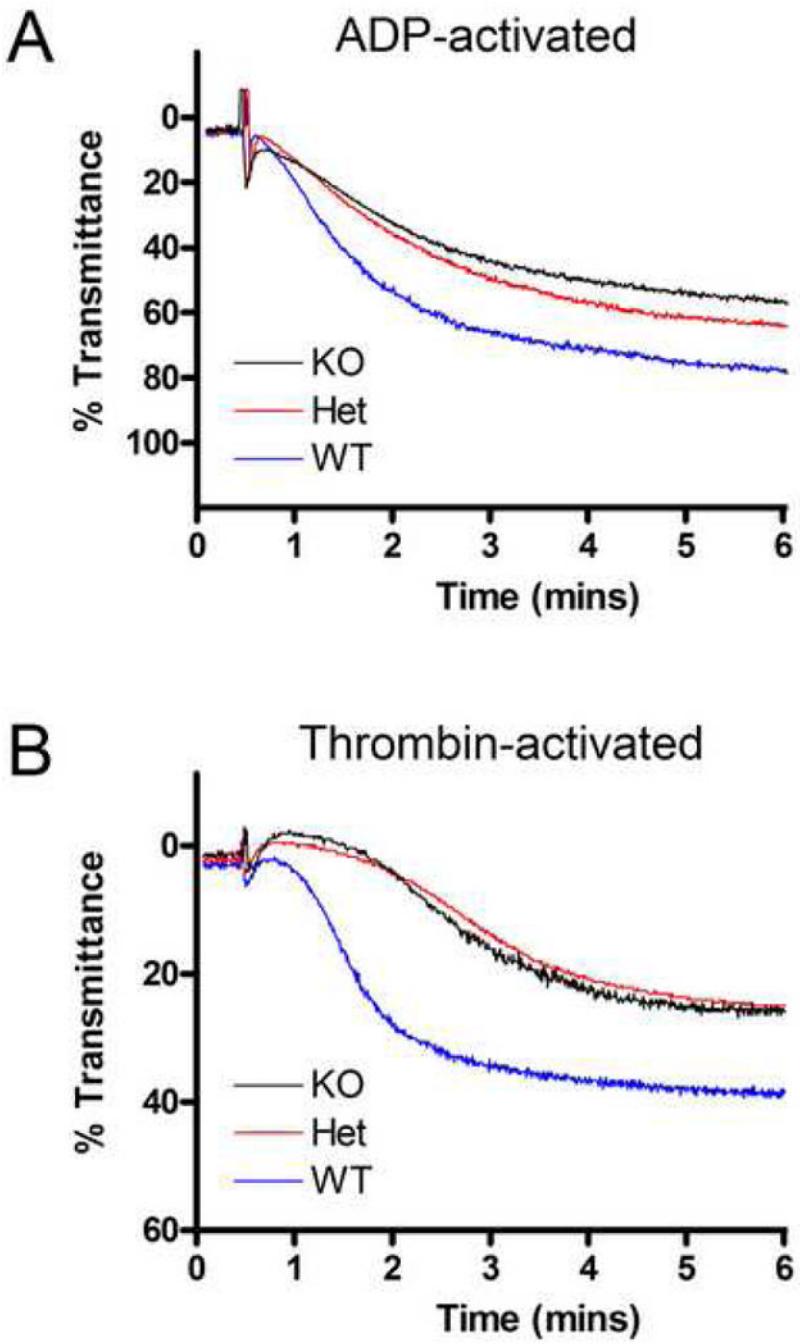

Figure 2. Effects of LRP8-deficiency on platelet aggregation

Representative *in vitro* platelet aggregation stimulated by either 0.5 μM ADP (A) or 50 mU/mL thrombin (B). Aggregation of gel filtered platelets was measured on a Chrono-log whole blood aggregometer, and data were recorded as percent transmittance (% aggregation).

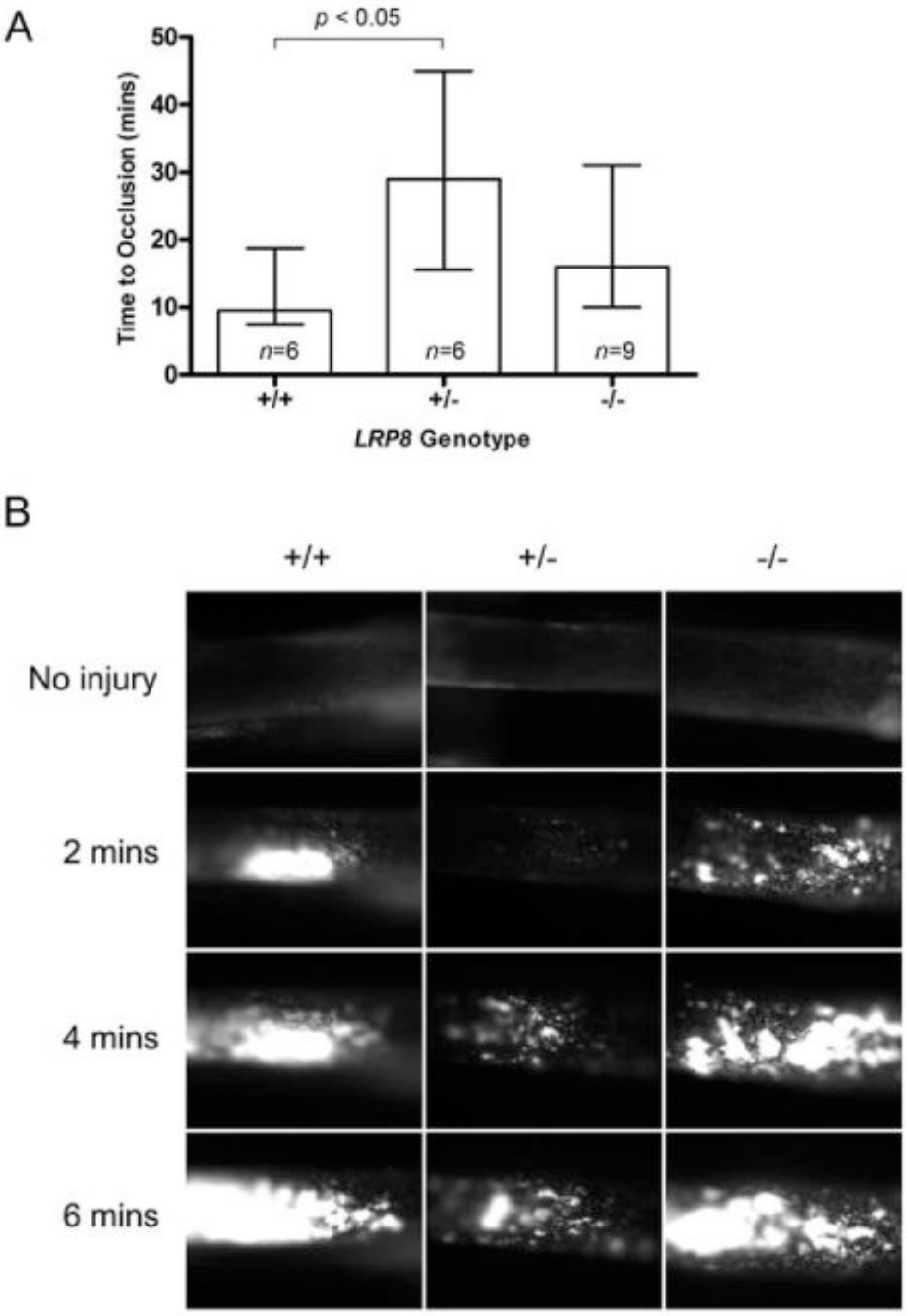

Figure 3. Thrombus formation assessed by intravital microscopy is altered *in vivo*

Platelets were labeled with Rhodamine 6G, and thrombosis was initiated with 12.5% $FeCl_3$ applied to the carotid artery. Time to cessation of flow is plotted as median ± Q1-Q3 (A). Representative images of thrombus formation are shown (B).

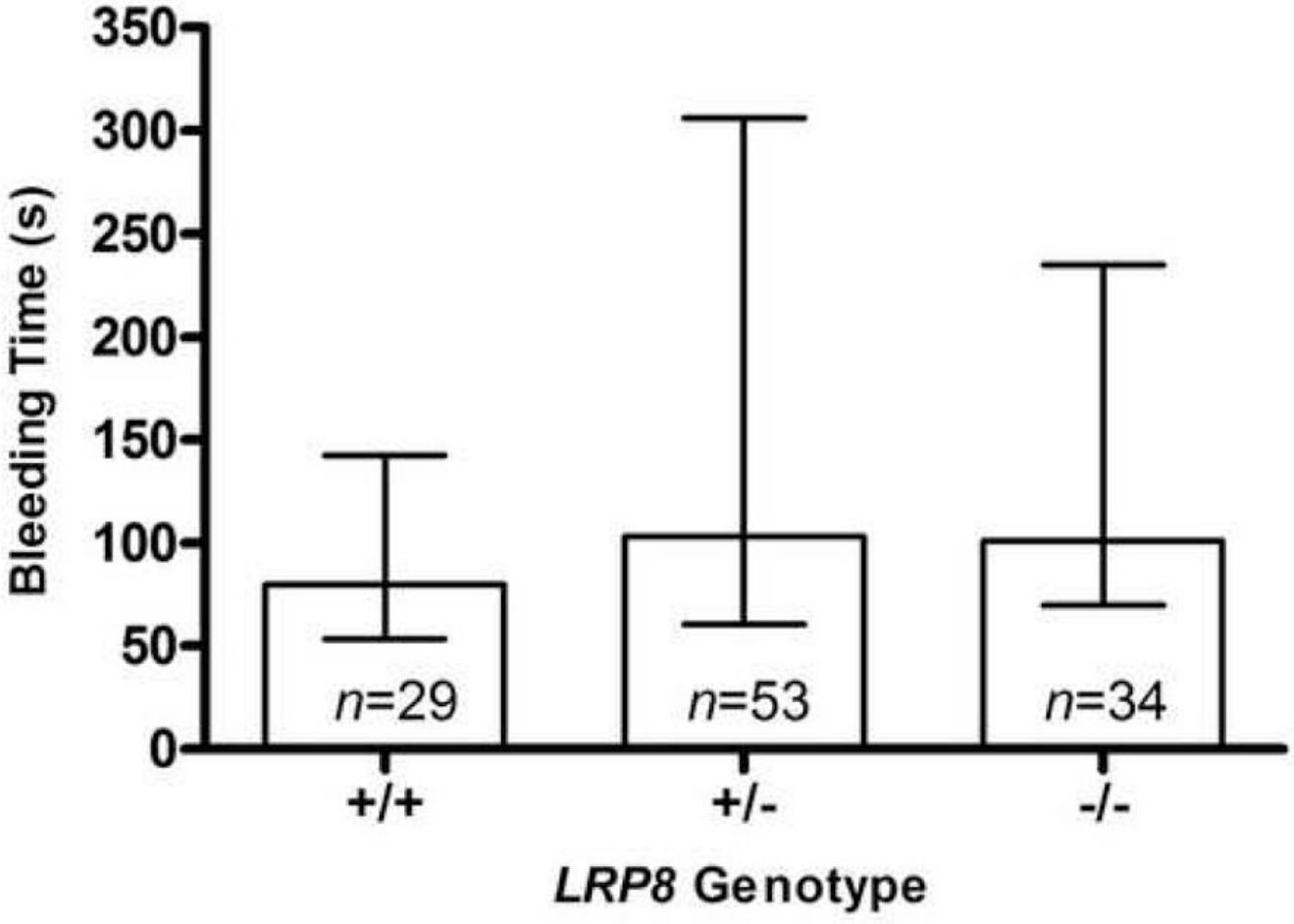

Figure 4. Effects of genotype on *in vivo* tail bleeding assays

The bleeding time was defined as time to cessation of initial bleeding. Plots show median ± Q1-Q3.

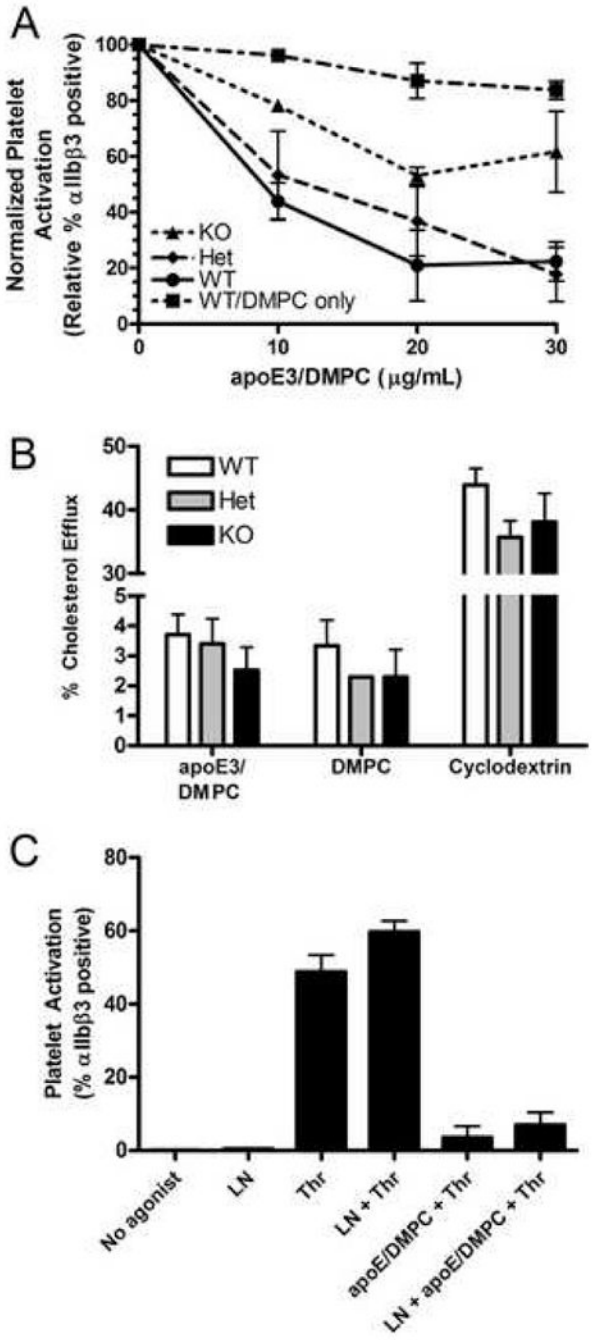

Figure 5. LRP8-deficiency attenuates apoE-mediated inhibition of activation of platelets

Gel filtered platelets were pre-incubated with lipidated apoE3 or equivalent DMPC before activation with thrombin and flow cytometric analysis (n=3, A). Genotype had no effect on cholesterol efflux from platelets, and efflux to lipid complexes with and without apoE3 was not significantly different (n=2, B). ApoE-mediated inhibition of platelet activation is not NO-dependent (n=4, C). LN, L-NAME.

Table 1

Platelet aggregometry analytic metrics

	[1] $LRP8^{+/+}$	[1] $LRP8^{+/-}$	[1] $LRP8^{-/-}$	[2] Overall p-value	[3] Dunnett's p-value
ADP (*n*=5)					
[4] Slope	1.45 (0.25)	0.85 (0.23)	0.85 (0.21)	0.016	<0.05
[5]% Aggregation	47.40 (8.70)	33.20 (10.07)	31.40 (7.41)	0.022	<0.05
Thrombin (*n*=4)					
[4] Slope	1.18 (0.48)	0.33 (0.15)	0.24 (0.14)	0.033	<0.05
[5]% Aggregation	42.50 (17.70)	25.25 (16.33)	20.75 (13.95)	0.013	<0.05

[1]Values expressed as mean (SEM)

[2]*P*-values generated using a repeated measures ANOVA

[3]*P*-values for $LRP8^{+/-}$ and $LRP8^{-/-}$ vs. $LRP8^{+/+}$ by Dunnett's post-test.

[4]Absolute value of the slope of the results between minutes 1-2 of aggregation calculated by linear regression

[5]Maximum aggregation in response to agonist