Platelet IκB Kinase-β Deficiency Increases Mouse Arterial Neointima Formation via Delayed Glycoprotein Ibα Shedding

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Objective—On the luminal surface of injured arteries, platelet activation and leukocyte–platelet interactions are critical for the initiation and progression of arterial restenosis. The transcription factor nuclear factor-κB is a critical molecule in platelet activation. Here, we investigated the role of the platelet nuclear factor-κB pathway in forming arterial neointima after arterial injury.

Methods and Results—We performed carotid artery wire injuries in low-density lipoprotein receptor-deficient (LDLR−/−) mice with a platelet-specific deletion of IκB kinase-β (IKKβ) (IKKβfl/fl/PF4cre/LDLR−/−) and in control mice (IKKβfl/fl/LDLR−/−). The size of the arterial neointima was 61% larger in the IKKβfl/fl/PF4cre/LDLR−/− mice compared with the littermate control IKKβfl/fl/LDLR−/− mice. Compared with the control mice, the IKKβfl/fl/PF4cre/LDLR−/− mice exhibited more leukocyte adhesion at the injured area. The extent of glycoprotein Ibα shedding after platelet activation was compromised in the IKKβ-deficient platelets. This effect was associated with a low level of the active form of A Disintegrin And Metalloproteinase 17, the key enzyme involved in mediating glycoprotein Ibα shedding in activated IKKβ-deficient platelets.

Conclusion—Platelet IKKβ deficiency increases the formation of injury-induced arterial neointima formation. Thus, nuclear factor-κB–related inhibitors should be carefully evaluated for use in patients after an arterial intervention. (Arterioscler Thromb Vasc Biol. 2013;33:241-248.)

Key Words: arterial injury • leukocytes • NF-κB • platelets • restenosis

Neointimal hyperplasia after percutaneous interventions, such as balloon angioplasty or stenting, is the principal cause of arterial restenosis. Platelet deposition and subsequent leukocyte–platelet interactions on the injured luminal surface are critical in the repair process after arterial damage. Although platelets are anucleate cells, they contain nearly all of the nuclear factor-κB (NF-κB) family members. NF-κB plays a complex role in platelet activation. NF-κB inhibitors impair platelet aggregation mediated by ADP, collagen, and thrombin, and reduce ATP release, thromboxane B2 formation, and P-selectin expression stimulated by thrombin. However, arachidonic acid-induced platelet activation is not affected by NF-κB inhibitors. The common pathway that regulates the activation of NF-κB is based on the degradation of IκB from the NF-κB complex, a process initiated by IκB phosphorylation. The latter is catalyzed by a multisubunit protein kinase called IκB kinase (IKK). IKKβ is more active than other IKK subunits in catalyzing IκB phosphorylation. The loss of IKKβ dramatically inhibits NF-κB activation, resulting in embryonic lethality in mice.

We were interested in determining whether the inhibition of platelet NF-κB activation could suppress neointima formation after arterial injury. Using floxed IKKβ (IKKβfl/fl) mice, we generated mice with IKKβ deficiency specifically in platelets by breeding PF4-Cre (PF4cre) mice with these floxed mice, and breeding the resulting mice with low-density lipoprotein

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receptor-deficient (LDLR−/−) mice. The role of platelet IKKβ in arterial neointima formation was evaluated by comparing the size of the neointima and leukocyte–platelet interactions in the injured arteries. Furthermore, the underlying mechanisms contributing to the change in the leukocyte–platelet interactions were explored.

Materials and Methods

The PF4fl/fl mice13 and the floxed-IKKβ mice,14 provided by the groups of Drs Radek C. Skoda and Michael Karin, were bred. A mouse 384 single-nucleotide polymorphism panel (markers spread across the genome at approximately 7-Mbp intervals; Charles River Laboratories International, Inc, Wilmington, MA) was used to characterize the genetic background of the breeders. Polymorphic markers demonstrated that the IKKβ0/0/PF4+/− breeders were 98.17% C57BL/6. The LDLR−/− mice (002207) from the Jackson Laboratory were bred with IKKβ0/0/PF4+/− to generate IKKβ0/0/PF4+/−LDLR−/− mice, and their littermate IKKβ0/0/LDLR−/− mice. The breeding scheme is detailed in Figure I in the online-only Data Supplement.

The mice were subjected to a guide wire injury in the carotid artery, and the size of the arterial neointima was examined using a method previously described.1,13,14 Leukocyte interactions with the injured arteries were studied by immunostaining the arteries with specific leukocyte markers or directly observing the injured arteries in vivo by intravital epifluorescence microscopy.1 All of the animal experiments and care were approved by the Georgia Health Sciences University Animal Care and Use Committee, in accordance with The Association for Assessment and Accreditation of Laboratory Animal Care International guidelines. The data are presented as the mean±SEM and were analyzed by either 1-way ANOVA followed by a Bonferroni correction post hoc test or Student t test to evaluate 2-tailed levels of significance. The null hypothesis was rejected at P<0.05.

Results

Platelet IKKβ Deficiency Increases Neointimal Formation in LDLR−/− mice

IKKβ0/0/PF4+/−/LDLR−/− mice and their littermate control IKKβ0/0/LDLR−/− mice were fed a Western diet for 2 weeks, followed by carotid artery wire injury. Four weeks later, the arteries were excised and processed for analysis. The 2 groups of mice were identical in body weight, blood cholesterol levels, and number of peripheral leukocytes (Tables I and II in the online-only Data Supplement). The number of platelets in the IKKβ0/0/PF4+/−/LDLR−/− mice that received bone marrow from IKKβ0/0/PF4+/−/LDLR−/− mice compared with the IKKβ0/0/PF4+/−/LDLR−/− mice (Figure 1A). The size of the media was also increased in the IKKβ0/0/PF4+/−/LDLR−/− mice compared with the IKKβ0/0/LDLR−/− mice (Figure 1A). Notably, the ratio of the intima to the media (I/M) was markedly elevated in the IKKβ0/0/PF4+/−/LDLR−/− mice compared with the IKKβ0/0/LDLR−/− mice (Figure II in the online-only Data Supplement). Macrophages in the injured carotid arteries were stained with F4/80 and found to be significantly increased in the IKKβ0/0/PF4+/−/LDLR−/− mice compared with the control mice (Figure 1B).

To exclude the effect of IKKβ deficiency in other PF4-expressing cells on the formation of arterial neointima,15 we used bone marrow transplantation to generate LDLR−/− chimeric mice that lacked IKKβ only in their platelets and the control mice that retained IKKβ in their platelets. The carotid arteries of both groups were injured with a guide wire. Four weeks after the injury, we observed that the neointimal lesions were 64% larger in the LDLR−/− mice that received bone marrow from the IKKβ0/0/PF4+/−/LDLR−/− mice compared with the control mice. The arterial neointimas from the LDLR−/− mice that received bone marrow from IKKβ0/0/PF4+/−/LDLR−/− mice also stained more strongly with a macrophage marker (Figure IIIA and IIIB in the online-only Data Supplement).

Figure 1. IκB kinase-β (IKKβ) deficiency in platelets augments injury-induced arterial neointima formation in low-density lipoprotein receptor-deficient (LDLR−/−) mice. A, Movat pentachrome staining of the arterial neointima 4 weeks after injury; the size of the neointima and media were measured (n=12 for each group). B, Immuno-staining (with anti-F4/80) of the infiltrating macrophages in the arterial neointima. Twelve cross sections of each mouse carotid artery were analyzed. The percentage positive area was calculated by dividing the positive area by the measured lesion area. Twelve injured carotid arteries were included for each group.
LDLR−/− and IKKβ0/0/LDLR−/− mice (Figure 2A). However, considerably more neutrophils were adhered to the injured area of the carotid arteries in the IKKβ0/0/PF4+/−/LDLR−/− mice compared with the IKKβ0/0/LDLR−/− mice (Figure 2B). Seven days after the wire injury, more infiltrating macrophages were observed in the injured carotid arteries of the IKKβ0/0/PF4+/−/LDLR−/− mice than in the IKKβ0/0/LDLR−/− mice (Figure 2C).

Next, we examined the interactions of the leukocytes with the injured mouse carotid arteries in vivo by using intravital epifluorescence microscopy. The circulating leukocytes, which were labeled with rhodamine 6G, rolled on and adhered to the injured vessel wall after the wire injury. The number of leukocytes rolling on the arterial wall did not differ between the IKKβ0/0/PF4+/−/LDLR−/− and IKKβ0/0/LDLR−/− mice (Figure 2D). During the early stage after the arterial injury, the number of leukocytes adhering to the arterial wall was not markedly different between the 2 groups. Twenty minutes later, more leukocytes had adhered to the arterial walls of the IKKβ0/0/PF4+/−/LDLR−/− mice compared with the IKKβ0/0/LDLR−/− mice (Figure 2D).

To further define the binding affinity of the IKKβ-deficient and control platelets to leukocytes, an ex vivo microflow chamber was used.14 The chamber was coated with thrombin-activated, IKKβ-deficient and control platelets, which were isolated from IKKβ0/0/PF4+/− or IKKβ0/0 mice. Then, whole blood from the wild-type mice was allowed to flow through the chamber. Interestingly, the number of rolling wild-type leukocytes on the chamber coated with activated IKKβ-deficient platelets was low, but these levels did not reach significance when compared with the chamber coated with control platelets. However, the number of adhering leukocytes was much greater in the chamber coated with IKKβ-deficient platelets compared with the chamber coated with control platelets (Figure 2E).

To evaluate the role of platelet IKKβ deficiency in mediating platelet–leukocyte interactions in the circulation, we examined the mouse blood leukocyte population by flow cytometry. Both platelet–neutrophil and platelet–monocyte aggregates were increased 4 to 5 times in the IKKβ0/0/PF4+/−/LDLR−/− mice compared with the IKKβ0/0/LDLR−/− mice (Figure IV in the online-only Data Supplement). Within those aggregates, the level of glycoprotein (GP) Ibα expression was remarkably higher in IKKβ0/0/PF4+/−/LDLR−/− mice (Figure IV in the online-only Data Supplement). Leukocyte Mac-1 (CD11b/CD18) and platelet GPIbα are critically involved in the formation of leukocyte–platelet aggregates.16-18 This result suggests that platelet IKKβ deficiency enhances platelet–leukocyte aggregation resulting from the elevated level of GPIbα on IKKβ-deficient platelets.

Platelet IKKβ Deficiency Decreases Platelet Activation, Secretion, and Aggregation
To evaluate the role of IKKβ deficiency in platelet function, platelets from IKKβ0/0/PF4+/− mice and their IKKβ0/0 littermates were stimulated with thrombin. Flow cytometry revealed that there was no significant difference in P-selectin expression between the resting IKKβ-deficient platelets and the control platelets (Figure 3A). However, P-selectin expression induced with 0.1 U/mL thrombin was diminished in the IKKβ-deficient platelets compared with the control platelets (Figure 3A). In addition, Lumi-Aggregometer–based experiments demonstrated that IKKβ-deficient platelets exhibited reduced ATP release and aggregation compared with control platelets, when stimulated with thrombin (Figure 3B). Electron microscopy also indicated that the release of α-granules and platelet aggregation were attenuated in the IKKβ-deficient platelets compared with the control platelets, after stimulation with thrombin for 10 minutes (Figure 3C).

IKKβ Deficiency Attenuates GPIbα Shedding in Platelets
Platelet GPIbα plays an important role in the interaction between platelets and leukocytes.16-18 We examined the level of GPIbα in IKKβ-deficient platelets. Platelets were isolated from IKKβ0/0/PF4+/− mice and their littermate IKKβ0/0 mice. Flow cytometry (Figure 4A) and Western blot analyses (Figure 4B) demonstrated that there was no significant difference in GPIbα expression between the resting IKKβ-deficient...
platelets and the control platelets. Stimulation with thrombin caused GP shedding from the surface of the platelets. Indeed, thrombin treatment led to rapid GPIbα shedding from the IKKβfl/fl platelets in a time-dependent manner. However, IKKβ deficiency significantly attenuated the extent of GPIbα shedding (Figure 4A and 4B). Flow cytometry demonstrated slow shedding of GPV in IKKβ-deficient platelets compared with control platelets (Figure VA in the online-only Data Supplement). Interestingly, stimulation with thrombin did not change the surface levels of GPV1 (Figure VB in the online-only Data Supplement), GPIX (Figure VC in the online-only Data Supplement), or αIIbβ3 (Figure VD in the online-only Data Supplement) on the platelets. ADP (5 μM) and collagen IV (20 μg/mL) also caused GPIbα shedding, but no difference was observed between the IKKβ-deficient and control platelets (data not shown). No difference was observed in the protein levels of GPV, GPVI, GPIX, or αIIbβ3 on the membrane of the platelets after stimulation with ADP or collagen (data not shown). These results demonstrate that platelet IKKβ plays a critical role in GPIbα and GPV shedding after thrombin stimulation.

**Figure 3.** IKKβ kinase-β (IKKβ) deficiency in platelets decreases platelet activation, secretion, and aggregation. The washed platelets from the IKKβfl/fl/PF4cre and the IKKβfl/fl mice were stimulated with or without thrombin. A, Platelet P-selectin expression was analyzed by flow cytometry after stimulation with 0.1 U/mL thrombin for 0, 10, and 60 minutes at 22°C, and the relative expression of P-selectin (mean fluorescence intensity) was compared (n=5). B, ATP release and platelet aggregation were examined in a Lumi-Aggregometer after the platelets were incubated with 0.025 U/mL thrombin. The data represent 5 independent experiments. C, Platelet aggregation and α-granule release (arrowhead) were evaluated by electron microscopy after incubation with 0.1 U/mL thrombin for 10 minutes at 22°C. The data represent 5 independent experiments.

**Figure 4.** IKKβ kinase-β (IKKβ) deficiency inhibits thrombin-induced glycoprotein (GP) Ibα shedding in platelets. Platelets isolated from IKKβfl/fl/PF4cre and IKKβfl/fl mice were stimulated with 0.1 U/mL thrombin. A, Representative flow cytometric histograms and the relative expression of GPIbα at different times after stimulation (n=5). B, An immunoblot of platelet GPIbα at different times after stimulation (n=5).
antibody that selectively blocks GPIbα-binding site on Mac-1 in mouse injury models.18 The carotid arteries collected from mice at 1 hour after the carotid arterial injury were examined by histology. The number of platelets covering the injured area was not significantly different between the IKKβfl/fl/PF4cre/LDLR−/− and the IKKβfl/fl/LDLR−/− mice treated with the control immunoglobulin G (IgG) or the anti-M2 antibody (Figure 5A). However, treatment with the anti-M2 antibody significantly reduced neutrophil adherence to the injured area of the carotid arteries in both the IKKβfl/fl/PF4cre/LDLR−/− mice and the IKKβfl/fl/LDLR−/− mice compared with mice treated with control IgG (Figure 5B). More importantly, the increased leukocyte adhesion observed in the control IgG-treated IKKβfl/fl/PF4cre/LDLR−/− mice compared with the control IgG-treated IKKβfl/fl/LDLR−/− mice was abolished when these mice were treated with the anti-M2 antibody (Figure 5B). The same results were observed when we directly examined leukocyte interactions with the injured arteries by intravital epifluorescence microscopy (Figure 5C). In addition, in an ex vivo microflow chamber, the rolling of leukocytes on the activated IKKβ-deficient platelets or the control platelets did not differ significantly. In line with the in vivo data for leukocyte adhesion, the adhesion of the IgG-treated leukocytes to the activated IKKβ-deficient platelets in this ex vivo model was much greater than that observed in the activated control platelets. However, treatment with the anti-M2 antibody eliminated the increased leukocyte adhesion on the activated IKKβ-deficient platelets (Figure 5D).

### Discussion

The initiation and progression of the neointima formation that underlies restenosis involves the adhesive interactions of thrombin with the αIIbβ3 complex. The shedding of the αIIbβ3 integrin from platelet membranes by the disintegrin and metalloproteinase 17 (ADAM17) is critically involved in platelet surface receptor shedding.19,20 To test the involvement of ADAM17 in our system, we stimulated IKKβ-deficient and control platelets with thrombin and detected immature (or pro) and mature (or active) forms of ADAM17 using Western blot analysis. The proform of ADAM17 was present in the resting control platelets and transiently increased by 2.7-fold during platelet activation. This finding is consistent with earlier studies.20,21 IKKβ-deficient platelets had a similar level of active ADAM17 in the resting platelets, but a much lower level of active ADAM17 during platelet activation compared with the control platelets (Figure 6A). The level of phosphorylated p38 mitogen-activated protein kinase (MAPK) has been shown to play a critical role in ADAM17 activation.22–24 Thus, we tested the effect of IKKβ deficiency on p38 MAPK phosphorylation. Thrombin remarkably increased p38 MAPK phosphorylation in the IKKβfl/fl platelets, but no significant increase in p38 MAPK phosphorylation was observed in the IKKβ-deficient platelets after 30 minutes of thrombin stimulation (Figure 6B). These data indicate that IKKβ is a key molecule in ADAM17 maturation via the regulation of p38 MAPK phosphorylation.
platelets with leukocytes, and leukocytes with the denuded vessel wall. NF-κB is expressed in platelets and plays a critical role in platelet activation. Here, we showed, in vivo, that platelet IKKβ deficiency increases neointima formation after arterial injury as a result of enhanced leukocyte–platelet interactions. Furthermore, we found that IKKβ deficiency inhibits ADAM-17 maturation, resulting in delayed GPIbα shedding in platelets.

Wire injury–induced neointima formation in the mouse carotid artery is a widely used model to mimic the pathology of arterial neointima formation in patients with arterial restenosis. An excessive leukocyte–platelet interaction and leukocyte infiltration after artery injury has been shown to exaggerate the repair mechanisms and augment neointima formation. After endothelial denudation by mechanical injury, platelets immediately adhere to and accumulate on the injured luminal surface of arteries. P-selectin and many GPs on activated platelets mediate leukocyte rolling and localization, and further support leukocyte recruitment. The binding of platelet P-selectin to its leukocyte ligand P-selectin glycoprotein ligand-1 initiates leukocyte recruitment to the activated platelets. The interaction of P-selectin and P-selectin glycoprotein ligand-1 rapidly activates the leukocyte integrin Mac-1. The binding of leukocyte Mac-1 to platelet GPIbα subsequently strengthens the firm adhesion and transmigration of leukocytes to sites of platelet deposition. The loss of Mac-1/GPIbα binding leads to reduced leukocyte accumulation after arterial injury, and further results in the inhibition of neointima thickening. In this study, increased neointima formation in the IKKβ−/−/PF4−/−/LDLR−/− mice was accompanied by an increase in leukocyte adhesion to the injured area. This increased neointima was eliminated by neutrophil depletion (Figure VI in the online-only Data Supplement), indicating that this phenotypic change in neointima formation is because of increased leukocyte adhesion. Furthermore, this increased leukocyte adhesion was abrogated by a blocking antibody that specifically inhibits the binding of leukocyte Mac-1 with platelet GPIbα. These results demonstrate that high levels of GPIbα on activated platelets contribute to the increased neointima formation in IKKβ−/−/PF4−/−/LDLR−/− mice.

The dynamic change in GPIbα on activated platelets occurs mainly through ADAM17-mediated GPIbα shedding. ADAM17 is a sheddase on the cell surface that cleaves a variety of substrates, such as heparin-binding epidermal growth factor, transforming growth factor-α, tumor necrosis factor receptor, epidermal growth factor receptor, vascular cell adhesion molecule-1, and L-selectin. After cellular activation, the proform (134 kDa) of ADAM17 is proteolyzed to yield the mature (active) form (98 kDa). The latter active form cleaves its substrates. ADAM-17 activation in leukocytes occurs through p38 or the extracellular signal-regulated kinases MAPK pathway. ADAM17 is a major sheddase involved in platelet GPIbα shedding. Inactivation led to a 90% reduction in GPIbα shedding in platelets. Brill et al reported that oxidative stress activates ADAM-17 in platelets in a p38-dependent fashion. Through a similar mechanism, IKKβ deficiency inhibited ADAM-17 maturation, resulting in delayed shedding of platelet GPIbα. This result is consistent with the observation that, in oral squamous cell carcinoma cells, NF-κB inhibition suppresses ADAM-17 maturation.

Platelet IKKβ deficiency did not cause a significant change in platelet adhesion and accumulation on the injured arteries. In this study, we found that IKKβ deficiency delayed GPIbα and GPV shedding. GPIbα and GPV are able to bind von Willebrand factor and collagen, respectively. High levels of GPIbα and GPV may enhance platelet adhesion to the subendothelial area of the injured arteries. However, many other molecules that are important for platelet adhesion to the subendothelial area, including GPVI, GPIX, and αIIbβ3, did not differ between the IKKβ−/−/PF4−/−/LDLR−/− mice and the control platelets (Figure V in the online-only Data Supplement). Among these studies, from other groups using NF-κB inhibitors have shown that broad NF-κB inhibition causes a defect in platelet adhesion and spreading. All of these factors may explain why decreased shedding of GPIbα and GPV does not result in an increase in platelet adhesion on the injured arteries.

NF-κB is a double-edged sword for activated platelet-mediated pathologies. NF-κB is a key regulator of inflammation, immunity, apoptosis, and cell proliferation in all types of nucleated cells. NF-κB affects the progression of inflammatory...
diseases, such as myocardial ischemia, bronchial asthma, arthritis, and cancer. The suppression of NF-κB activation has been shown to inhibit the expression of adhesion molecules, and the release of chemokines and cytokines by various inflammatory cells, eventually suppressing the progression of inflammation. However, NF-κB activation during the late stage of inflammation is associated with the resolution of inflammation and anti-inflammatory gene expression. A few reports have indicated that blockade of the NF-κB pathway accelerates the pathology of inflammatory diseases. For example, Kanter et al revealed that the inhibition of NF-κB activation in macrophages increased atherosclerosis in LDLR−/− mice. Zaph et al reported that deficient NF-κB activation in the intestinal epithelium is associated with increased inflammation in vivo. Consistent with its effect on inflammation, NF-κB activation initiates platelet activation. This process is evident by the reduced level of P-selectin, and the suppressed release of granules and ATP in IKKβ-deficient platelets. However, NF-κB activation appears to be necessary for activated platelets to shed their GPs, and the inactivation of NF-κB led to sustained activation of platelets, as demonstrated by much higher levels of GPIbα and GPV on the surface of the activated IKKβ-deficient platelets compared with the wild-type platelets. NF-κB has been shown to be involved in the transcriptional regulation of >150 genes, a significant proportion of which exhibit proinflammatory properties. Therefore, approaches to specifically inhibit the NF-κB pathway are under active development as possible therapeutic interventions. In this study, we demonstrated that platelet IKKβ deficiency enhances leukocyte–platelet interactions, resulting in aggregated neointima formation after arterial injury. The underlying mechanism is that NF-κB inactivation delays ADAM17-mediated GPIbα shedding, thus strengthening the interaction of leukocytes with platelets. Because the inhibition of the NF-κB pathway is likely to be a promising therapeutic strategy and because tissue-specific therapies are not currently available, the application of NF-κB inhibitors for diseases that have pathologies in which activated platelets are extensive participants, such as arterial injury, sepsis, and thrombosis, should be carefully evaluated.

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Disclosures

None.

References


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Supplemental materials

Platelet IKKβ Deficiency Increases Mouse Arterial Neointima Formation via Delayed Glycoprotein Ibα Shedding

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Extended methods

Mice

PF4-cre\(^1\) and floxed IKKβ mice\(^2\) (C57BL/6 background) were received from Dr. Radek C. Skoda and Dr. Michael Karin. The C57BL/6J and LDLR\(^{-/-}\) mice were received from The Jackson Laboratory (Bar Harbor, ME). PF4-cre and floxed IKKβ mice were crossed to generate platelet-specific IKKβ-deficient mice -IKKβ\(^{fl/fl}\)/PF4\(^{cre}\) mice and their IKKβ\(^{fl/fl}\) littermates (Supplemental Figure VII). A mouse 384 SNP panel (including markers spread across the genome at approximately 7 Mbp intervals) was used to characterize the genetic background of the breeding IKKβ\(^{fl/fl}\)/PF4\(^{cre}\) mice (Charles River Laboratories International, Troy, NY).

IKKβ\(^{fl/fl}\)/PF4\(^{cre}\) and LDLR\(^{-/-}\) mice were crossed to generate IKKβ\(^{fl/fl}\)/PF4\(^{cre}\)/LDLR\(^{-/-}\) mice and
their IKKβfl/fl/LDLR−/− littermates. Supplemental Figure I shows the breeding scheme. All of the animal experiments and care were approved by the Georgia Health Sciences University Animal Care and Use Committee, in accordance with AAALAC guidelines.

**Carotid artery wire injury model**

Eight-week-old male mice were fed a Western diet (42.0% kcal fat, 42.7% kcal carbohydrate, 15.2% kcal protein) (Harlan Teklad, Madison, WI) for 2 weeks. A guide wire injury was then administered to the carotid artery. To quantify the neointimas, each carotid artery was serially sectioned from the bifurcation to the common carotid artery, resulting in 100 slides; each slide had 3 serial sections. Among these slides, 12 slides, including slides 1, 10, 19, 28, 37, 46, 55, 64, 73, 82, 91 and 100, were stained with Movat pentachrome (Sigma, St. Louis, MO). For each carotid artery, 10 sections (one section on each above slide) were analyzed. The areas of the lumen, internal elastic lamina, and external elastic lamina were determined by planimetry using NIH Image Software, and the intimal area, medial area and intima to media (I/M) ratio were calculated.

**Neutrophil depletion**

After being fed a Western diet for 2 weeks, a carotid artery wire injury was performed on the IKKβfl/fl/PF4cre/ LDLR−/− mice and the IKKβfl/fl/ LDLR−/− control mice. A neutrophil-depleting anti-PMN antibody (Accurate Chemical & Scientific, Westbury, NY) was diluted in PBS (1:5), and 0.1 ml of the diluted antibody was injected into the mouse peritoneal cavity daily for 4 weeks after the wire injury. For the control mice, rabbit IgG (Accurate Chemical & Scientific) was injected at the same dose. The efficacy of the anti-PMN antibody on
neutrophil depletion was confirmed by flow cytometry and HESKA CBC-Diff Veterinary Hematology System (data not shown).

**Bone marrow transplantation**

The bone marrow transplantation was performed as previously described. Bone marrow-derived cells from the IKKβ^fl/fl^/PF4^cre^/LDLR^-/-^ mice or IKKβ^fl/fl^/LDLR^-/-^ controls were transplanted into lethally irradiated LDLR^-/-^ mice. Three weeks after the bone marrow transplantation, the mice were fed a Western diet for 2 weeks and then subjected to carotid artery wire injury.

**Histological analysis of the injured arteries**

Cross sections of the injured arteries were stained with monoclonal antibodies to identify the platelets (MWReg30; Santa Cruz Biotechnology, Santa Cruz, CA), macrophages (F4/80, clone CI:A3-1; Accurate Chemical, Westbury, NY) and neutrophils (anti-mouse neutrophil, clone 7/4; Accurate Chemical). The images were analyzed with Image-Pro Plus.

**In vivo examination of leukocyte interactions with the injured arterial wall**

The left carotid arteries of the mice were injured with a guide wire to induce vascular damage. After 10 min, the leukocyte interactions with the injured vessel were recorded and analyzed with an intravital epifluorescence microscopy system.

**Leukocyte interactions with activated platelets in a micro-flow chamber**
The mouse platelets were isolated from the IKKβ^{fl/fl}/PF4^{cre} mice and their littermate IKKβ^{fl/fl} mice by gel filtration. The micro-flow chamber was prepared as previously described. Specifically, 2 x 10^9/ml of isolated platelets were loaded into a rectangular glass capillary tube. After adhering to the surface of the capillary tube, the platelets were activated with thrombin (0.1 U/ml). The wild type mice were anesthetized and injected with 1 mg/ml rhodamine 6G (50 µl/30 g body weight) via the tail vein. Then, the blood from the carotid artery was passed through the micro-flow chamber. For some experiments, the mice were first treated with anti-M2 antibody and IgG. Leukocyte rolling and adhesion on the activated platelets was observed and recorded with an intravital epifluorescence microscope system.

### Blocking of the GPIbα binding site on Mac-1

An affinity purified peptide-specific polyclonal antibody (termed anti-M2) to the Mac-1 binding site for GPIbα was kindly supplied by Dr. Daniel I. Simon. A total of 100 µg anti-M2 or 100 µg IgG (Accurate Chemical & Scientific) was injected via the tail vein of IKKβ^{fl/fl}/PF4^{cre}/LDLR^{−/−} mice and the IKKβ^{fl/fl}/LDLR^{−/−} control mice that had been fed a Western diet for 2 weeks. Following antibody or IgG treatment, these mice were subjected to wire injury.

### Platelet activation, aggregation and secretion

The mouse platelets were isolated by gel filtration. The purity of the platelets was determined by flow cytometry (Supplemental Figure VIII). Platelet activation was achieved by treating the platelets with thrombin (Sigma, St. Louis, MO) at the indicated concentrations, followed by neutralization with an equimolar dose of hirudin (Sigma) if necessary. As previously described, platelet aggregation was measured in a Lumi-Aggregometer model 700 (Chronolog)
at 37°C with stirring (1000 rpm). In parallel with platelet aggregation, platelet secretion was monitored as ATP release with the addition of the luciferin-luciferase reagent to the platelet suspension. Quantification was performed using ATP standards.

**Flow cytometric analysis**

The anti-Ly6G, anti-CD41 and anti-P-selectin antibodies were purchased from BD Biosciences. The anti-CD115 antibody was from eBioscience (San Diego, CA). The anti-GPIbα, anti-GPV, anti-GPVI, anti-GPIX and anti-αIIbβ3 antibodies were obtained from Emfret Analytics (Wurzburg, Germany). For whole blood flow cytometry, Ly6G and CD115 were used to identify mouse circulating neutrophils and monocytes, respectively. CD41 was used to detect platelets. Flow cytometry was performed using a FACSCalibur (BD Biosciences). The data were analyzed using CellQuest (Tampa, FL) software.

**Platelet electron microscopy**

Platelets was isolated from whole blood and fixed in glutaraldehyde in White’s saline. The platelets were then placed in 1% osmic acid in Zetterquist’s buffer, dehydrated with alcohol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate to enhance the contrast, and the sections were then examined with a Philips 301 electron microscope (F.E.I. Co., Hillboro, OR, USA).

**Western blot analysis**

The platelets were lysed in a modified RIPA lysis buffer. Primary antibodies against GPIbα (Emfret Analytics, Wurzburg, Germany), ADAM17, active ADAM17 (Abcam,
Cambridge, MA), P38 MAPK, p-P38 (phosphorylated P38 MAPK), and GAPDH (Cell Signaling, Danvers, MA) were added, followed by incubation with alkaline phosphatase–conjugated secondary antibodies. The membranes were developed with a chemiluminescence reagent. The band intensities were quantified using the NIH Image J program.

**Blood lipid and leukocyte analysis**

Plasma triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and total cholesterol were measured via an automated enzymatic technique (Boehringer Mannheim GmbH). Total and differential leukocyte counts in the blood were quantified using an automated blood cell counter (Hemavet 850FS, CDC Technologies, Oxford, CT).

**Statistical analysis**

The data are presented as the mean ± SEM. The data were analyzed by either one-way ANOVA followed by a Bonferroni correction post-hoc test or Student’s *t*-test to evaluate two-tailed levels of significance. The null hypothesis was rejected at *P* < 0.05.
### Supplemental Table I and II

Blood cell analysis (Table I) and lipid profile (Table II) of the

\( \text{IKK}\beta^{0/0}/\text{LDLR}^{-/-} \) mice and the control \( \text{IKK}\beta^{0/0}/\text{LDLR}^{-/-} \) mice

#### Table I

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>WBC (k/μL)</th>
<th>NE (k/μL)</th>
<th>LY (k/μL)</th>
<th>MO (k/μL)</th>
<th>NE%</th>
<th>LY%</th>
<th>MO%</th>
<th>PLT (k/μL)</th>
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<tbody>
<tr>
<td>( \text{IKK}\beta^{0/0}/\text{LDLR}^{-/-} )</td>
<td>10</td>
<td>10.7±0.9</td>
<td>2.8±0.5</td>
<td>5.4±0.8</td>
<td>0.5±0.1</td>
<td>30.0±1.8</td>
<td>58.6±1.5</td>
<td>4.3±0.6</td>
<td>780.8±123.5</td>
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<tr>
<td>( \text{IKK}\beta^{0/0}/\text{PF4}^{cre}/\text{LDLR}^{-/-} )</td>
<td>10</td>
<td>9.8±0.6</td>
<td>2.9±0.8</td>
<td>5.1±0.9</td>
<td>0.5±0.1</td>
<td>31.9±1.3</td>
<td>56.8±1.4</td>
<td>5.4±0.5</td>
<td>598.6±95.8</td>
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#### Table II

<table>
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<tr>
<th>Genotype</th>
<th>BW (g)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dL)</th>
<th>LDL (mg/dl)</th>
<th>VLDL (mg/dL)</th>
<th>HDL (mg/dL)</th>
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<tbody>
<tr>
<td>( \text{IKK}\beta^{0/0}/\text{LDLR}^{-/-} )</td>
<td>28.4±7.7</td>
<td>863.4±58.2</td>
<td>338.3±21.2</td>
<td>636.3±47.3</td>
<td>68.4±9.7</td>
<td>159.0±10.4</td>
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<tr>
<td>( \text{IKK}\beta^{0/0}/\text{PF4}^{cre}/\text{LDLR}^{-/-} )</td>
<td>27.2±6.4</td>
<td>907.7±57.1</td>
<td>368.7±19.4</td>
<td>667.5±56.4</td>
<td>61.3±8.2</td>
<td>144.5±8.7</td>
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</tbody>
</table>
Supplemental Figure I. The breeding scheme of experimental mice.
Supplemental Figure II.

Supplemental Figure II. The size of the neointima and media of the injured carotid arteries. The arterial neointima at 4 weeks after injury was stained with Movat pentachrome, and the ratio of intima (I) to media (M) was calculated (I/M, n = 12 for each group)
Supplemental Figure III. Platelet IKKβ deficiency increased neointima formation in the wire-injured carotid arteries from the LDLR⁻/⁻ mice following bone marrow transplantation. The LDLR⁻/⁻ mice were lethally irradiated and then received bone marrow from the IKKβ⁺/⁺/PF4<sup>cre</sup>/LDLR⁻/⁻ or IKKβ⁺/⁺/LDLR⁻/⁻ mice. Three weeks after bone marrow transplantation, the mice were fed a Western diet for 2 weeks and then subjected to carotid artery wire injury. A, Movat pentachrome staining of cross sections of the injured artery at 4 weeks after injury. The size of the neointima (I) and media (M) and the ratio of the intima to media (I/M) were quantified (n = 10 for each group). B, The infiltrated macrophages were immunostained with anti-F4/80 antibody in the arterial neointima. The percentage of the area that was positive was calculated by dividing the positive area by the measured lesion area (n = 10 for each group).
Supplemental Figure IV. Platelet IKKβ deficiency increased platelet-leukocyte aggregates in vivo. IKKβ^{fl/fl}/PF4^{cre}/LDLR^{-/-} and IKKβ^{fl/fl}/LDLR^{-/-} mice were fed a Western diet for 4 weeks. Whole blood was collected from the submandible and used for flow cytometry. Platelet-neutrophil aggregates were defined as cells positive for both CD41 (platelet marker) and Ly6G (neutrophil marker), whereas platelet-monocyte aggregates were defined as cells positive for CD41 and CD115 (monocyte marker). Representative flow cytometry results showing platelet-neutrophil aggregates (A), levels of GPIbα for platelet-leukocyte aggregates (B and D) and platelet-monocyte aggregates (C) (n = 5 for each group).
Supplemental Figure V. Effects of IKKβ deficiency on GPV shedding in platelets. The platelets were isolated from the IKKβ<sup>fl/fl</sup>/PF4<sup>cre</sup> and IKKβ<sup>fl/fl</sup> mice and stimulated with thrombin at 0.1 U/ml. Representative flow cytometric histograms show the level of GPV (A), GPVI (B), GPIX (C) and αIIbβ3 (D) on the activated platelets. Three separate experiments with different donors were performed.
Supplemental Figure VI. Neutrophil depletion decreased neointima formation in wire-injured carotid arteries of both IKKβ^{fl/fl}/PF4^{cre}/LDLR^{−/−} mice and IKKβ^{fl/fl}/LDLR^{−/−} mice. The mice were fed a Western diet for 2 weeks and then subjected to carotid artery wire injury. Control rabbit IgG and anti-PMN antibody were intraperitoneally injected into these mice daily. Four weeks later, the carotid arteries were collected, and the size of the neointima and media was analyzed with Movat pentachrome staining. Ten mice were included in each group. *P<0.05 vs. IKKβ^{fl/fl}/LDLR^{−/−} mice treated with control IgG; #P<0.05 vs. IKKβ^{fl/fl}/PF4^{cre}/LDLR^{−/−} mice treated with control IgG.
Supplemental Figure VII. Western blot of isolated platelets showing that PF4-cre effectively knocked out platelet IKKβ expression in IKKβ^{fl/fl}/PF4^{cre} mice.

Supplemental Figure VIII. The purity of isolated platelets was determined by flow cytometry. Representative data showing that more than 99% platelets are CD41 positive.
References


