Platelet Extracellular Regulated Protein Kinase 5 Is a Redox Switch and Triggers Maladaptive Platelet Responses and Myocardial Infarct Expansion

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Background—Platelets have a pathophysiologic role in the ischemic microvascular environment of acute coronary syndromes. In comparison with platelet activation in normal healthy conditions, less attention is given to mechanisms of platelet activation in diseased states. Platelet function and mechanisms of activation in ischemic and reactive oxygen species–rich environments may not be the same as in normal healthy conditions. Extracellular regulated protein kinase 5 (ERK5) is a mitogen-activated protein kinase family member activated in hypoxic, reactive oxygen species–rich environments and in response to receptor-signaling mechanisms. Prior studies suggest a protective effect of ERK5 in endothelial and myocardial cells after ischemia. We present evidence that platelets express ERK5 and that platelet ERK5 has an adverse effect on platelet activation via selective receptor-dependent and receptor-independent reactive oxygen species–mediated mechanisms in ischemic myocardium.

Methods and Results—Using isolated human platelets and a mouse model of myocardial infarction (MI), we found that platelet ERK5 is activated post-MI and that platelet-specific ERK5−/− mice have less platelet activation, reduced MI size, and improved post-MI heart function. Furthermore, the expression of downstream ERK5-regulated proteins is reduced in ERK5−/− platelets post-MI.

Conclusions—ERK5 functions as a platelet activator in ischemic conditions, and platelet ERK5 maintains the expression of some platelet proteins after MI, leading to infarct expansion. This demonstrates that platelet function in normal healthy conditions is different from platelet function in chronic ischemic and inflammatory conditions. Platelet ERK5 may be a target for acute therapeutic intervention in the thrombotic and inflammatory post-MI environment. (Circulation. 2015;132:47-58. DOI: 10.1161/CIRCULATIONAHA.115.015656.)

Key Words: blood platelets ■ echocardiography ■ infarction ■ microcirculation

Platelet activation occurs at sites with vascular inflammation and in the presence of increased concentrations of reactive oxygen species (ROS). Platelet agonists, inflammatory molecules, and ROS are all greatly increased in the ischemic microenvironment after myocardial infarction (MI) or stroke. This results in sustained platelet activation and the secretion of inflammatory molecules and degradative enzymes such as matrix metalloproteinases (MMPs), leading to more leukocyte recruitment, extracellular matrix degradation, infarct expansion, and a decline in heart function beyond the primary infarct event. Acute coronary syndromes (ACSs) are a continuum with acute thrombosis at one end of the spectrum (ST-segment–elevation myocardial infarction) and chronic, ongoing myocardial ischemia at the other (unstable angina).

Platelet responses also represent a continuum in this process, beginning with plaque rupture and major vessel thrombosis, and continuing with microvascular occlusion and vascular inflammation in the peri-infarct tissue. This contributes to infarct expansion and a continued decline in heart function. The development of pharmacological agents to blunt platelet activation is largely based on platelet function in normal states, with less attention paid to ongoing platelet activation after an ischemic event.

Clinical Perspective on p 58

Aspirin and clopidogrel are the major platelet inhibitors used to treat ACS, yet 1 prospective study showed only a 20% reduction in adverse vascular events and increased bleeding
complications with the addition of clopidogrel to aspirin.\textsuperscript{6,7} Variability in the patient response to platelet inhibitors is attributed in part to polymorphisms in drug metabolism\textsuperscript{8,9} and can result in suboptimal treatment regimens for patients with ACS. Approximately one-third of patients experiencing a ST-segment–elevation myocardial infarction, even with coronary stenting, develop a no-reflow phenomenon thought to arise from microvascular obstruction. Enhanced platelet activity or inadequate platelet inhibition at the time of MI is more common in patients with no reflow. A more complete understanding of mechanisms leading to dysregulated platelets in disease states may reveal additional therapeutic strategies.\textsuperscript{10}

Extracellular regulated protein kinase 5 (ERK5; also called BMK1) is a mitogen-activated protein kinase family member activated in the myocardium and endothelial cells on ROS exposure.\textsuperscript{11-14} Targets of ERK5 are largely nuclear,\textsuperscript{13,15} but ERK5 also has nontranscriptional, cell-signaling functions. We have identified ERK5 in platelets, and, with the use of human platelets and platelets isolated from platelet-specific ERK5\textsuperscript{−/−} mice, we have investigated whether ERK5 activation after exposure to ROS and specific platelet ligands is elevated in ischemic tissues and contributes to myocardial dysfunction and remodeling. Our data establish a potential mechanistic link between platelet function in ischemic myocardial tissue and adverse myocardial remodeling after MI. We reveal that ERK5 is an ischemic sensor in platelets, contributing to ongoing post-MI platelet activation that accelerates ventricular remodeling and systolic dysfunction. Our data also demonstrate that ERK5 regulates platelet protein expression post-MI. Altogether, this study indicates that platelet ERK5 has a central role in platelet activation in the ischemic post-MI environment.

**Methods**

Complete reagent list, buffers, and methods are detailed in the online-only Data Supplement.

**Antibodies and Reagents**

A list if the antibodies and reagents used is indicated in the online-only Data Supplement.

**Platelet Isolation**

Human platelets were obtained by using laboratory protocols approved by the University of Rochester School of Medicine and Dentistry Institutional Review Board, with the isolation protocol as noted in the online-only Data Supplement.

**Protein Studies**

Primary antibody was 1:1000 overnight at 4°C in 3% bovine serum albumin/Tris-buffered saline–Tween 20. Secondary antibody (GE Healthcare, Buckinghamshire, UK) was used in a 1:2000 titer in 5% milk/Tris-buffered saline–Tween 20 for 1 hour at room temperature. Final autoradiographic films (Bioblot BXR, Laboratory Product Sales, Rochester NY) were quantified by densitometry using ImageJ software (National Institutes of Health).

**MMP Activity Assay**

Platelets were isolated from 120 μL of whole blood, and ventricular apex isolates were homogenized in extraction buffer (online-only Data Supplement). Final supernatants were protein normalized, lysates were centrifuged for 15 minutes at 4°C, and supernatants were placed in 50% vol/vol 2x nondenaturing sample buffer (online-only Data Supplement). Fifty micrograms of total cell lysate per lane was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the gel was renatured by gently rocking in 2.5% Triton X-100 for 30 minutes at room temperature, then allowed to equilibrate at room temperature with gentle rocking in zymogram buffer (online-only Data Supplement). The zymogram buffer was decanted, and the gel was rocked at room temperature for 4 hours in Simply Blue Safestain (Invitrogen). MMP activity was noted by clear bands in the final gel (a reverse image). Total MMP activity in each lane was quantified by densitometry with the use of ImageJ software (National Institutes of Health).

**Mouse Colony**

ERK5\textsuperscript{floxed/floxed} mice on a C57Bl/6 (B6) background were mated with B6 PF4-Cre mice. Animal studies were performed in accordance with the University Committee on Animal Research at University of Rochester Medical Center.

**Intravital Microscopy (Thrombosis Model)**

In brief, wild-type (WT) or ERK5\textsuperscript{−/−} mice were anesthetized; platelets were labeled in vivo with platelet-specific fluorescent antibody (Emfret). Mesenteric arterioles (80–100 μm in diameter) were selected and damaged by the addition of Whatman paper soaked in 15% FeCl\textsubscript{3}, to the vessel surface for 45 s. Thrombus formation was captured with a digital camera (Nikon).

**Pulmonary Thromboembolism Model**

Mice were briefly anesthetized and injected with anti-GPIb beta anti-body (Emfret) conjugated to Alexa 750 (Invitrogen). After 20 minutes, the external jugular vein was isolated and collagen (Chrono-log #385, 0.8 mg/kg) and epinephrine (60 μg/kg) were injected into the external jugular vein to induce thromboembolism. Three minutes later the animal was perfused and the lungs were harvested. The harvested lungs were imaged on an Odyssey CLx Imager (LI-COR Biotech.). The pulmonary embolism (PE) burden was expressed as mean fluorescence intensity.

**Tail Bleeding Assay (Hemostasis Model)**

Mice were anesthetized, and 3 mm at the tail tip was amputated with a scalpel and a steady stream of blood was visualized in 37°C phosphate-buffered saline until the time of hemostasis.

**Myocardial Infarction Model**

Chronic MI was induced by permanent ligation the left anterior descending (LAD) coronary artery. The anesthetized mouse was placed on a heating pad and endotracheal intubated for mechanical ventilation (inspiratory tidal volume of 250 μL at 130 breaths/min). A left thoracotomy was performed in the fourth intercostal space. The mouse heart was exposed, and the LAD coronary artery was ligated 2 mm from its ostial origin with 9-0 silk suture. A sham operation involved the same procedure, but a suture was passed under the LAD coronary artery without ligation.

**Left Ventricular Infarct Assessment**

Hearts were harvested at the end of the study after perfusion and fixation in methanol/acetic acid fixative. Parasternal short-axis sections were cut before mounting and staining with Masson Trichrome reagent. Slides were analyzed and photographed by using an Olympus light microscope (model BX41). The infarcted area (blue collagen staining for scar tissue) was expressed as percentage of left ventricular (LV) surface area by using ImageJ software (National Institutes of Health).

**Echocardiography**

Echocardiographic analysis using M-mode was performed with the use of a Vevo2100 echocardiography machine (VisuaLSonics, Toronto, Canada) and a linear-array 40-MHz transducer (MS-550D).
LV systolic and diastolic measurements were captured in M-mode from the parasternal short axis. Heart function analysis is completely described in the online-only Data Supplement.

Statistical Analysis

Data were analyzed by using SAS software (version 9.4, SAS Institute, Cary, NC). For experiments involving repeated measurements for a given subject, the Friedman $\chi^2$ test, a nonparametric test, was computed. Additionally, pairwise comparisons between the baseline and various concentration or time point values were made by using the paired $t$ test. When independent samples were compared, the standard Student $t$ test was used as noted. A $P$ value of <0.05 was considered statistically significant.

Our in vivo animal studies report data from a relatively small population size. A limitation is that normality cannot be assumed in the sample distribution. For experiments involving repeated measurements for a given subject, the Friedman $\chi^2$ test, a nonparametric test, was computed and reported to provide a more robust analysis.

Results

Expression and Function of ERK5 in Platelets

ERK5 is phosphorylated in the activated state (p-ERK5). To demonstrate that platelets express ERK5 that becomes activated, washed human platelets were isolated and left resting, or stimulated with multiple concentrations of the thrombin receptor agonist peptide. p-ERK5 was determined by Western blot and quantified as a ratio of total pERK5/ERK5. The thrombin receptor agonist peptide induced platelet ERK5 activation (Figure 1A). Platelet ERK5 was also strongly activated by the thromboxane receptor agonist U46619 (Figure 1B). However, the P2 receptor agonist 2-methyl-ADP and the collagen receptor (GPVI) agonist convulxin only weakly activated ERK5 (Figure 1C and 1D, blots overexposed for emphasis). The same concentrations of 2-methyl-ADP and convulxin activated platelet ERK1 (Figure 1 in the online-only Data Supplement). These data indicate that ERK5 is selectively activated by specific platelet receptor agonists.

Global ERK5-deficient mice (ERK5$^{-/-}$) are embryonic lethal.15 To determine the role of ERK5 in platelet function, platelet ERK5$^{-/-}$ mice were created by crossing PF4-Cre mice with ERK5 flox mice (Figure 2A). No change in ERK5 protein expression was noted in other major organs (data not shown). Washed platelets from WT and platelet ERK5$^{-/-}$ mice were isolated and stimulated with thrombin. ERK5$^{-/-}$ platelets had dramatically attenuated thrombin-mediated platelet activation as shown by decreased activated GPIIbIIIa (JON/A antibody binding) and decreased surface P-selectin expression in comparison with WT platelets (Figure 2B and 2C). ERK5$^{-/-}$ platelets also had attenuated thromboxane receptor–mediated platelet activation (Figure 2D). Similar to human platelet ERK5 activation, WT and ERK5$^{-/-}$ platelets had no significant change in activation in response to ADP or convulxin in (Figure 2E). These data confirm that platelet ERK5 is activated by select platelet agonists.

ERK5 can be activated by redox stress in ischemic pathological states.12,17,18 To determine whether platelet ERK5 is activated by redox stress, we incubated human platelets with hydrogen peroxide ($H_2O_2$) as a surrogate exogenous ischemia mediator. $H_2O_2$ activated human platelet ERK5 (Figure 3A). To determine whether platelet-derived (endogenous) ROS generated in hypoxic conditions, such as in ischemia, activates platelet ERK5, human and mouse platelets were incubated under normoxic (21% $O_2$) or reduced oxygen (5% $O_2$) conditions and immunoblotted for p-ERK5. p-ERK5 is acutely increased by hypoxia in both human (Figure 3B) and mouse (Figure 3C) platelets. These data demonstrate that ERK5 is

![Figure 1. ERK5 is expressed in human platelets and activated by thrombin and thromboxane. Washed human platelets were stimulated with 1 to 5 μmol/L TRAP (A), 0.01 to 10 μmol/L U46619 (B), 1 to 10 μmol/L 2-methyl adenosine diphosphate (ADP; C), or 5 to 5000 ng/mL convulxin (D), each for 10 minutes. Western blotting was performed for p-ERK5 (blots overexposed for emphasis in C and D, and total ERK5 as loading control). ERK5 activation reported as mean p-ERK5/ERK5 (± SEM, n=4–7, *P<0.05 vs 0, paired t test). $P=0.08$ for A, $P=0.06$ for B, $P=0.35$ for C, and $P=0.85$ for D by the Friedman test. CVX indicates convulxin; EXK, extracellular regulated protein kinase 5; p-ERK5, phosphorylated ERK5; SEM, standard error of the mean; and TRAP, thrombin receptor agonist peptide.](http://circ.ahajournals.org/doi/10.1161/CIRCULATIONAHA.116.024400)
a mediator of platelet activation in response to thrombin and thromboxane, and that platelet ERK5 is activated directly by exogenous ROS and relative hypoxia.

Platelet ERK5 In Vivo Function
To investigate whether ERK5 is a terminal platelet effector or an intermediary, molecules controlling platelet activation that are known ERK5 targets in other tissues were examined. Platelets were isolated from WT and platelet ERK5–/– mice and RAC phosphorylation was determined by Western blot.19–21 Phosphorylation of RAC1 at serine 71, a signature of decreased RAC1 activity, is implicated in regulating cellular activity including ROS responses.22–25 Basal RAC1 phosphorylation at inhibitory serine 71 was increased in ERK5–/– mouse platelets by almost 5-fold in comparison with WT mouse platelets (Figure 4A), consistent with ERK5 maintaining platelets in an activation-responsive state. This is supported by ERK5–/– platelets having reduced basal JON/A binding and P-selectin expression on isolation and decreased plasma platelet factor 4 (PF4) and thromboxane in ERK5–/– mice under resting conditions (Figure II in the online-only Data Supplement). These data indicate that ERK5 plays a role in regulating downstream platelet activation pathways.

To determine whether platelet ERK5 deficiency affects hemostasis and thrombosis, we performed timed tail bleeding studies in WT and platelet ERK5–/– mice and found no difference (Figure 4B). We also performed thrombosis studies using a mesenteric injury model that induces oxidative vessel injury.26 The time to ferric chloride–induced vessel occlusion was significantly prolonged in platelet ERK5–/– mice in comparison with WT mice (Figure 4C), but platelet ERK5–/– mice still exhibited vessel occlusion. To explore thrombus formation in a non–ROS-dependent model, WT and ERK5–/– mice were injected with collagen/epinephrine via the jugular vein to induce PE.27 PE size was determined by using an infrared fluorescent tagged platelet antibody. WT and platelet ERK5–/– mice had similar PE size (Figure 4D).

Together these data indicate that platelet ERK5 mediates thrombus formation in response to some forms of vessel injury, but is not definitively necessary for physiological thrombosis.

Post-MI Cardiac Function in Platelet ERK5–/– Mice
Because platelet ERK5 is activated by ROS, thrombin, and thromboxane, ERK5 activation in an ischemic environment was explored by using a mouse MI model in which these mediators have significant pathological roles.28–30 As a chronic MI model, the LAD coronary artery was permanently ligated. LAD ligation in WT C57Bl/6J (B6) mice lead to increased ERK5 activation in platelets isolated on day 3 postligation in comparison with sham mice (open chest, no LAD ligation; Figure 5A). Similarly,
ERK5 activation was noted after LAD ligation in platelets from mice on a mixed-strain background (B6/129; Figure III in the online-only Data Supplement). This demonstrates that persistent cardiac ischemia promotes platelet ERK5 activation.

Platelet inhibitors improve patient outcomes after transmural myocardial ischemia. LAD coronary ligation induces transmural ischemia as shown by clear ST-segment elevation on the ECG in both WT and platelet ERK5−/− mice within 30 s of ligation (Figure 5B). Cardiac function was assessed after permanent coronary artery ligation in WT and platelet ERK5−/− mice. Baseline (pre-LAD ligation) echocardiograms of WT and platelet ERK5−/− mice showed no difference in LV dimensions or function (Figure IV in the online-only Data Supplement). The ischemic area at risk was also very similar between WT and platelet ERK5−/− mice after LAD coronary ligation (Figure V in the online-only Data Supplement). Platelet ERK5−/− mice had improved heart function starting on day 3 post-LAD coronary artery ligation as evidenced by echocardiographic parameters of LV dimension and myocardial contractility. These include improved LV ejection fraction, improved fractional shortening, a less dilated LV cavity, thicker LV walls, and lower LV volume indices (LV end-diastolic volume, LV end-systolic volume, LV internal diameter end diastole, and LV internal diameter end systole; Figure 5 and Figure VI in the online-only Data Supplement). Hearts from WT mice were more dilated, congested, and remodeled in comparison with platelet ERK5−/− mouse hearts after LAD coronary ligation (Figure 5C and 5D, Movies I and II in the online-only Data Supplement). Histological data demonstrated that the infarction pattern spread far beyond the anterior wall of the LV in WT mice and this pattern was reduced and patchy in platelet ERK5−/− mice (Figure 5E and 5F). These data indicate that ERK5-dependent platelet activation promotes infarct expansion, whereas platelet ERK5 deficiency has a post-MI cardioprotective effect.

Platelet activation was assessed after coronary artery ligation in WT and platelet ERK5−/− mice by measuring the concentrations of plasma PF4 and the stable thromboxane metabolite thromboxane B2. WT and ERK5−/− mice had very similar platelet counts pre- and post-MI (Figure VII in the online-only Data Supplement), but both PF4 and thromboxane B2 plasma concentrations were significantly greater in the blood of WT mice than in platelet ERK5−/− mice (Figure 6A). Day 3 post-MI, heart tissue was immunostained by using a platelet-specific marker CD42c to assess relative platelet localization, particularly at the edges of the infarcted tissue. There was increased platelet immunostaining in WT hearts in comparison with platelet ERK5−/− mouse hearts (Figure 6B and Figure VIII in the online-only Data Supplement). To further confirm that the phenotype in platelet ERK5−/− mice is not related to genetic background owing to PF4-Cre, LAD ligation was performed in WT and B6 PF4-Cre mice. Both LV function and infarct size were similar in these strains of mice (Figure IX in the online-only Data Supplement).

Platelet activation may promote infarct expansion by releasing proinflammatory molecules that recruit and activate white blood cells, or by platelet release of degradative enzymes such as MMPs. To assess post-MI leukocyte infiltrates, WT and platelet ERK5−/− mouse hearts were immunostained for CD45- and CD45-positive cells quantified in the periflcnt zone. Leukocyte infiltrates were unchanged in platelet

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**Figure 3.** Platelet ERK5 is activated by ROS. (A) Dose-dependent platelet ERK5 ROS activation. Washed human platelets were stimulated with H2O2 (0.5–1000 μmol/L) for 5 minutes and p-ERK5 determined at multiple time points by p-ERK5 immunoblotting normalized to total ERK5 (*P<0.05 vs 0, paired t test; P=0.12 by Friedman test). (B) Washed human platelets were incubated under normoxic or reduced oxygen (5% O2) conditions for 2 to 10 minutes (P<0.05 vs 0, paired t test and P=0.02, Friedman test). (C) Washed mouse platelets were incubated under normoxic or reduced oxygen (5% O2) conditions for 2 to 10 minutes. (P<0.05 vs 0, paired t test; P=0.02, Friedman test). ERK5 activation reported by Western blotting as mean p-ERK5/ERK5 (± SEM, n=4–6). ERK5 indicates extracellular regulated protein kinase 5; p-ERK5, phosphorylated ERK5; ROS, reactive oxygen species; and SEM, standard error of the mean.
ERK5–/– mice in comparison with WT mice (Figure X in the online-only Data Supplement). MMPs, including MMP9, are found in platelets and their secretion may contribute to tissue remodeling and loss. Platelet MMP9 content was decreased in platelets from WT mice on day 3 after LAD artery ligation in comparison with sham mice, indicating that platelets release MMP9 post-MI (Figure 6C). In the comparison of platelet MMP9 expression and activity between WT and ERK5–/– mice post-MI, total MMP9 content is greater, yet MMP activity less in ERK5–/– mouse platelets (Figure 6D), consistent with less platelet activation and secretion of MMPs. Basal MMP9 expression is also greater in platelet ERK5–/– mice (Figure XI in the online-only Data Supplement), but basal MMP activity is not different between WT and ERK5–/– mice (Figure XII in the online-only Data Supplement). Consistent with less platelet activation and less MMP release in platelet ERK5–/– mice, heart tissue isolated from the LV apex (myocardial region supplied by the LAD coronary artery) of WT mice had increased total MMP9 and TIMP1 (a MMP inhibitor increased by elevated local concentrations of MMP) and increased total MMP activity in comparison with platelet ERK5–/– mice (Figure 6E). These data indicate that reduced platelet MMP release into the post-MI coronary vasculature and subsequent reduced myocardial remodeling may contribute to the protective phenotype observed in platelet ERK5–/– mice.

To determine whether increased ERK5 activation results in increased platelet agonist sensitivity, washed platelets were isolated from WT and platelet ERK5–/– mice under sham operation or LAD coronary ligation on day 6 post-MI and platelets were then thrombin stimulated. WT mice had augmented post-MI thrombin-mediated activation in comparison with control WT mouse platelet activation. This effect was not observed in ERK5–/– platelets after MI (Figure 7A). While exploring potential downstream ERK5-signaling proteins, we observed increased post-MI expression of several proteins also known to mediate platelet activation in comparison with platelets isolated from sham-operated mice. This included: ERK5, P70S6K, and RAC (Figure 7B). The increased protein expression is specific to some proteins rather than a general effect, because the expression of proteins such as TIMP1 and GAPDH did not change post-MI (Figure 7B). WT and ERK5–/– platelets had very similar basal protein expression including RAC (Figure 4A), TIMP1, and GAPDH (Figure XIII in the online-only Data Supplement). However, in post-MI conditions, platelets taken from ERK5–/– mice had greatly reduced P70S6K and RAC expression in comparison with WT mice (Figure 7C).
Because ERK5 is a regulator of ubiquitination in other cells, and the ubiquitin-proteasome protein degradation pathway is active in platelets, potentially accounting for decreased protein expression, we compared protein ubiquitination in platelets from WT and platelet ERK5−− mice pre- or 24 hours post-LAD ligation. ERK5−− platelets had more protein ubiquitination both pre- and post-MI (Figure 7D), a signature for proteasome targeting and degradation. This indicates that platelets...
Figure 6. Post-MI platelet activation is reduced in platelet ERK5<sup>−/−</sup> mice. A, Plasma PF4 or TxB<sub>2</sub> were measured by ELISA as markers of ongoing platelet activation 3 days after LAD coronary artery ligation (mean±SD, n=6. *P<0.05 vs WT, t test for unequal variances). B, Representative parasternal short-axis sections of the heart on day 3 after LAD coronary ligation. CD42c (platelet) immunostaining is less in platelet ERK5<sup>−/−</sup> mouse hearts than in WT hearts at the infarct border (additional images are shown in S11). C, Platelet MMP content by Western blotting on day 3 after LAD coronary ligation is reduced in comparison with sham-operated mice (mean MMP9/GAPDH±SEM, n=4–5. *P=0.02 vs sham-operated, t test for unequal variances). D, LV MMP9 and TIMP1 content is reduced in platelet ERK5<sup>−/−</sup> mice after MI (mean intensity±SEM, n=5 each group, *P=0.02 vs WT, t test for unequal variances). LV extracts on day 3 after LAD coronary artery ligation were protein-normalized and run in duplicate on nonreducing gels with a gelatin matrix. Gelatinase activity is shown as light bands on the final zymogram. Total LV MMP activity (all isoforms) was calculated for each lane (mean intensity±SEM, n=6–10, *P<0.001 vs WT, t test for unequal variances). Washed platelet MMP9 content is increased in platelet ERK5<sup>−/−</sup> mice in comparison with WT mice on day 3 after LAD coronary artery ligation (mean MMP9/GAPDH±SEM, n=5. *P<0.05 vs WT mouse platelets). E, Platelet MMP9 content and MMP activity are reduced in platelet ERK5<sup>−/−</sup> mice. Washed platelets were isolated on day 3 after LAD coronary artery ligation, homogenized, protein-normalized, and immunoblotted for MMP9 content (mean intensity±SEM, n=3–5, *P<0.01 vs WT, t test for unequal variances). F, Washed platelets were isolated on day 3 after LAD coronary artery ligation and were also run on nonreducing gels with a gelatin matrix. Gelatinase activity is shown as light bands on the final zymogram. Total MMP activity (all isoforms) was calculated for each lane. (Protein expression normalized to GAPDH and gelatinase activity is demonstrated by light bands on the final zymogram [total platelet MMP activity, mean±SEM, n=8–10, *P=0.04 vs WT, t test for unequal variances]). ELISA indicates enzyme-linked immunosorbent assay; ERK5, extracellular regulated protein kinase 5; LAD, left anterior descending; LV, left ventricle; MI, myocardial infarction; MMP, matrix metalloproteinase; PF4, plasma platelet factor 4; SD, standard deviation; SEM, standard error of the mean; TbxB<sub>2</sub>, thromboxane B<sub>2</sub>; and WT, wild type.
may sense and respond to a pathological ischemic environment and that platelet ERK5 may be a key regulator of the expression of platelet proteins and platelet activity after MI.

Our data demonstrate that acute MI and subsequent chronic myocardial ischemia lead to ongoing platelet activation, infarct expansion, LV remodeling, and severe contractile dysfunction, that is in part platelet ERK5 dependent. ERK5 promotes platelet activation in the dysregulated post-MI ischemic environment by initiating platelet activation pathways and regulating the expression of proteins important in platelet function. Inhibition of platelet ERK5 may therefore confer protection from ischemic ventricular remodeling by limiting platelet activation and targeting for degradation specific molecules important for platelet activation, thus limiting platelet MMP release (Figure 8).

Discussion

Our study indicates that, in normal healthy conditions, ERK5 mediates platelet activation in response to some agonists. Furthermore, in ischemic post-MI conditions, platelet ERK5 is activated leading to increased platelet activation, and ERK5
regulates the expression of some proteins important for platelet activation. Our data provide important evidence that platelet function and platelet protein expression is not static in diseased states. Current therapies may be severely limited by not accounting for a potential moving therapeutic target that is apparent in platelets from the post-MI environment.

Platelet ERK5 activity contributes to ongoing myocardial dysfunction and tissue remodeling after MI. ERK5 is likely not the final effector of increased platelet activation, but serves as an upstream platelet sensor of ROS in ischemic states and mediates downstream thrombin and thromboxane receptor signaling. ERK5 also has a central role in regulating the expression of platelet proteins post-MI, in part, through altered protein ubiquitination. These findings strongly underscore the concept that mechanisms of platelet activation differ in pathological states in comparison with normal healthy conditions. Targeting platelet ERK5 may be another strategy for antplatelet therapy after acute MI.

ERK5 has been implicated in cell cycle progression and cellular transformation in many tissues. Because platelets are anucleate, the expression of ERK5 in platelets was initially somewhat surprising, although other members of the mitogen-activated protein kinase family regulate platelet maturation and activation. Various studies indicate that ERK5 can regulate cellular function via important cytosolic substrates including BAD, Cx43, peroxisome proliferator-activated receptor γ, and P90RSK. It is reported that downstream P70S6K and RAC1 are integral signal transduction components required for platelet activation. Our study affirms this observation, because ERK5-deficient platelets have attenuated activation coincident with reduced P70S6K and RAC1 expression only after MI. This adds evidence that ERK5 is not the final effector for ischemia-mediated platelet dysregulation, but rather ERK5 may act as an upstream ischemic sensor or intermediary signaling molecule. How platelet ERK5 deficiency prevents infarct expansion and contractile dysfunction is especially important, because this study is the first to ascribe a deleterious role for ERK5 after ischemia in the cardiovascular system. Endothelial ERK5 inhibition increases tissue damage after ROS exposure with a consequent protective effect on the heart and the vasculature. The tissue-specific difference in ERK5 functions underscores the need to better understand platelet activation in disease states. It also may make therapeutic targeting of ERK5 difficult, because it must be targeted in a cell-specific manner. However, great advances are being made in drug delivery that may make specifically targeting platelets or downstream platelet ERK5 substrates possible.

ERK5 activation is agonist specific. ERK5 deficiency alters platelet activation via protease-activated receptor and the thromboxane receptor signaling, but not via collagen or P2Y12 receptors (Figures 1 and 2). A common downstream connection in protease-activated receptor and thromboxane-mediated signaling in platelets is the Gqα subunit of G-protein–coupled receptors. This is consistent with reports in other cell systems where ERK5 activation by surface agonists occurs in a Gqα-dependent manner. 

Microvascular occlusion and inflammatory cell infiltrates in the tissue adjacent to the initial infarcted tissue contributes significantly to infarct expansion and a decline in myocardial function. Because platelets are important immune modifiers, we anticipated that white blood cell infiltration in myocardial sections after LAD ligation may differ in WT and platelet ERK5−/− hearts; however, we found no difference. MMP-mediated tissue damage and remodeling also contribute to infarct expansion. Platelets isolated from WT mice 3 days after LAD artery ligation had reduced MMP9 content and increased MMP activity in comparison with platelet ERK5−/− mice. An inverse pattern was noted in the LV apex. This suggests that platelet MMP release into the microvasculature of the myocardium leads to infarct expansion, at least in part in a platelet ERK5-dependent manner. Platelet MMP-mediated myocardial remodeling was proposed by Gresele et al in a clinical study that showed a striking increase in platelet activity, and MMP concentration, as well, in samples from the coronary sinus of patients during ACS in comparison with matched patients without ACS.

In our LAD ligation mouse model there is a marked decrease in P70S6K and RAC1 protein expression in ERK5−/− platelets after MI (Figure 7), whereas other proteins are unchanged. This suggests that ERK5 modulates either the synthesis or degradation of proteins important for sustained platelet activation in ischemic pathologies. Because ERK5 regulates the ubiquitin-proteasome pathway, and the platelet proteasome has been implicated in regulating platelet production and function, we
focused on this mechanism and found that ERK5+/− platelets have increased protein ubiquitination and decreased expression of some proteins within 24 hours of MI. This further emphasizes the need to consider mechanisms of platelet activation in pathological states as fundamentally different than healthy conditions. Our studies focused on nonnuclear roles for ERK5 in circulating platelets, but ERK5 also has transcription regulatory functions that may influence gene expression patterns at the megakaryocyte level. Such ERK5 gene regulatory functions cannot be ruled out. Future studies are needed to fully consider megakaryocyte gene expression in healthy and post-MI conditions to determine whether ERK5 is also an ischemic sensor in the bone marrow, remote from the site of tissue injury.

Overall, these data indicate that ERK5 promotes ongoing platelet activation that augments myocardial damage after MI. Targeting ERK5 or its downstream mediators may represent a novel strategy for decreasing the thrombotic burden in patients with ischemic diseases such as MI and stroke.

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Disclosures

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References

7. Acknowledgments
We discovered that platelet extracellular regulated protein kinase 5, a protein traditionally found in the nucleus of other cells, is present in platelet protein expression likely represent enhanced protein synthesis from platelet mRNA, impaired degradation, or both.

In summary, antiplatelet medications such as aspirin and clopidogrel are used to treat myocardial infarction. For unclear reasons, these drugs are not always efficacious. For example, a significant number of patients experiencing ST-segment–elevation myocardial infarction develop the no-reflow phenomenon after percutaneous intervention even though high-dose antiplatelet medications are not always efficacious. For example, a significant number of patients experiencing ST-segment–elevation myocardial infarction develop the no-reflow phenomenon after percutaneous intervention even though high-dose antiplatelet medications are not always efficacious. For example, a significant number of patients experiencing ST-segment–elevation myocardial infarction develop the no-reflow phenomenon after percutaneous intervention even though high-dose antiplatelet medications are not always efficacious.
Platelet Extracellular Regulated Protein Kinase 5 Is a Redox Switch and Triggers Maladaptive Platelet Responses and Myocardial Infarct Expansion
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Supplemental Material

Supplemental Methods:
Antibodies and reagents

TRAP and Thrombin (Cayman Chemicals), ADP (Tocris, Bristol, UK), Convulxin (Santa Cruz Biotechnology), hydrogen peroxide (Millipore), U46619 (Cayman Chemical), PD184325 (Promega Biosciences), gelatin (Fisher Scientific). ERK5 antibody, p-ERK5 antibody, p-P70S6K antibody, P70S6K antibody, RAC antibody, p-RAC antibody, MMP2 antibody, ubiquitin antibody, actin antibody, and tubulin antibody were all purchased from Cell Signaling Technology, GAPDH antibody (Santa Cruz Biotechnology), MMP9 antibody (Millipore), TIMP1 antibody (Abcam). Anti-mouse and anti-rabbit secondary antibody (GE healthcare, UK).

Protein Biochemistry

Platelet Isolation: For human platelets, whole blood was collected into citrate plasma tubes and thoroughly mixed. The sample was centrifuged at 1100 rpm for 15 minutes using a bench top centrifuge. The supernatant was then added in a 1:1 (vol/vol) mix of supernatant/Tyrodes solution with final concentration 10 μM prostaglandin I₂ (Cayman Chemical) and centrifuged at 2600 rpm for 5 minutes using a bench top centrifuge. The supernatant was discarded, and the washed platelet pellet was carefully resuspended in fresh Tyrodes solution, and the experiments were immediately commenced. For mouse platelets, two drops of retro-orbital venous blood was collected using a capillary tube into heparinized Tyrodes solution: 950 μL Tyrodes and 50 μL 1000U/mL heparin in PBS (Sigma) which was divided into 330 μL aliquots. The final sample was centrifuged at
1000 rpm for 5 minutes using a bench top centrifuge. The erythrocyte pellet was discarded and the supernatant then centrifuged again at 1000 rpm for 5 minutes using a bench top centrifuge. The pellet was discarded and the supernatant was then added in a 1:1 (vol/vol) mix of supernatant/Tyrodes with final concentration 10μM prostaglandin I₂ and centrifuged at 2600 rpm for 5 minutes using a bench top centrifuge. The final supernatant was discarded and the washed platelet pellet from each mouse was carefully resuspended in 50 μL fresh Tyrodes solution, and immediately used for cell signaling studies.

**Protein studies:** Cell lysis and cell protein extraction, SDS PAGE, and Western blotting were conducted using buffers and techniques as described previously. Blocking buffer was 3% BSA (Sigma Aldrich) dissolved in Tris-buffered saline at pH 8.0 (Fisher Scientific) with 0.1% Tween-20 (TBS-T) at room temperature for 1 hour. Primary antibody was 1:1000 overnight at 4 °C in 3% BSA/TBS-T. Secondary antibody (GE Healthcare, Buckinghamshire, UK) was used in a 1:2000 titer in 5% milk/TBS-T for 1 hour at room temperature. ECL reagent used was Supersignal West Pico (Thermo Scientific) for each antibody, or supersignal West Femto (Thermoscientific) for the MMP2 and ERK5 antibodies. Final autoradiographic films (Bioblot BXR, Laboratory Product Sales, Rochester NY) were quantified by densitometry using ImageJ software (NIH).

**MMP activity assay:** Platelets were isolated on day 3 post-LAD coronary ligation from 120 μL whole blood as described above and ventricular apex isolates were homogenized in extraction buffer with a protease inhibitor cocktail (Roche) in the following
concentrations at pH 7.6: 1mM PMSF, 1mM sodium orthovanadate, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 10 mM Tris-HCl, 1% NP40, then final supernatants were protein normalized using a commercially-available kit (Thermo Scientific). Lysates were centrifuged for 15 mins at 4°C, and supernatants were placed in 50% vol/vol 2x non-denaturing sample buffer at the following final concentration: Tris-HCl 250 mM, 0.5% SDS, 1% glycerol, 0.05% bromophenol blue for 10 minutes without boiling. 50 μg of total cell lysate per lane was separated by SDS-PAGE (12% bis-acrylamide containing 1mg/mL final concentration gelatin within the matrix) at 125V (constant voltage, room temperature). The gel was renatured by gently rocking in 2.5% Triton-X-100 for 30 mins at room temperature, then allowed to equilibrate at room temperature with gentle rocking in zymogram buffer with the final concentrations: Tris-base 50mM, NaCl 0.2M, CaCl$_2$ 5mM, Tween-20 0.02% for 30 mins before decanting, and incubating in fresh zymogram buffer for 12 hours overnight at 37°C. The zymogram buffer was decanted, and the gel was rocked at room temperature for 4 hours in Simply Blue Safestain (Invitrogen). MMP activity was noted by clear bands in the final gel (a reverse image). Total MMP activity in each lane was quantified by densitometry using ImageJ software (NIH).

Animal models

**Mouse colony:** ERK5-flox mice were provided by Dr. Jun-ichi Abe (University of Rochester). These mice have loxP sites inserted at the 5’ and 3’ position of exon 4 in the ERK5 gene—a coding region in which the critical kinase activation domain is found. ERK5flox/flox mice on a C57Bl6 (B6) background were mated with B6 mice expressing Cre under PF4 promoter to generate platelet-specific ERK5 knockout mice (platelet
ERK5\(^{-/-}\) mice. Animal studies were performed in accordance with the University Committee on Animal Research at University of Rochester Medical Center.

**Intravital microscopy (thrombosis model):** Ferric chloride was used to induce mesenteric artery thrombosis in mice as described by us previously\(^2\). Briefly, WT or ERK5\(^{-/-}\) mice were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg). Platelets were labeled *in vivo* with platelet specific fluorescent antibody (Emfret). Mesentery was exteriorized arterioles (80–100 \(\mu\)m in diameter) were selected, and the mouse mesenteric arteriole damaged by the addition of Whatman’s paper soaked in 15% FeCl\(_3\) to the vessel surface for 45 sec, and images of thrombus formation were captured with a digital camera (Nikon). The time to lumen occlusion was then quantified.

**Pulmonary Thromboembolism Model:** Mice were briefly anesthetized with isoflurane to facilitate a retro-orbital injection of mouse anti GPIb beta (Emfret) conjugated to Alexa 750 (Invitrogen). After 20 minutes, the mice were anesthetized with ketamine/xylazine (80mg/12mg per kg i.m.). A skin incision was made over the external jugular (EJ) vein and the vessel was isolated. Collagen (Chrono-log #385, 0.8mg /kg) and epinephrine (60ug/kg) were then injected into the EJ vein to induce thromboembolism. Three minutes later, if the animal was still living, it was euthanized and the chest was opened. The right ventricle was perfused with heparinized saline followed by 10% formalin. The lungs were harvested and placed into PBS. The harvested lungs are kept at 4°C overnight and imaged on an Odyssey CLx Imager (Li-Cor Biotech.). The PE burden was expressed as mean fluorescence intensity (MFI).

**Tail bleeding assay (hemostasis model):** Mice were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg). A length of 3 mm at the tail tip was amputated with a
scalpel and a steady stream of blood was visualized in 37°C PBS until the time of hemostasis. The experiment was terminated at 15 minutes if hemostasis had not yet occurred. Mice were monitored for another 15 minutes post-experiment to assess for re-bleeding.

**Myocardial Infarction Model**: Chronic myocardial ischemia was induced by permanent ligation the left anterior descending (LAD) coronary artery: The mouse was placed on a heating pad and the airway was stabilized by endotracheal intubation and mechanical ventilation (inspiratory tidal volume of 250μl at 130 breaths/min). Maintenance anesthesia used was 1.5% isoflurane by inhalation. A left thoracotomy was performed in the fourth intercostal space. The mouse heart was exposed, and the LAD coronary artery was ligated 2 mm from its ostial origin with 9-0 silk suture. Transmural ischemia was assured by color loss on the LV wall and ST-segment elevation on the electrocardiogram (ECG). The chest was closed with 6-0 coated vicryl suture, the skin was closed using 6-0 nylon, anesthesia was stopped and the mouse recovered before extubation. A sham operation involved the same procedure, but a suture was passed under the LAD coronary artery without ligation. Aseptic technique was used throughout. Coronary artery branch points were visualized under 10x magnification prior to ligation which can be subjective and difficult to maintain consistency between animals due to variations in anatomy, and so any mouse (WT or genetically-modified) with an LVEF > 65% after 3 days of coronary artery ligation was deemed to have unsatisfactory infarction and was excluded from the study. LV Infarct assessment: Hearts were harvested at the end of the study (day 9) after perfusion and fixation in methanol/acetic acid fixative. Parasternal short axis section were cut (5 μm thickness) before mounting and staining with Masson’s
trichrome reagent. Slides were analyzed and photographed using an Olympus light microscope (Model BX41). The infarcted area (blue collagen staining for scar tissue) was expressed as percentage of LV surface area using ImageJ software (NIH). Area at risk: the LAD coronary artery was ligated, and then one minute later 0.3% methylene blue was infused retro-orbitally and five minutes of perfusion was allowed prior to animal sacrifice. The hearts were excised, fixed, and sectioned. The ischemic area of LV at risk was noted by pallor, and expressed as percentage of LV surface area using ImageJ software (NIH). Echocardiography: Echocardiographic analysis using M-mode was performed using a Vevo2100 echocardiography machine (VisualSonics, Toronto, Canada) and a linear-array 40MHz transducer (MS-550D). Image capture was undertaken in mice under general isoflurane anesthesia. LV systolic and diastolic measurements were captured in M-mode from the parasternal short axis. Fraction shortening (FS) was assess as follows: %FS = (end diastolic diameter - end systolic diameter)/(end diastolic diameter) x100%. Since pronounced apical wall motion abnormalities are anticipated from LAD coronary ligation, left ventricular ejection fraction (EF) was measured and averaged in both the parasternal short axis (M-Mode) using the simplified Quinones calculation: %EF=Dd^2-Ds^2/Dd^2, and by the Simpson’s method of LV endocardial border tracing of the end diastolic dimension (EDD) and end systolic dimension (ESD) in the parasternal long axis: % EF=(EDD-ESD)/EDD. The pooled data were analyzed for statistical significance.

Supplemental Figures
**Figure S1.** Human platelets were stimulated with increasing concentrations of convulxin (CVX) to stimulate the collagen receptor or 2-methyl ADP (ADP) to stimulate the P2Y12 receptor. These agonists did not strongly activate human platelet ERK5 (p-ERK5, Fig. 1), but they did activate ERK1 (p-ERK1) by Western blotting using a phospho-specific antibody (total ERK-1/2 as a loading control). ERK1 activation is expressed as ratio of P-ERK1/ERK1/2 (± SEM, n=3-4 in each group). *P<0.05 vs. 0 concentration convulxin, paired t-test; P=0.08, Friedman’s test; #P=0.056 vs. 0 concentration ADP, paired t-test; P=0.19, Friedman’s test).

**Figure S2.** Basal external P-selectin (PF4) and thromboelastography (TEG) function in WT and ERK5−/− mice by TTE. Baseline LV dimensions and myocardial function is similar in WT and platelet ERK5−/− mice (mean ± S.D.). * P < 0.05, t-test.

**Figure S3.** Sustained activation of platelet ERK5 (p-ERK5) was noted by Western blotting 48 hrs following LAD coronary ligation in a mixed background strain of mice (C57Bl/6J and 129S1/SvImJ). Activation of ERK5 in washed mouse platelets was expressed as mean ratio of p-ERK5/ERK5 (mean ± S.D., n=3).
**Figure S5.** The LV region at risk is similar in WT and platelet ERK5−/− mice. 5 mins after LAD coronary ligation mice were injected intravenously (retro-orbitally) with methylene blue (2%). Perfused tissue is blue and ischemic tissue pink. LV region at risk quantified as % LV area (± SEM, n=13, and quantified using NIH ImageJ software).

**Figure S6.** Representative parasternal long axis and parasternal short axis still TTE images of WT and ERK5−/− mice on day 0 (baseline) and 7 days post LAD coronary ligation.
**Figure S7.** Platelet count in WT and ERK5−/− mice at baseline and following LAD coronary ligation (mean ± SEM, *P* < 0.05 day 3 vs. baseline and day 7 vs. baseline for WT and ERK5−/−; *P* = not significant (NS) between WT and ERK5−/− on each day, t-test for unequal variances, *n*=4-7).

**Figure S8.** Representative parasternal short axis sections of the heart on day 3 after LAD coronary ligation. Staining for CD42c is shown as brown precipitate in WT, WT (flox/flox), and in platelet ERK5−/− mouse hearts. Less CD42c staining in platelet ERK5−/− mouse hearts is suggestive of less intramyocardial (microvascular) thrombus formation. These additional sections are supportive of similar data in other mice shown in Fig. 6B.
**Figure S9.** Cardiac function in sham WT (C57BL/6) mice after LAD coronary ligation showed similar MI function to WT-PF4-Cre (C57BL/6). Hemodynamic data (mean ± SEM, n=3-5 * P< 0.05 vs. B6 Sham, t-test for unequal variances). Cardiac morphology (LV infarct size) by Masson Trichrome staining (blue=infarct) is similar on day 7 following LAD coronary ligation in WT and WT-PF4-Cre mice (both C57BL/6 strain). Quantification of Masson Trichrome positive staining (mean % of LV area, N=5 ± SEM, P=NS, t-test for equal variances).

**Figure S10.** Myocardial white blood cells infiltrates. CD45+ staining on day 3 following LAD coronary ligation in WT and platelet ERK5−/− mouse hearts. Data are shown as mean WBC count per high powered field (mean ± SEM, n=5; * p < 0.05 vs baseline, t-test for unequal variances).
Figure S11. Baseline platelet MMP-9 expression in WT and ERK5<sup>−/−</sup> platelets. Isolated washed mouse platelet extracts were separated by SDS-PAGE then immunoblotted for MMP-9. Blots are representative of at least 4 different individual mice for each strain. The arrowhead indicates the expected molecular mass of MMP-9. MMP-9 content is expressed as mean MMP-9/GAPDH (mean ± SEM, n=3; **P<0.05 vs WT, t-test for equal variances).

Figure S12. Baseline platelet MMP activity in WT and ERK5<sup>−/−</sup> mice. Washed mouse platelet extracts were protein-normalized and run in duplicate on non-reducing gels with a gelatin matrix. Gelatinase activity is shown as light bands on the final zymogram. Total MMP activity (all isoforms) was calculated for each lane (mean ± SEM, N=3, *p < 0.05 vs. WT).
Figure S13. Platelet P70S6K and TIMP1 content is similar in WT and platelet ERK5$^{-/-}$ mice under resting conditions. Protein content is expressed as a ratio of P70S6K/GAPDH (± SEM, n=3-6 in each group, $P=\text{NS}$, t-test for equal variances).

**Movie 1.** Parasternal long axis view of a WT mouse heart on day 9 following MI.

**Movie 2.** Parasternal long axis view of a platelet ERK5$^{-/-}$ mouse heart on day 9 following MI.
Supplemental References
