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Noncanonical Matrix Metalloprotease 1–Protease-Activated Receptor 1 Signaling Drives Progression of Atherosclerosis

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Abstract

Objective——Protease-activated receptor-1 (PAR1) is classically activated by thrombin and is critical in controlling the balance of hemostasis and thrombosis. More recently, it has been shown that noncanonical activation of PAR1 by matrix metalloprotease-1 (MMP1) contributes to arterial thrombosis. However, the role of PAR1 in long-term development of atherosclerosis is unknown, regardless of the protease agonist.

Approach and Results——We found that plasma MMP1 was significantly correlated (R=0.33; P=0.0015) with coronary atherosclerotic burden as determined by angiography in 91 patients with coronary artery disease and acute coronary syndrome undergoing cardiac catheterization or percutaneous coronary intervention. A cell-penetrating PAR1 pepducin, PZ-128, currently being tested as an antithrombotic agent in the acute setting in the TRIP-PCI study (Thrombin Receptor Inhibitory Pepducin-Percutaneous Coronary Intervention), caused a significant decrease in total atherosclerotic burden by 58% to 70% (P<0.05) and reduced plaque macrophage content by 54% (P<0.05) in apolipoprotein E–deficient mice. An MMP1 inhibitor gave similar beneficial effects, in contrast to the thrombin inhibitor bivalirudin that gave no improvement on atherosclerosis end points. Mechanistic studies revealed that inflammatory signaling mediated by MMP1–PAR1 plays a critical role in amplifying tumor necrosis factor α signaling in endothelial cells.

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Disclosures

None.

Conclusions——These data suggest that targeting the MMP1–PAR1 system may be effective in tamping down chronic inflammatory signaling in plaques and halting the progression of atherosclerosis.

Visual Overview——An online visual overview is available for this article.

Keywords

acute coronary syndrome; atherosclerosis; coronary artery disease; percutaneous coronary intervention; thrombin

Patients being treated for acute coronary syndromes (ACS) in either an elective or urgent basis incur a high risk for occlusive thrombus formation because of disruption of atherosclerotic plaques which can lead to life-threatening ischemia and myocardial infarction (MI).[1] Despite highly prevalent use of statins, antithrombotics, and therapies directed against ADP receptor and thromboxane pathways in platelets, recurrent atherothrombosis of vulnerable plaques remains an area of major unmet medical need. This was underscored by a pivotal prospective study of patients treated for ACS, where the 3-year cumulative rate of major adverse coronary events after percutaneous coronary intervention (PCI) remained at 20%.[2] Notably, 63% of the adverse events were related to the original culprit lesion, and the remaining events from other lesions[2] indicating the need for new therapeutics that can halt or reverse progression of culprit and nonculprit atherosclerotic lesions without unduly affecting safety.

An emerging new target in the prevention of both acute and chronic ischemic events in subjects with atherothrombotic disease is protease-activated receptor-1 (PAR1).[3–5] PAR1 was originally identified as the high-affinity thrombin receptor in platelets and is enriched in endothelium overlying atherosclerotic plaques.[6] PAR1 is classically activated by thrombin through proteolysis and cleavage of the N-terminal extracellular domain of the receptor at the $R_{41}\downarrow S_{42}$ site.[7] This cleavage leads to the generation of a new amino terminus which functions as a tethered ligand that binds to extracellular loops of the receptor, leading to a major conformational change and trans-membrane signaling to intracellular G proteins.[8] Many of the resulting cellular effects are mediated via induction of a host of secondary intracellular mediators, including intracellular Ca^{2+} flux, activation of protein kinase C, rho GTPase, PI3 (phosphatidylinositol-3)-kinase, and mitogen-activated protein kinases with pleiotropic effects on gene transcription, inflammation, and proliferation depending on the protease agonist.[9]

The role of PAR1 signaling in the blood vessel wall has been investigated in the context of intimal hyperplasia and restenosis involving physical injury of the endothelium[10,11] or in the control of endothelial barrier function.[12] Thrombin and exposed tissue factor are acutely elevated at the site of vascular injury, and inhibition with thrombin inhibitors reduces neointimal thickening induced by balloon angioplasty in animal models.[13–16] However, clinical trials have surprisingly failed to demonstrate any significant effect of thrombin inhibitors on restenosis after angioplasty.[17–19] Long duration use of the direct thrombin inhibitor, dabigatran, showed a small but significantly increased risk of MI or ACS when combining the safety outcome data from multiple trials.[20] Moreover, thrombin is rapidly

inactivated by ATIII (anti-thrombin III) and heparin to form the thrombin–ATIII complex (TAT) complex and is not readily exposed to subendothelial cells in intact atherosclerotic plaques. Thus, thrombin may play a more important role in hemostasis and repair processes involving acute physical disruption of endothelium and the underlying vessel wall, but its long-term effects may be more complex in impacting chronic inflammatory diseases of the blood vessel, such as atherosclerosis.[6,12,21,22]

As PAR1 is abundant at the site of atherosclerotic plaques but active thrombin is mostly absent, this raises the possibility that other PAR1 protease activators may exist in close proximity during the process of plaque formation. In this regard, we made the finding that the inflammatory/tissue remodeling matrix metalloprotease-1 (MMP1) can activate PAR1 by noncanonical signaling at a $D_{39}{\downarrow}P_{40}$ site on PAR1 distinct from thrombin[23] and that MMP1 and PAR1 colocalize at the site of atherosclerotic lesions in humans.[11] To test the effect of chronic blockade of PAR1 in atherosclerotic plaque development, we used a cell-penetrating pepducin, PZ-128, which is being evaluated for efficacy and safety in subjects undergoing PCI in the TRIP-PCI study (Thrombin Receptor Inhibitory Pepducin-Percutaneous Coronary Intervention).[5] Here, we report a significant correlation between systemic MMP1, tumor necrosis factor α (TNFα), and coronary atherosclerotic lesion burden in patients with coronary artery disease (CAD) and ACS. We found that chronic treatment with PZ-128 caused a marked decrease in total atherosclerotic burden and macrophage infiltrates in the aorta, carotids, and other arteries of apolipoprotein E–deficient ($ApoE^{-/-}$) mice. Likewise, an MMP1 inhibitor, FN-439, gave similar beneficial effects as the PAR1 pepducin, suggesting that the MMP1–PAR1 axis may be a relevant target in long-term suppression of atherothrombotic disease.

Materials and Methods

Atherosclerotic Burden Assessment and Plasma MMP1, TAT, and TNFα From Patients Undergoing Cardiac Catheterization/PCI

TRIP-PCI is a multicenter, double-blinded, placebo-controlled phase 2 study conducted in the United States to evaluate the safety and efficacy of PZ-128 in preventing arterial thrombosis in patients undergoing cardiac catheterization±PCI for stable CAD or ACS (non–ST-segment–elevation ACS: unstable angina and non–ST-segment–elevation MI). Institutional review board approval was obtained before study initiation of TRIP-PCI at the clinical sites in April 2016. All adult patients who met the inclusion criteria for the study provided written informed consent before enrollment/randomization and had the option to participate in additional blood sampling for this biomarker analysis of baseline plasma MMP1, TAT, and TNFα. Patients were eligible if they were aged ≥18 years with stable CAD or non–ST-segment–elevation ACS and were scheduled to undergo nonemergent PCI or nonemergent coronary angiography with the intention to undergo PCI if clinically indicated and if elective use of an intravenous glycoprotein IIb/IIIa inhibitor was not planned (full clinical trial inclusion and exclusion criteria detailed at ClinicalTrials.gov NCT02561000). Cardiovascular angiography and interventions were performed according to professional practice guidelines (AHA [American Heart Association], SCAI [Society for Cardiovascular Angiography and Interventions], ACCF [American College of Cardiology Foundation]).

Animal Models

Male *ApoE*$^{-/-}$ mice (B6.129P2-ApoE^{tm1Unc}/J, 6–7 weeks old) were purchased from Jackson Labs and maintained using IACUC (Institutional Animal Care and Use Committee)-approved protocols. To minimize potential PAR1-influenced sex differences observed in *ApoE*$^{-/-}$ mice,[24] we did not use female animals in the current study because we found that PAR1 can downregulate estrogen receptor expression in tissue culture and in mouse models.[25] Conversely, PAR1 has 3 functional estrogen-responsive elements in its promoter region, and estradiol can induce up to 4-fold increases in PAR1 expression.[26] Furthermore, recent work showed that estrogen may strongly influence transcriptional regulation of inter-cellular adhesion molecule-1 (ICAM-1) in endothelial cells.[27] Although potentially interesting, given the confounding and complex effects of estrogen on PAR1-dependent transcriptional regulation, we decided to study only male mice in the current study. The *ApoE*$^{-/-}$ mice were fed a high-fat/high-cholesterol Western atherogenic diet (40% fat, 0.21% cholesterol; D12079B, Research Diets) for 16 weeks and permitted food and water consumption ad libitum. Treatments were delivered 6 days per week by 100 μL subcutaneous injection. At the end of the 16-week treatment period, whole blood was collected and separated for plasma. The mice were euthanized with ketamine/xylazine and perfused/fixed with 10% formalin via cardiac puncture. The aorta and the internal carotids of each mouse were isolated, removed, and fixed in 10% formalin.

Histology, Immunohistochemistry, and Cell Culture

Adventitial fat was removed from the isolated aortas which were splayed open and pinned down on dissecting black wax for en face staining with Oil-Red-O. The stained sections were imaged using a Canon PowerShot SX20IS digital camera. The acquired images were then analyzed according to the AHA guidelines for experimental atherosclerosis en face staining technique.[28] Primary human umbilical vein endothelial cells and human pulmonary artery endothelial cells, EA.hy926 human umbilical vein endothelial cells,[29] and the MS-1 C57BL6 mouse endothelial cell line were purchased from Lonza or American Type Culture Collection. MMP1a-antibody was made by previously described methods.[30,31]

A detailed Material and Methods Section is available in the online-only Data Supplement.

Results

Circulating MMP1 Correlates With Coronary Atherosclerotic Burden in Patients Being Evaluated for Presumptive PCI

In an analysis of 97 patients with CAD/ACS evaluated for presumptive PCI in the TRIP-PCI study, angiographic CAD burden was quantified based on a CASS (Coronary Artery Surgery Study) Registry 29-segment scoring system of the number and location of diseased coronary arteries with ≥50% stenosis. The goal was to determine whether there was a potential relationship between angiographic atherosclerotic plaque burden and baseline systemic levels of MMP1, TAT, and TNFα. Patients had several baseline cardiovascular risk factors, including dyslipidemia (89%), hypertension (82%), obesity (73%), and smoking history (62%; Table). Patients were nearly equally split between ACS (48%) and stable CAD (52%) and had an average TIMI (Thrombolysis In Myocardial Infarction) risk score of 3.3±1.3. As

shown in Figure 1A, there was a significant correlation (R=0.33; P=0.00149) between circulating plasma MMP1 levels and CAD burden and between MMP1 and circulating thrombin (TAT; R=0.29; P=0.004; Figure IA in the online-only Data Supplement). In contrast, there was no significant correlation between TAT levels and CAD burden as assessed by coronary angiography (Figure IB in the online-only Data Supplement). Likewise, systemic TNFα was not correlated with CAD burden (Figure IC in the online-only Data Supplement). However, TNFα was significantly correlated with both MMP1 (R=0.32; P=0.00153; Figure 1B) and TAT levels (R=0.41; P=0.00005; Figure ID in the online-only Data Supplement) in the patients with CAD and ACS.

We used heparin rather than sodium citrate or EDTA as anticoagulant in the main baseline blood collection tubes because the Zn-dependent MMP1 assay requires active metal-loenzyme and heparin does not interfere with its activity, unlike the aforementioned metal chelating agents. To examine the possibility that heparin may have affected TAT levels, baseline blood was also collected from 53 of the patients using EDTA as anticoagulant. As shown in the Figure IIA in the online-only Data Supplement, plasma TAT levels measured from whole-blood anticoagulated with EDTA versus heparin were highly correlated with each other R=0.76 (P<0.00001) and had a R=0.68 (P<0.00001) for the LN-transformed values (Figure IIB in the online-only Data Supplement). TAT levels or LN(TAT) from the EDTA-anticoagulated samples did not correlate with CAD burden (Figure IIC and IID in the online-only Data Supplement) in the 53 subjects with CAD or ACS. This served to validate that the choice of anticoagulant did not significantly affect the TAT analysis in this patient data set.

Chronic Inhibition of MMP1 and PAR1 Decreases Atherosclerotic Lesion Burden

We then investigated the effect of chronic treatment with MMP1, PAR1, and the thrombin inhibitor bivalirudin in the development of atherosclerotic lesions in mice. Male *ApoE*$^{-/-}$ mice were fed a high-fat/high-cholesterol Western diet for 16 weeks. Mice were treated subcutaneously daily 6 d/wk with the PAR1 pepducin PZ-128 (10 mg/kg), MMP1 inhibitor FN-439 (5 mg/kg), thrombin antagonist bivalirudin (10 mg/kg), versus PAR2 pepducin PZ-235 (6.3 mg/kg) or vehicle. FN-439 was previously shown to be specific for MMP1 and inhibits >94% of MMP1 activity, 0% to 7% of MMP2, MMP3, and MMP8, 21% of MMP9, and 36% of MMP13 activity but has no inhibitory effects against thrombin.[11] PZ-128, PZ-235, and FN-439 inhibitor dose levels were selected based on previous pharmacokinetic and pharmacodynamic efficacy experiments conducted in mice.[11,31–34] High-dose bivalirudin (10 mg/kg) was confirmed to block in vivo thrombin activity in the mice by causing a significant prolongation of the tail bleeding time and suppression of TAT levels (Figure IIIA and IIIB in the online-only Data Supplement). PZ-128 was also confirmed to block PAR1 activity in mice using chemotactic migration of peripheral blood mononuclear cells (PBMCs) toward gradients of the mouse PAR1 agonist, MMP1a, as a pharmacodynamic measure of in vivo efficacy (Figure IIIC in the online-only Data Supplement). The fasting total plasma cholesterol increased by 2.5-fold from a mean of 350 mg/dL at baseline to ≈900 mg/dL after 16 weeks of high-fat/high-cholesterol diet, with no significant differences in the change in total plasma cholesterol from baseline between the different treatment groups (Figure IV in the online-only Data Supplement).

The formation of atherosclerotic lesions in the thoracic/abdominal and infrarenal/iliac sections of the aorta markedly decreased in mice treated with the PAR1 pepducin PZ-128 or MMP1 inhibitor FN-439 compared with the vehicle group (Figure 2A). Total lesional area of the descending aorta was expressed as the percentage of the combined area of the thoracic/abdominal and infrarenal/iliac sections of the aorta. Treatment with PZ-128 significantly reduced the mean lesional area by 58% (thoracic/abdominal; $P<0.001$) to 70% (iliac; $P<0.05$) and FN-439 by 42% (thoracic/abdominal; $P<0.01$) to 63% (iliac; $P<0.05$; Figure 2B). Conversely, chronic treatment with the thrombin inhibitor bivalirudin had no significant effects on mean aortic plaque formation. Similar effects were seen in the aortic arch segment[28] where PZ-128 and FN-439 gave a significant 25% suppression of plaque area, whereas bivalirudin had no effect (Figure V in the online-only Data Supplement). Because PAR2 can also be transactivated by thrombin cleavage of PAR1 on the surface of endothelial cells under inflammatory conditions,[12] we also tested the effects of chronic PAR2 inhibition. The PAR2 pepducin inhibitor, PZ-235,[34] had no effects on total aorta athero-sclerotic plaque burden at the 16-week end point (Figure 2A and2B), aortic arch plaque (Figure V in the online-only Data Supplement), or cholesterol levels (Figure IV in the online-only Data Supplement) and was not examined further.

Perivascular angiogenesis is a prominent feature of advanced atherosclerosic lesions and potentially contributes to plaque instability and rupture.[35] Neovascularization of plaques was examined in the vasa vasorum at the right and left renal artery branch points from the aorta of $ApoE^{-/-}$ mice fed the high-fat/high-cholesterol diet and treated with PZ-128, FN-439, bivalirudin or vehicle as described above. The aorta-renal artery vasa vasorum segment was whole mount immunostained for CD31, a marker for endothelial cells. In the vehicle cohort, angiogenesis (Figure VIA in the online-only Data Supplement) appeared in distinct areas corresponding to the location of atherosclerotic plaques to the luminal side of the aorta. Chronic treatment with PZ-128 and FN-439 significantly ($P<0.05$) decreased mean plaque neovascularization as compared with the vehicle-treated mice by 78% and 59%, respectively (Figure VIB in the online-only Data Supplement). Treatment with PZ-128 and FN-439 also significantly ($P<0.05$) reduced the number of vascular bundles in the vasa vasorum of the renal artery branches by 75% and 50%, respectively (Figure VIC in the online-only Data Supplement). Chronic treatment with bivalirudin, however, had no effect on neovascularization of the vasa vasorum underlying the renal artery lesions or on the number of the vascular bundles as compared with the vehicle cohort.

MMP1–PAR1 Mediates TNFα-Induced Expression of Leukocyte Adhesion Molecules on Endothelial Cells

To gain insight into the potential mechanism of the MMP1–PAR1 system in promoting macrophage infiltration into atherosclerotic lesions, we tested the hypothesis that MMP1 may be activating PAR1 on endothelial cells to promote expression of the adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) which are critical for leukocyte adhesion, infiltration, and lesion development in atherosclerosis.[36] Because TNFα plays a major initiating role in inducing endothelial inflammatory signaling in atherosclerosis and significantly correlated with MMP1 levels in our patients (Figure 1B), we first demonstrated that TNFα causes a dose-dependent release (3–7 fold) of MMP1

protein into the media from 3 different human endothelial cell types (Figure 3A; Figure VII in the online-only Data Supplement) and up to a 7- to 8-fold increase in *MMP1* gene expression (Figure VIII in the online-only Data Supplement). Treatment of human umbilical vein endothelial cells (primary venous), human pulmonary artery endothelial cells (primary arterial), and EA.hy926 (venous) endothelial cells with TNFα also caused an increase in ICAM-1 and VCAM expression which was suppressed by pharmacological blockade of PAR1 with PZ-128 or RWJ-56110 (RWJ), or MMP1 with FN-439, but not by negative control pepducin, P1pal-19EE (Figure 3B–3F). Furthermore, silencing *Mmp1* gene expression with short hairpin RNA (shRNA; Figure 3D) or *PAR1* with short interfering RNA (siRNA; Figure 3E) caused significant decreases in TNFα-induced expression of adhesion molecule ICAM-1. Similar inhibitory effects on both ICAM-1 and VCAM-1 expression were observed by silencing *Mmp1a* gene expression in mouse MS-1 endothelial cells (Figure 3G), indicating a prominent involvement of the MMP1(a)–PAR1 system in the expression of leukocyte adhesion molecules induced by TNFα in both human and mouse endothelial cells.

MMP1–PAR1 Triggers Transmigration of Monocytes Through Endothelial Monolayers

Given the marked inhibitory effects of blocking the MMP1–PAR1 system in suppressing both expression of endothelial leukocyte adhesion molecules and macrophage infiltration into atherosclerotic lesions, we determined whether exogenous MMP1(a) could also trigger transmigration of monocytes through endothelial monolayers. First, we found that transmigration of human PBMCs through primary human umbilical vein endothelial cell (Figure 4A) and human pulmonary artery endothelial cell (Figure 4B) monolayers toward MMP1a in media was significantly inhibited by pharmacological inhibitors of PAR1 (PZ-128, RWJ) and MMP1a (FN-439) but not with negative control P1pal-19E PAR1 pepducin. Evidence that PZ-128 was suppressing the MMP1–PAR1 system in mouse PBMCs was shown by ex vivo transmigration of PBMCs isolated from mice treated with vehicle versus 10 mg/kg PZ-128. As shown in Figure 4C, the PZ-128–treated mice had 70% inhibition of PBMC migration through confluent monolayers of either human or mouse endothelial cells toward gradients of MMP1a. Likewise, exposure of endothelial cells to recombinant human MMP1 caused a 1.75-fold increase in monocyte transmigration through human umbilical vein endothelial cell monolayers for 1 hour which was inhibited by PZ-128 but not by the negative control pepducin, P1pal-19E (Figure 4D). MMP1a from mouse fibroblasts also caused a significant 14-fold increase ($P<0.001$) in transmigration of monocytes for a 16-hour period that was nearly completely blocked ($P<0.001$) by pharmacological inhibitors of PAR1 (PZ-128, RWJ) and MMP1a (FN-439) and by siRNA silencing of *PAR1* in the endothelial cells but not with negative control scrambled siRNA or with P1pal-19E (Figure 4E). Likewise, immunodepletion of MMP1a from the fibroblast media completely prevented ($P<0.001$) transmigration of monocytes through the endothelial monolayers as compared with control IgG (Figure 4E). Together, these data indicate that the MMP1(a)–PAR1 system drives effective monocyte transmigration through endothelial cell monolayers.

Inhibition of MMP1 and PAR1 Suppresses Macrophage Infiltrates in Carotid Artery Lesions

We quantified macrophage infiltrates at the site of plaque lesions in the internal carotid arteries. Mean macrophage content was significantly ($P<0.05$) decreased by 54% and 47%, respectively, in the lesions from the carotid arteries of mice treated with the PAR1 pepducin PZ-128 or the MMP1 inhibitor FN-439 as compared with the vehicle cohort (Figure 4F and4G). In contrast, the bivalirudin treatment cohort demonstrated a significant 51% increase ($P<0.05$) in mean macrophage infiltrates at the site of the carotid artery lesions as compared with vehicle-treated mice (Figure 4G). Carotid plaque cross-sectional area was significantly decreased by 33% ($P<0.001$) in the PZ-128 and FN-439 but not bivalirudin-treated animals (Figure IXA in the online-only Data Supplement). Collagen content of the intimal area was also quantified in the carotid artery lesions and showed 20% to 35% numerically higher but nonsignificant increases in collagen content in the PZ-128 and FN-439 mice and no effect in the bivalirudin-treated mice as compared with vehicle (Figure IXB in the online-only Data Supplement).

TNFα-Induced Expression of ICAM-1 Is Mediated by Transactivation of MMP1–PAR1–NF-κB Signaling in Endothelial Cells

Unlike the MMP1 inhibitor FN-439, the thrombin inhibitor bivalirudin had no suppressive effects on atherosclerotic lesion development or macrophage infiltrates in the 16-week mouse model. In concordance with these in vivo observations, treatment of endothelial cells with 3 different thrombin inhibitors (bivalirudin, hirudin, and PPACK [D-phenylalanyl-prolylarginyl chloromethyl ketone]) had no effect on TNFα-induced expression of ICAM-1 or VCAM-1 (Figures 3B and5A) or nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a major proinflammatory transcription factor in endothelial cells (Figure 5A; Figure XA in the online-only Data Supplement). In contrast, blockade of either PAR1 or MMP1 with PZ-128 and FN-439, respectively, gave major suppressive effects on TNFα induction of ICAM-1 expression and nuclear translocation of NF-κB (Figure 5A). Similarly, knockdown of *Par1* or *Mmp1* with siRNA or shRNA suppressed TNFα-induced nuclear translocation of NF-κB (Figure XB in the online-only Data Supplement). We also confirmed that the *Mmp1* shRNA did not have off-target suppression of interleukin-1β induction of ICAM-1 and VCAM-1 (Figure XC in the online-only Data Supplement). Inhibition of PI3-kinase (with LY294002 or wortmannin), a downstream effector of PAR1, gave a similar pattern of inhibition as blocking MMP1–PAR1 on TNFα-induced ICAM-1 expression (Figures 3B and5A), nuclear translocation of NF-κB (Figure 5A), and complete blockade of AKT phosphorylation (Figure 5A). TNFα also induced a 10- to 20-fold increase in mRNA expression of *Icam-1* and *Vcam-1*, both of which were significantly ($P<0.001$) inhibited with PZ-128, FN-439, and LY294002 (Figure XD in the online-only Data Supplement).

To further delineate the mechanism of action of noncanonical activation of PAR1 by MMP1, we examined the direct effects of the canonical versus noncanonical PAR1 agonists on nuclear translocation of transcription factors NF-κB and AP-1 (activator protein 1) in endothelial cells. Both MMP1 and PRSFLLRN (noncanonical peptide ligand generated by MMP1 cleavage of PAR1) activated nuclear translocation of NF-κB and AP-1 to a similar extent as TNFα (Figure 5B; Figure XA in the online-only Data Supplement). Likewise,

thrombin also activated NF-κB and AP-1 signaling; however, SFLLRN (canonical peptide ligand generated by thrombin cleavage of PAR1) was less efficacious than PRSFLLRN (Figure 5B; Figure XA in the online-only Data Supplement).

TNFα has been shown to be a potent activator of JNK (c-Jun N-terminal kinase)–AP1 pathways through the mitogen-activated protein kinase MEKK1 (mitogen-activated protein kinase kinase), and ERK1/2 (extracellular signal-regulated kinases) through Ras/Raf, however, it has not been established how TNFα activates p38 mitogen-activated protein kinase inflammatory signaling in endothelial cells.[37,38] TNFα-induced robust activation of phospho-ERK1/2, phospho-JNK, and phospho-p38 in endothelial cells as expected (Figure 6A; Figure XI in the online-only Data Supplement). Treatment with either the PAR1 inhibitor, PZ-128, or the MMP1 inhibitor, FN-439, had no effect on TNFα activation of phospho-ERK1/2 or phospho-JNK. However, PZ-128 and FN-439 treatment gave nearly complete suppression of phospho-p38. Likewise, PZ-128 and FN-439 also nearly completely blocked ICAM-1, VCAM-1 expression, and nuclear translocation of NF-κB in the endothelial cells (Figure 6A). These data, together with the results from Figures 3 and 5, are consistent with the mechanism depicted in Figure 6B, namely that TNFα activates NF-κB/AP-1 inflammatory signaling and expression of the key leukocyte adhesion molecules ICAM-1 and VCAM-1, in part, through transactivation of MMP1–PAR1 signaling pathways.

Discussion

Although MMPs are recognized as key regulators of plaque structure and stability, the potential contribution of MMPs as direct signaling molecules in the local plaque milieu that perpetuate the chronic proinflammatory[39] and tissue-remodeling events in atherogenesis[40] has not been explored. The long-standing view is that MMPs, such as MMP1, mainly contribute to plaque development and architecture by cleaving interstitial collagen and other matrix proteins.[41,42] MMP1 is also significantly elevated in patients after MI,[43,44] in subjects with diabetes mellitus[45] and ACS,[46] and those with morbid carotid artery disease,[47] suggesting that MMP1 could reflect a poorer prognosis in CAD and ACS; but whether it is a direct contributor to poor cardiovascular outcome is not known.

In the present study, we show that targeting the MMP1–PAR1 system with inhibitors of either MMP1 or PAR1 significantly decreased total atherosclerotic burden, macrophage infiltration, and plaque neoangiogenesis in mouse models of atherosclerosis. In contrast, the thrombin inhibitor bivalirudin had no salutary effects on reducing aortic plaque formation, neoangiogenesis of the vasa vasorum, or macrophage infiltrates in arterial lesions, consistent with earlier data from *ApoE*$^{-/-}$ mice treated with warfarin.[48] Moreover, we found that plasma levels of MMP1 but not thrombin (TAT) significantly correlate with total coronary atherosclerotic burden as determined by 29-segment angiography in patients with CAD and ACS enrolled in the TRIP-PCI phase 2 study. Together, these findings indicate that the PAR1 activator MMP1 promotes the development of atherosclerosis and that preventing PAR1 inflammatory signaling downstream of MMP1 may be effective in suppressing atherosclerotic plaque formation and progression. By comparison, a significant correlation was previously detected between circulating TAT and severity of CAD (P<0.001; R=0.225)

based on a 16-segment AHA classification in a study of 295 patients with stable CAD.[49] A possible reason that may explain the different outcomes in TAT correlations is that our enrolled patients had much more extensive atherosclerosis/CAD on average with 48% of the population comprising patients with ACS.

We previously demonstrated that MMP1 activates distinct signaling outcomes from thrombin in platelets and smooth muscle cells[11,23] by noncanonical cleavage of the PAR1 extra-cellular domain between residues Asp 39 and Pro 40. Likewise, activation of endothelial PAR1 by MMP1 in cancer models greatly enhanced expression of inflammatory cytokines and leukocyte-attracting chemokines (especially monocyte chemoattractant protein 1, interleukin-8, TNFα) as compared with thrombin[50] and stimulates angiogenesis.[32,51,52] This is consistent with the observation here that inhibition of MMP1–PAR1 with FN-439 or PZ-128, but not the thrombin inhibitor bivalirudin, suppresses renal artery plaque angiogenesis in the vaso vasorum. Unlike a recent study showing a reduction in cross-sectional area of brachiocephalic artery lesions in *ApoE*$^{-/-}$ mice globally deficient in PAR2,[53] we did not observe any detectable effects of a PAR2 pepducin inhibitor on total aorta or aortic arch lesion burden. By comparison, the PAR1 pepducin gave large average reductions in plaque lesions in all vessels/segments examined. Although a different vascular model, we note that we previously showed that global PAR2 deficiency[10] caused a significant increase in the hyperplastic response to carotid artery wire injury relative to wild-type mice in the C57BL/6 background indicating a potential negative feedback role for PAR2 under certain pathophysiologic conditions or other compensatory effects because of genetic deletion of PAR2.

We also made the surprising observation that TNFα-induced expression of leukocyte adhesion molecules, ICAM-1 and VCAM-1, was substantially diminished with pharmacological inhibitors or gene silencing of PAR1 and MMP1(a) in both mouse and human endothelial cells. We used endothelial cells and leukocytes from both human and mice as the mouse ortholog of collagenase-1, namely MMP1a, has a less stable prodomain as compared with human MMP1.[54,55] Nonetheless, targeting the MMP1(a)–PAR1 axis markedly reduced PBMC infiltration through endothelial monolayers from both human and mouse sources in vitro and ex vivo and resulted in substantial drops in macrophage content in mouse carotid artery lesions. Inhibitors of the MMP1(a)–PAR1 system gave large suppressive effects on macrophage infiltrates and plaque size but small effects on collagen content. This pattern seems to be in contrast to the results with MMP8/MMP13-deficient[56] and MMP13-deficient[57] mice or animals treated with the MMP13i-A inhibitor[58] which had significantly more organized collagen as compared with wild-type or vehicle mice in the *ApoE*$^{-/-}$ background but similar plaque size and macrophage content. Interestingly, MMP9 deficiency[59] resulted in yet another pattern that showed both macrophage and collagen decreases in the atherosclerotic plaques of *ApoE*$^{-/-}$ mice. The MMP1 inhibitor used in this study, FN-439, has partial inhibitory effects on MMP9 and MMP13, therefore, it is possible that the effects of blocking MMP1a on atherosclerotic plaque development and architecture may be either partly masked or enhanced by minor effects on these other 2 opposing MMPs.

Evidence was provided that MMP1–PAR1 activates Akt and p38 and triggers nuclear localization of the transcription factors NF-κB and AP-1 to greatly amplify TNFα

inflammatory signaling in endothelium. Although we did not detect a contribution of thrombin to TNFα upregulation of ICAM-1 or VCAM-1 in endothelial cell cultures, exogenously added thrombin was able to independently activate NF-κB signaling in endothelial cell culture. Thrombin has been shown to stimulate both pro- and anti-inflammatory pathways in endothelial cells via PAR1[12,50] depending on the concentration of protease and the cellular context and environment.[60,61] Thrombin is also a potent activator of endothelial barrier disruption and release of vWF (Von Willebrand factor) from Weibel–Palade bodies via PAR1-rho GTP pathways[12] and is a strong regulator of hemostatic responses. Recent reports[22,62,63] have shown that blockade of thrombin with dabigatran decreases atherosclerosis in *ApoE*$^{-/-}$ mice, as also seen in the *F2*$^{-/+}$/*ApoE*$^{-/-}$ mouse.[22] We used bivalirudin in this study because we previously showed that it is highly specific for thrombin exosite 1 and effectively prevents cleavage and activation of the high-affinity thrombin receptor PAR1 in humans and in animal models.[64,65] Moreover, the high dose of bivalirudin used here inhibited in vivo thrombin activity in mice by both bleeding time measurements and reduction in circulating TAT levels. Although speculative, one possibility to explain our lack of effect of bivalirudin on atherosclerotic plaque development in the *ApoE*$^{-/-}$ model is that the peptide inhibitor bivalirudin has strong interactions with exosite 1 but relatively weak interactions with the thrombin active site and does not as efficiently block the low-affinity PAR4 thrombin receptor or other thrombin substrates which lack a hirudin-like sequence.[7,64,66] Conversely, the small molecule dabigatran binds tightly to the thrombin active site but does not interact with exosite 1 and should equally block both PAR1 and PAR4 and prevent cleavage of all other thrombin substrates. Thus, different modes of inhibition of thrombin and different effects on the target PAR1 which binds with high affinity to exosite 1 of thrombin in competition to bivalirudin but weakly to the active site could potentially account for the differences that we observe in our mouse model versus the dabigatran inhibitor.

Indeed, thrombin activation of PAR1 is a potent activator of platelet aggregation and acute arterial thrombosis,[6] and expression of PAR1 in atherosclerotic coronary arteries leads to a marked vasoconstrictory response to thrombin.[67] Moreover, PAR1 may play a role in cardiovascular remodeling mediated by an angiotensin II–independent mechanism.[68] This has led to a major effort to develop PAR1 antagonists such as vorapaxar to reduce ischemic event occurrence and sequelae in CAD and ACS. In this regard, the efficacy and long-term safety of the oral PAR1 inhibitor vorapaxar for 1- to 2.5-year treatment periods was evaluated in 2 large phase 3 randomized trials. The ability of vorapaxar to prevent MI and stroke in subjects (n=26 449) with high-risk atherothrombotic disease (either post-MI, history of stroke, or peripheral arterial disease) was tested in the TRA (thrombin receptor antagonist) 2°P-TIMI 50 study.[3] In addition, a second trial assessed the ability of vorapaxar to prevent MI and stroke in subjects (n=12 944) with chronic ACS (TRA-CER [thrombin receptor antagonist for clinical event reduction in acute coronary syndrome]).[69] Vorapaxar was associated with a reduction in risk of MI, stroke, cardiovascular death, and revascularization in patients with a previous MI or peripheral arterial disease and was approved by the Food and Drug Administration for these two indications.[70] However, these benefits occurred at the expense of more bleeding, including more intracranial bleeding, likely due in part to the extremely long pharmacodynamic effect of vorapaxar of ≥3 weeks.[70]

Although the direct effects of vorapaxar on atherosclerotic lesion reduction were not directly assessed in individual subjects per se, these overall long-term reductions in cardiovascular events in subjects with severe atherothrombotic disease are consistent with our findings that PZ-128 markedly reduces atherosclerotic burden in the *ApoE*$^{-/-}$ mouse model even in the absence of an antiplatelet effect, as unlike humans, mice lack PAR1 on their platelets.[71]

Contrary to thrombin inhibitors (bivalirudin, hirudin, argatroban, and dabigatran), intracellular PAR1 pepducins, such as PZ-128, do not directly interfere with the procoagulant activity of thrombin cleavage of fibrinogen[4] or affect bleeding parameters in patients with CAD or nonhuman primates as monotherapy or with aspirin.[5] Because thrombin-dependent fibrin generation is unaffected by inhibition of PAR1, a reversible PAR1 antagonist should have a favorable bleeding risk relative to a direct thrombin inhibitor in patients with severe atherothrombotic disease. PZ-128 is currently being assessed for prevention of acute ischemic and thrombotic complications of PCI versus standard-of-care in patients with CAD and ACS in the TRIP-PCI study. Unlike vorapaxar, the inhibitory effects of PZ-128 are reversible with 50% recovery of PAR1 activity by 24 hours and ≈75% recovery by 8 days in subjects receiving concomitant aspirin.[5] PZ-128 and other PAR1 inhibitors with similarly favorable pharmacodynamic half-lives may be well-worth testing as chronic treatment as a disease-modifying agent in suppressing or reversing endothelial inflammation and plaque burden.

Limitations of this study include caution in extrapolating from the findings of our small clinical trial size of 97 subjects to larger patient populations with CAD and ACS, and the use of pharmacological inhibitors which can have off-target effects, and that bivalirudin has a shorter half-life as compared with the new oral anticoagulants, such as dabigatran. Although widely used to model atherosclerosis in humans, the limitations of the *ApoE*$^{-/-}$ mouse strain include lesions that are more stable and less prone to rupture, a lack of coronary artery lesions, and effects on immunologic cells, lipid function, and metabolism.[24]

Supplementary Material

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Nonstandard Abbreviations and Acronyms

ACS	acute coronary syndromes
AP-1	activator protein 1

ApoE$^{-/-}$	apolipoprotein E–deficient
CAD	coronary artery disease
ICAM-1	intercellular adhesion molecule 1
MI	myocardial infarction
MMP1	matrix metalloprotease-1
PAR1	protease-activated receptor-1
PBMC	peripheral blood mononuclear cells
PCI	percutaneous coronary intervention
TAT	thrombin antithrombin III complex
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1

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Highlights

- Blocking noncanonical activation of protease-activated receptor-1 by a matrix metalloprotease-1 inhibitor results in significant protection against progression of atherosclerosis in mice.
- Targeting protease-activated receptor-1 with a first-in-class intracellular pepducin PZ-128 significantly decreases total atherosclerotic burden and macrophage infiltration in mice
- Plasma matrix metalloprotease-1 is significantly correlated with atherosclerotic burden in patients with coronary artery disease and acute coronary syndrome undergoing cardiac catheterization or percutaneous coronary intervention.
- The current work provides support for testing the effects of chronic treatment of coronary artery disease/acute coronary syndrome patients with inhibitors of the matrix metalloprotease-1–protease-activated receptor-1 axis

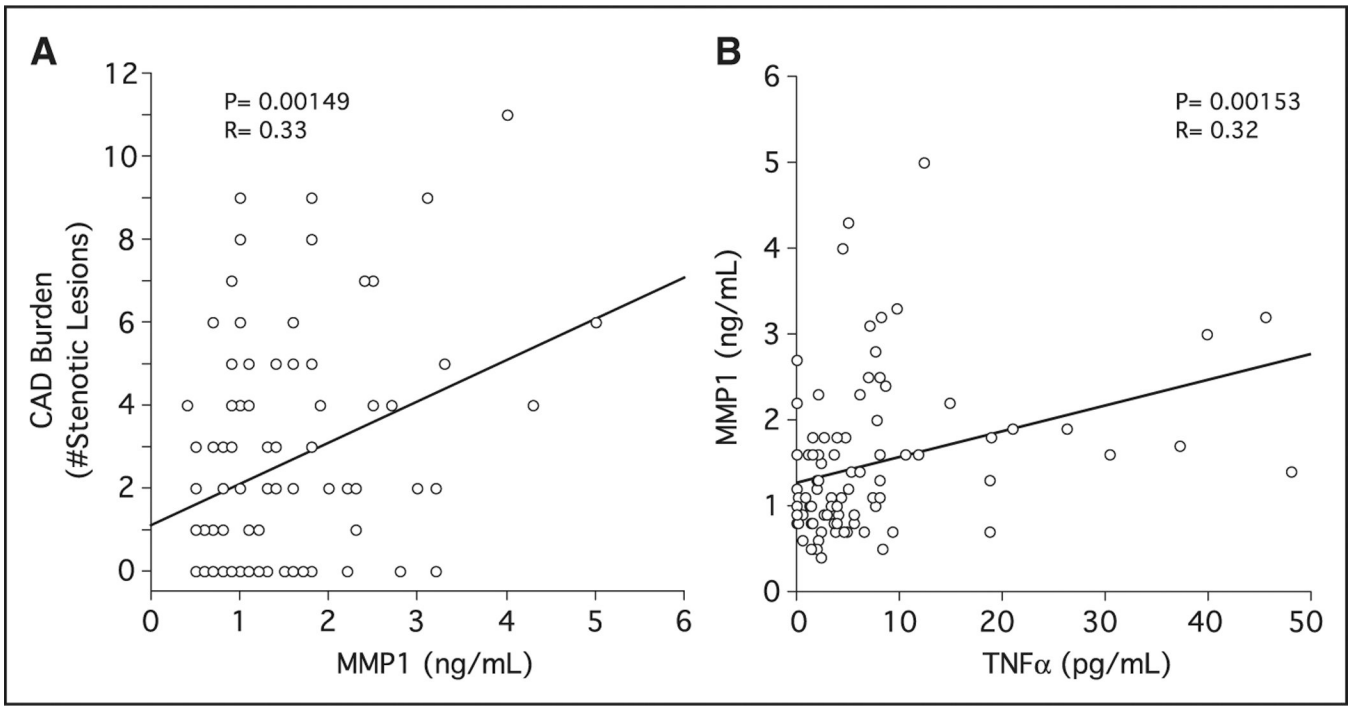

Figure 1.

Circulating matrix metalloprotease-1 (MMP1) levels correlate with coronary artery disease (CAD) burden (no. of coronary arteries with ≥50% stenosis) in patients undergoing coronary angiography for presumptive percutaneous coronary intervention (PCI). Patients (n=91) with symptoms of coronary artery disease or acute coronary syndromes were evaluated for PCI by coronary angiography to quantify number of coronary arteries with ≥50% stenosis from atherosclerotic lesions (CAD burden). **A**, Systemic plasma MMP1 was determined for each patient just before angiography and plotted vs CAD burden. **B**, Systemic plasma tumor necrosis factor α (TNFα vs MMP1 was plotted for each subject. Linear regression analyses were performed, and least-squares lines, *R* values, and *P* values for the slopes are shown.

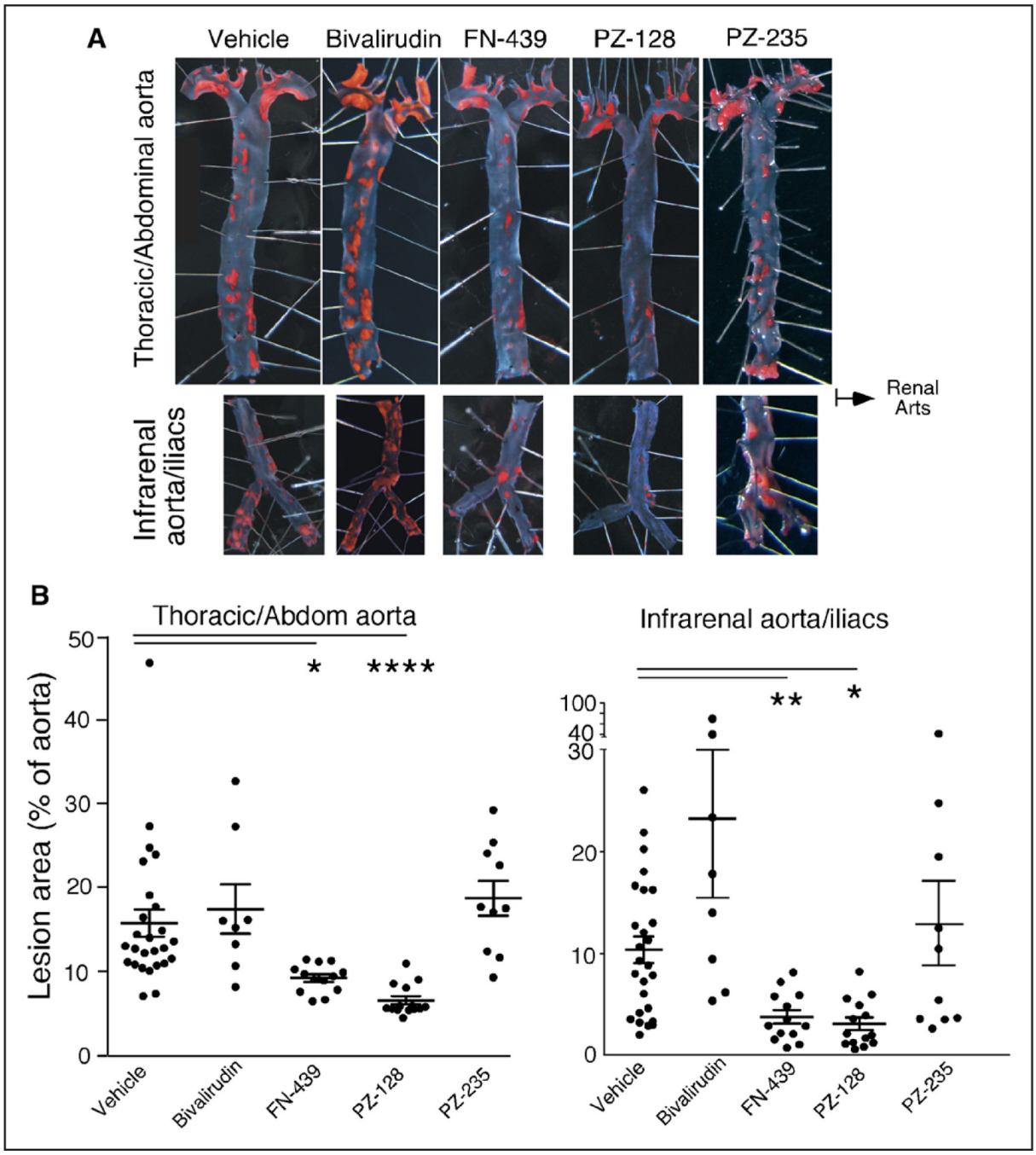

Figure 2.

Inhibition of protease-activated receptor 1 (PAR1) or matrix metalloprotease-1 (MMP1) attenuates the development of athero-sclerotic lesions in aorta and iliac arteries of apolipoprotein E–deficient (*ApoE*$^{-/-}$) mice. **A**, Male *ApoE*$^{-/-}$ mice (n=8–26) were fed a Western type diet for 16 wk and treated daily with subcutaneous vehicle, thrombin inhibitor bivalirudin (10 mg/kg), MMP1 inhibitor FN-439 (5 mg/kg), PAR1 pepducin PZ-128 (10 mg/kg), or PAR2 pepducin PZ-235 (6.3 mg/kg). After 16 wk, mice were euthanized and aortas stained with oil-red O. The renal artery segment of the aorta was removed for assessment of

angiogenesis (Figure VI in the online-only Data Supplement). **B**, Quantification of plaque area (% of total aortic surface). The D' Agostino & Pearson omnibus K2 normality test and Brown–Forsythe equal variance test gave a nonparametric distribution, therefore, the Kruskal–Wallis test followed by Dunn multiple comparisons test was performed.* $P<0.05$, ** $P<0.01$, **** $P<0.0001$.

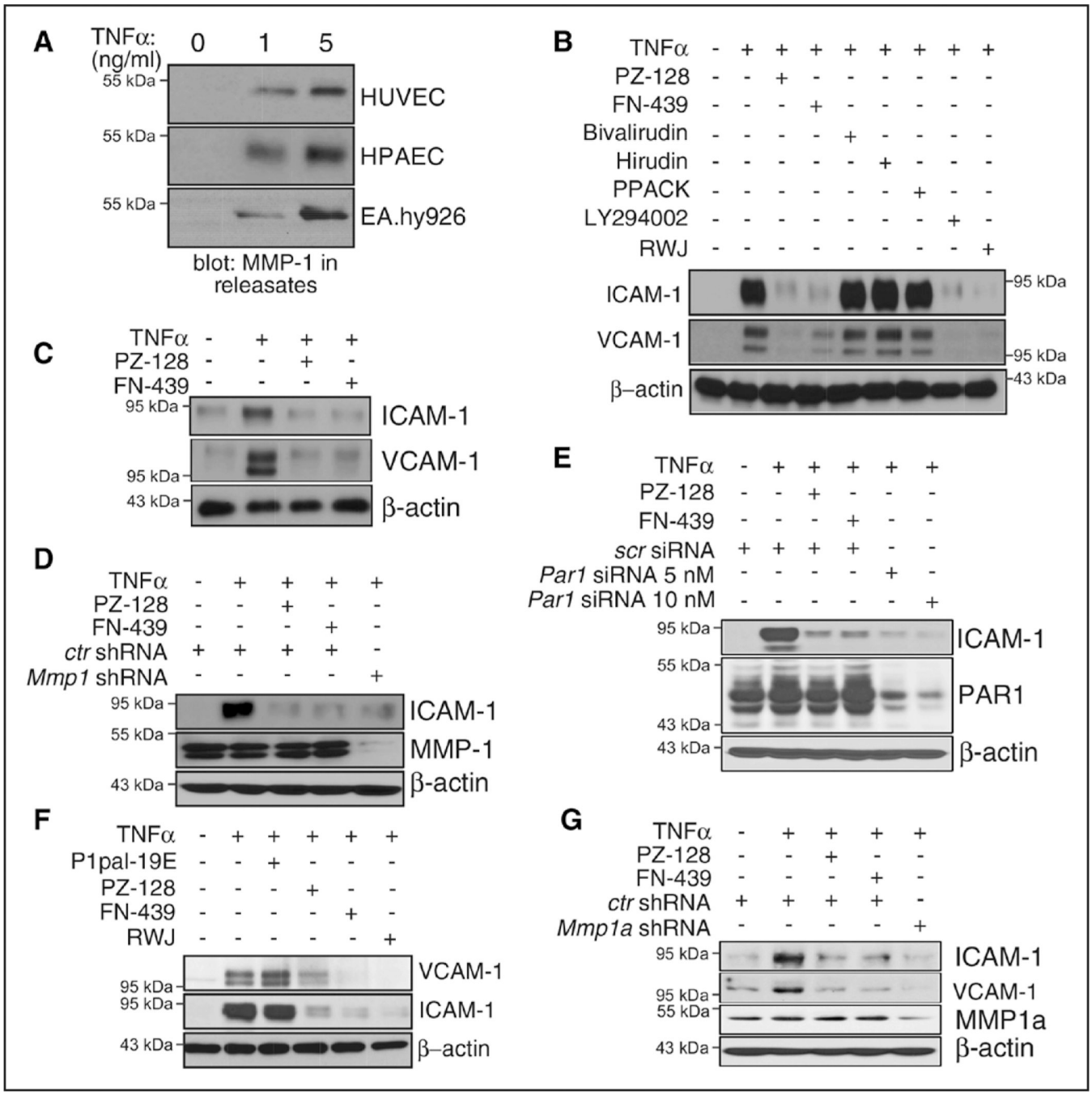

Figure 3.

Knockdown or inhibition of matrix metalloprotease-1 (MMP1) (a) or protease-activated receptor-1 (PAR1) suppresses tumor necrosis factor α (TNFα)–induced expression of adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule (ICAM)-1 in human and mouse endothelial cells. **A**, Human umbilical vein endothelial cells (HUVECs), human pulmonary artery endothelial cells (HPAECs), and EA.hy926 endothelial cells were grown as 70% confluent monolayers and treated with TNFα for 4 h. Extracellular media from endothelial supernatants (releasates) was then

harvested and analyzed for MMP1 protein levels by Western blot. Equal total protein loading (100 ng/lane) was determined by Bradford assay. **B**, HUVECs were serum starved for 16 h and treated with 1 ng/mL TNFα. Inhibitors to PAR1 (10 μmol/L PZ-128 and 5 μmol/L RWJ), MMP1 (20 μmol/L FN-439), thrombin (30 μg/mL bivalirudin, 0.5 U/mL hirudin, 5 μmol/L PPACK [D-phenylalanyl-prolyl-arginyl chloromethyl ketone]), and PI3K (phosphatidylinositol-3 kinase; 5 μmol/L LY294002) were added 1 h before addition of TNFα. Whole-cell lysates were collected after 2 and 4 h and probed for ICAM-1 and VCAM-1, respectively. **C**, HPAECs were serum starved for 16 h and activated for 4 h with 1 ng/mL TNFα and treated with PZ-128 or MMP1 inhibitor FN-439, and ICAM-1 and VCAM-1 assessed by Western blot. **D**, Human EA.hy926 endothelial cells were silenced with lentivirus short hairpin RNA (shRNA) against *Mmp1* or control lentivirus shRNA. After 2 d, cells were activated for 4 h with 1 ng/mL TNFα in the presence or absence of 10 μmol/L PZ-128 or FN-439 as indicated, and ICAM-1 and MMP1 expression assessed by Western blot. **E**, EA.hy926 endothelial cells were silenced with short interfering RNA (siRNA) to *Par1* or control siRNA for 48 h or treated with 10 μmol/L PZ-128 or FN-439 just before addition of 1 ng/mL TNFα and ICAM-1 or PAR1 Western blots performed. **F**, Inactive control pepducin, 10 μmol/L P1pal-19E has no effect on TNFα-induced expression of VCAM-1 and ICAM-1 as compared with 10 μmol/L PZ-128, 5 μmol/L RWJ, or 10 μmol/L FN-439 in EA.hy926 cells. **G**, shRNA silencing of *Mmp1a* (after 2 d) suppresses TNFα-induced expression of ICAM-1 and VCAM-1 as compared with 10 μmol/L PZ-128 or FN-439 in mouse MS-1 endothelial cells. β-Actin was used as loading control. Experiments were conducted 3× to 5× each and gave similar results.

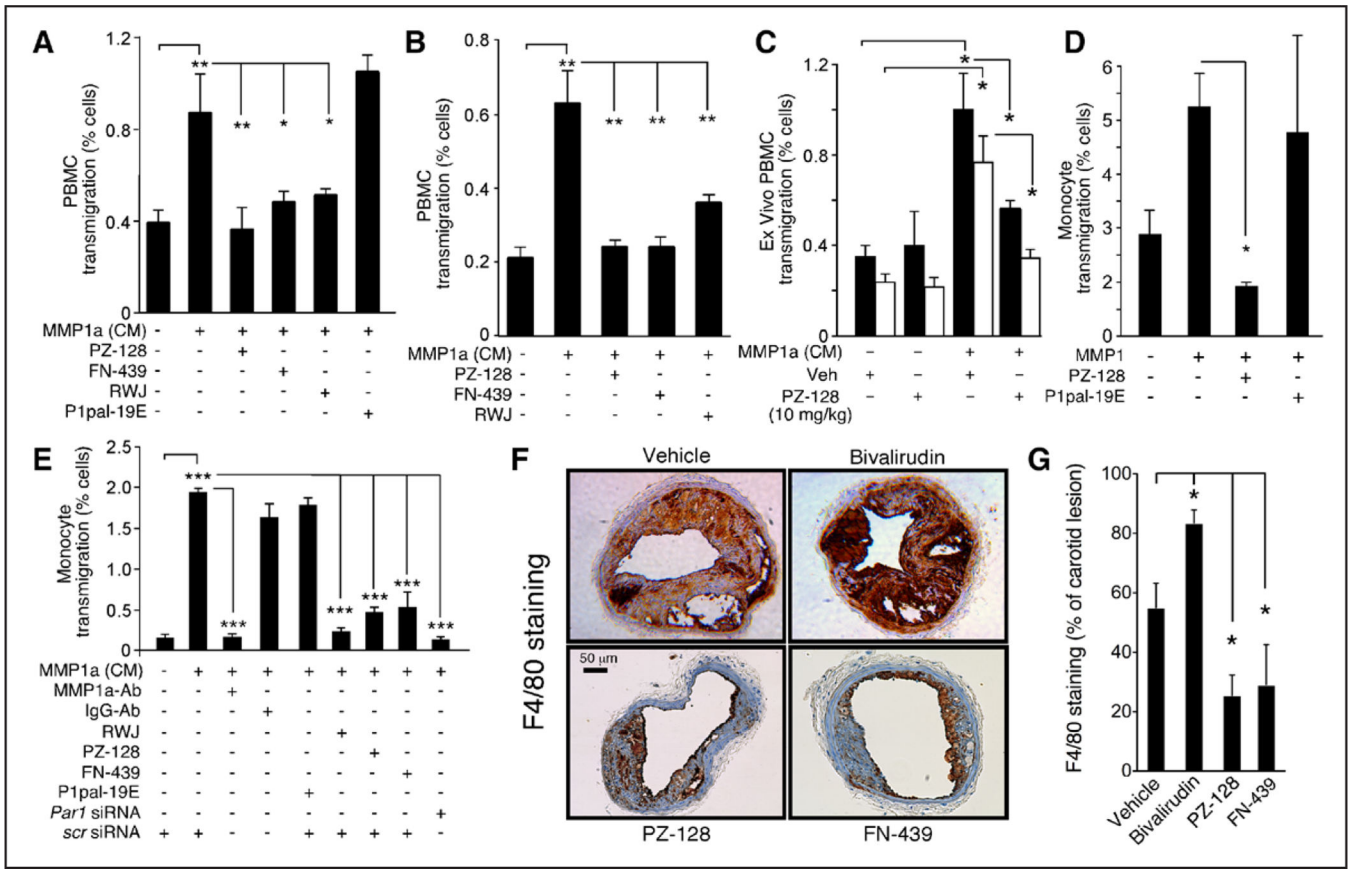

Figure 4.

Inhibition of matrix metalloprotease-1 (MMP1)–protease-activated receptor-1 (PAR1) signaling suppresses monocyte transmigration and macrophage infiltrates in carotid lesions. **A**, Transmigration of human peripheral blood mononuclear cells (PBMCs; 50 000/well) for 3 h through confluent monolayers of human umbilical vein endothelial cells (HUVECs; n=4) or (**B**) human pulmonary artery endothelial cells (HPAECS) to NIH-3T3 produced MMP1a (conditioned media [CM]) in the presence of PAR1 inhibitors PZ-128 (5 μmol/L in **A** and 10 μmol/L in **B**) and RWJ-56110 (RWJ, 5 μmol/L in **A** and 10 μmol/L in **B**), 20 μmol/L FN-439, or negative control pepducin, 5 μmol/L P1pal-19E (n=2). **C**, Ex vivo transmigration (3 h) of PBMCs isolated from whole blood from mice (n=5) treated with vehicle or 10 mg/kg PZ-128 through monolayers of human EA.hy926 (filled bars) or mouse MS-1 (open bars) endothelial cells toward gradients of MMP1a (CM). **D**, Transmigration of THP1 monocytes for 1 h through monolayers of HUVECs exposed to exogenous pure 3 nmol/L MMP1 in the presence of 3 μmol/L PZ-128 or P1pal-19E (n=3). **E**, Transmigration of THP1 monocytes for 16 h through monolayers of EA.hy926 cells to MMP1a (CM) either pre-depleted by MMP1a-antibody (Ab), IgG control, or with 5 μmol/L RWJ, 10 μmol/L PZ-128, or 10 μmol/L FN-439. Knockdown of *Par1* with short interfering RNA (siRNA) but not with scrambled (scr) siRNA in the EA.hy926 cells with siRNA gave similar inhibition of monocyte transmigration as pharmacological blockade of PAR1 (n=2). **F**, Internal carotid arteries were isolated from $ApoE^{-/-}$ mice in Figure 2 after 16 wk and stained with F4/80-Ab to detect activated macrophages with representative carotid artery sections shown from each

treatment group and quantification (**G**) of the % F4/80 lesion area (n=5–9 carotids). N was insufficient to perform normality tests, however, the Brown–Forsythe equal variance test was performed and passed, which was followed by ANOVA and Newman–Keuls multiple comparisons post hoc tests (*P<0.05, **P<0.01, ***P<0.001).

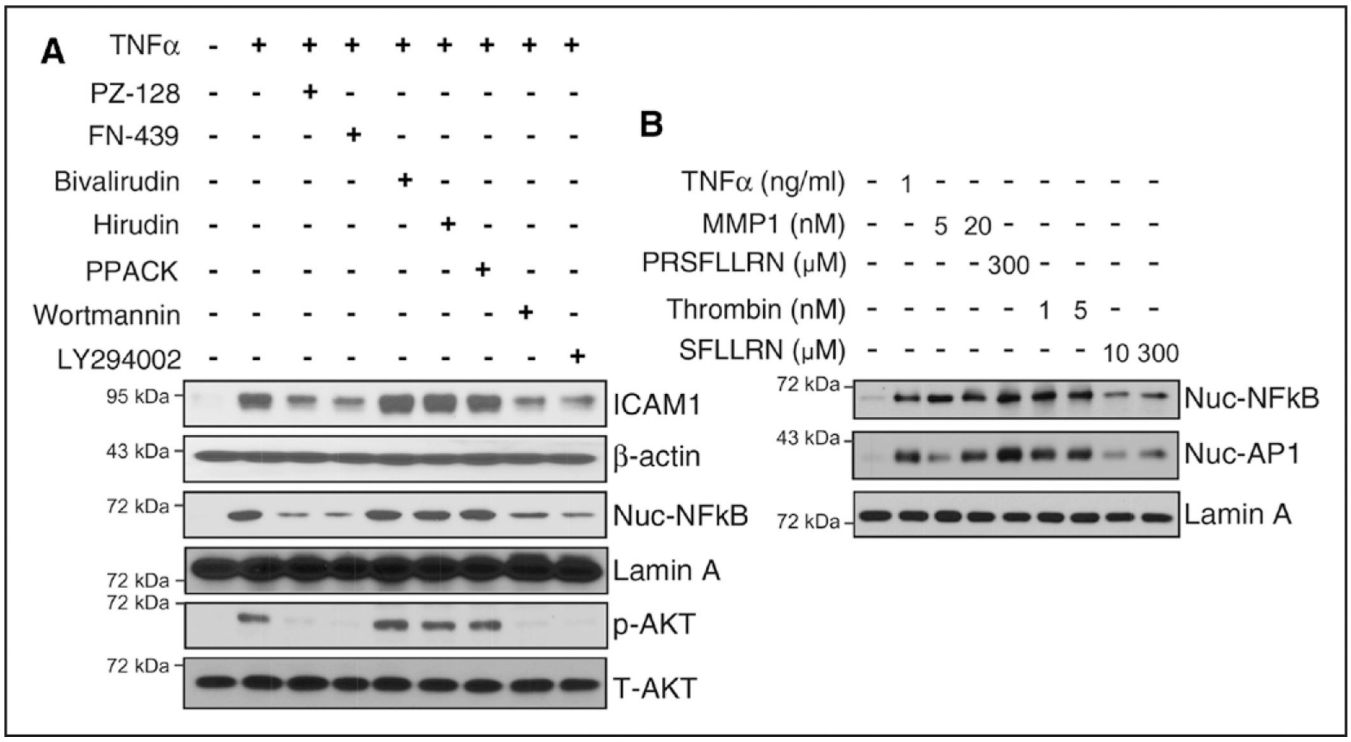

Figure 5.

Tumor necrosis factor α (TNFα)–induced expression of intercellular adhesion molecule-1 (ICAM-1) is mediated by noncanonical transactivation of matrix metalloprotease-1 (MMP1)–protease-activated receptor-1 (PAR1)–NF-κB signaling in endothelial cells. **A**, Pharmacological inhibition of PAR1 (10 μmol/L PZ-128) and MMP1 (10 μmol/L FN-439) but not thrombin (30 μg/mL bivalirudin, 0.5 U/mL hirudin, or 5 μmol/L PPACK [D-phenylalanyl-prolyl-arginyl chloromethyl ketone]) suppresses TNFα (1 ng/mL)-induced NF-κB nuclear localization, AKT activation, and ICAM-1 expression in endothelial cells. Similarly, PI3K (phosphatidylinositol-3 kinase) inhibitors wortmannin (100 nmol/L) and LY294002 (5 μmol/L) inhibited TNFα-induced NF-κB nuclear localization, AKT activation, and ICAM-1 expression. β-Actin and lamin A were used as total cell and nuclear extract loading controls, respectively. **B**, Effect of MMP1 and the MMP1-generated PAR1 ligand, PRSFLLRN, thrombin, and the canonical SFLLRN peptide on nuclear translocation of NF-κB and AP-1 (activator protein 1). Lysates from endothelial cells were harvested at 15 min after addition of agonists for NF-κB and pAKT, at 1 h for AP-1, and at 4 h for ICAM-1 Westerns. Lamin A was used as nuclear loading control and experiments were conducted ≥3× each.

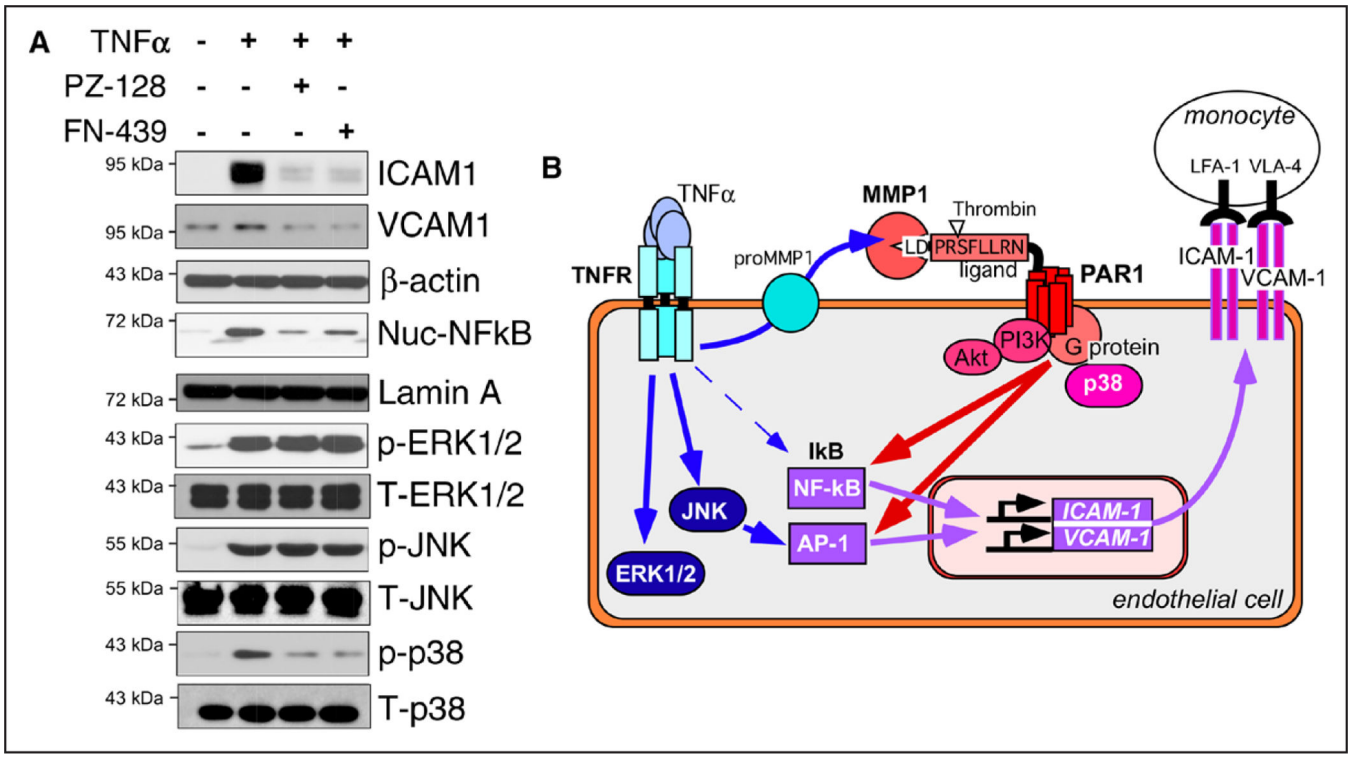

Figure 6.

Tumor necrosis factor α (TNFα) transactivates matrix metalloprotease-1 (MMP1)–protease-activated receptor-1 (PAR1)–nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory signaling in endothelial cells. **A**, Endothelial (EA.hy926) cytoplasmic protein extracts were probed with antibodies against intercellular adhesion molecule-1 (ICAM-1; 4 h), vascular cell adhesion molecule-1 (VCAM-1; 16 h), phospho (p) and total (T)-ERK1/2 (extracellular signal-regulated kinases), p- and T-JNK (c-Jun N-terminal kinase), p- and T-P38 (15 min), and nuclear (Nuc) extracts for NF-κB (15 min), with β-actin serving as loading control. TNFα (1 ng/mL), 10 μmol/L PZ-128, and 10 μmol/L FN-439 were used, and experiments were conducted at least 3× each. **B**, TNFα transactivation model of MMP1–PAR1 inflammatory signaling in endothelial cells.

Table.

Baseline Characteristics of Cardiac Catheterization/PCI Patients

Characteristic	All Patients (n=97)
Age, y	62±11
Range	32–84
Male, n	80 (82%)
Weight, kg	98±17
Range	60–139
Body mass index, kg/m^2	32±5
Range	20–49
Cardiovascular risk factors, n	
Hypertension	80 (82%)
Dyslipidemia	86 (89%)
Diabetes mellitus	32 (33%)
Smoking history	60 (62%)
Obesity *	71 (73%)
Age (male >45 y, female >55 y)	85 (88%)
Family history of premature coronary artery disease †	43 (44%)
Cardiovascular disease history, n	
Previous myocardial infarction	23 (24%)
Previous percutaneous coronary intervention	32 (33%)
Previous coronary artery bypass grafting	13 (13%)
Peripheral arterial disease	5 (5%)
Venous thrombotic disease	2 (2%)
Congestive heart failure	8 (8%)
Indication for cardiac catheterization/PCI, n	
Non-ST-segment-elevation myocardial infarction	17 (18%)
Unstable angina	29 (30%)
Stable angina	37 (38%)
Other ‡	14 (14%)
TIMI risk score §	3.3±1.3

Data represent mean±SD where indicated. BMI indicates body mass index; PCI, percutaneous coronary intervention; and TIMI, Thrombolysis In Myocardial Infarction.

* Subjects with BMI ≥30 kg/m^2.

† Includes any first-degree relative who have had any of the following diagnosed at <55 years for males or <65 years for females: angina, acute myocardial infarction, sudden cardiac death, coronary artery bypass grafting or percutaneous coronary intervention.

‡ Includes positive stress test, abnormal ECG, coronary artery calcifications.

§ The TIMI risk score for patients with unstable angina or non-ST-segment-elevation myocardial infarction ranges from 0 to 7, with higher scores indicating greater risk for ischemic events or mortality.