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PAR2 (protease-activated receptor 2) Deficiency Attenuates Atherosclerosis in Mice

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Objective—PAR2 (protease-activated receptor 2)-dependent signaling results in augmented inflammation and has been implicated in the pathogenesis of several autoimmune conditions. The objective of this study was to determine the effect of PAR2 deficiency on the development of atherosclerosis.

Approach and Results—PAR2 mRNA and protein expression is increased in human carotid artery and mouse aortic arch atheroma versus control carotid and aortic arch arteries, respectively. To determine the effect of PAR2 deficiency on atherosclerosis, male and female low-density lipoprotein receptor–deficient ($Ldlr^{-/-}$) mice (8–12 weeks old) that were $Par2^{+/+}$ or $Par2^{-/-}$ were fed a fat- and cholesterol-enriched diet for 12 or 24 weeks. PAR2 deficiency attenuated atherosclerosis in the aortic sinus and aortic root after 12 and 24 weeks. PAR2 deficiency did not alter total plasma cholesterol concentrations or lipoprotein distributions. Bone marrow transplantation showed that PAR2 on nonhematopoietic cells contributed to atherosclerosis. PAR2 deficiency significantly attenuated levels of the chemokines *Ccl2* and *Cxcl1* in the circulation and macrophage content in atherosclerotic lesions. Mechanistic studies using isolated primary vascular smooth muscle cells showed that PAR2 deficiency is associated with reduced *Ccl2* and *Cxcl1* mRNA expression and protein release into the supernatant resulting in less monocyte migration.

Conclusions—Our results indicate that PAR2 deficiency is associated with attenuation of atherosclerosis and may reduce lesion progression by blunting *Ccl2*- and *Cxcl1*-induced monocyte infiltration.

Visual Overview—An online visual overview is available for this article. (***Arterioscler Thromb Vasc Biol*. 2018;38: 1271-1282. DOI: 10.1161/ATVBAHA.117.310082.)**

Key Words: atherosclerosis ■ cholesterol ■ inflammation ■ macrophage ■ mice

Atherosclerosis is a chronic and progressive inflammatory disease defined as stenosing of blood vessels, which may lead to rupture or erosion of unstable plaques, resulting in acute atherothrombotic occlusion of blood flow to the brain (ischemic stroke) or heart (myocardial infarction).[1] The disease is characterized by infiltration of lipids, platelets, neutrophils, monocyte/macrophages (foam cells), T and B lymphocytes, and replication and migration of vascular smooth muscle cells (VSMCs) into the vessel walls of large arteries. This infiltration of immune cells results in local release of several classes of proteases implicated in all stages of atherosclerotic disease, including intimal thickening, plaque progression, and plaque rupture. Comprising a third of the protease compliment in an entire organism (degradome), serine proteases are crucial activators of the coagulation cascade and can activate cells through membrane-bound PARs (protease-activated receptors).[2]

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The 4 members of the PAR family (PAR1–4) are ubiquitously expressed by vascular cells and are activated via proteolytic cleavage of their N-terminal domain, exposing a tethered ligand.[3] PAR1, 3, and 4 are preferentially cleaved by the serine protease thrombin, an essential enzyme in hemostasis and thrombosis.[4] Studies using direct thrombin inhibitors or prothrombotic mice have shown that thrombin contributes to the initiation of atherosclerosis in murine models.[5–7] However, 1 study found that PAR4 deletion in mice has no effect on atherosclerosis.[8] Recent investigations have suggested that PAR1 may be the primary thrombin receptor for the initiation of atherosclerosis. Thrombin activation of PAR1 promotes the migration and proliferation of VSMCs, cytokine and chemokine expression, vascular calcification, and cellular apoptosis.[9–12] In addition, the binding of thrombin to PAR1 can result in transactivation

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Nonstandard Abbreviations and Acronyms

apoE	apolipoprotein E
FVIIa	factor VIIa
HDL-C	high-density lipoprotein cholesterol
IL	interleukin
LDL-C	low-density lipoprotein cholesterol
MCP-1	monocyte chemoattractant protein 1
MyD88	myeloid differentiation factor 88
oxLDL	oxidized low-density lipoprotein
PAR	protease-activated receptor
TF	tissue factor
TLR	toll-like receptor
TNFα	tumor necrosis factor α
TRIF	toll-like receptor adaptor molecule 1
VSMC	vascular smooth muscle cell

of PAR2.[13,14] PAR1 is also present and upregulated in both mouse and human atherosclerotic lesions.[15,16] Further analysis is required to better understand the role of thrombin activation of PARs in the development of atherosclerosis.

PAR2 is activated by the serine proteases trypsin, tryptase, and the TF (tissue factor)/FVIIa (factor VIIa) complex or FXa, but not by thrombin. PAR2 expression is increased in the atherosclerotic aorta of apolipoprotein E–deficient (*apoE*$^{-/-}$) mice compared with normolipidemic controls.[15–17] In addition, it is upregulated in atherosclerotic human coronary arteries and is localized to intimal VSMCs.[15–17] PAR2 activation in coronary artery VSMCs results in the secretion of multiple proatherogenic cytokines and chemokines, including IL (interleukin)-6, IL-8, and CCL2 (chemokine C-C motif ligand 2).[18] PAR2 deficiency is associated with reduced intimal VSMC hyperplasia in a carotid artery ligation model.[14] VSMC hyperplasia in this model results from VSMC dedifferentiation and proliferation, which is a common phenotype in early- and late-stage atherosclerotic lesion formation and progression, respectively.[19–21]

In this study, we investigated the role of PAR1 and PAR2 in diet-induced atherosclerosis using the low-density lipoprotein receptor–deficient (*Ldlr*$^{-/-}$) mouse model in conjunction with total PAR1 and PAR2 deficiency. Using bone marrow transplantation, we determined the relative contribution of hematopoietic or nonhematopoietic PAR2 in atherosclerotic disease. Finally, we examined the effects of PAR2 deficiency on atherosclerotic inflammation in mice and extended these findings to cell culture studies with primary murine VSMC.

Materials and Methods

Mice and Diet

Original *Par1*$^{-/-}$ and *Par2*$^{-/-}$ mice were obtained from R.W. Johnson Pharmaceutical Research Institute and were 11 and 6× backcrossed into C57BL/6J, respectively.[22,23]

Ldlr$^{-/-}$ mice (B6.129S7-*Ldlr*^{tm1Her}, stock no. 002207, N12) were obtained from The Jackson Laboratory (Bar Harbor, MA). *Ldlr*$^{-/-}$/*Par1*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par1*$^{-/-}$ cousin littermate mice (final N12) were generated by interbreeding *Ldlr*$^{-/-}$/*Par1*$^{+/-}$ mice, which were created by breeding *Ldlr*$^{-/-}$/*Par1*$^{-/-}$ onto the Jackson *Ldlr*$^{-/-}$ strain. A similar strategy was used to generate *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ littermates (final N8). Our original study with PAR2-deficient animals used both male and female mice (8–10 weeks of age). As there was no significant difference between the 2 sexes, we used male mice (8–10 weeks of age) for the remainder of our studies.

All mice were fed a normal mouse laboratory diet and water ad libitum. To induce hypercholesterolemia in the majority of studies, mice were fed a diet enriched with saturated milk fat (21% wt/wt) and cholesterol (0.15% wt/wt, diet TD.88137 from Harlan Teklad, produced by Purina Laboratory Diets, Land O' Lakes Inc). For the semisynthetic diet, we used a low-fat (4.3% fat wt/wt) modified AIN76A diet containing 0.15% cholesterol, as described by Teupser et al[24] (Purina Laboratory Diets, Land O' Lakes Inc).

Bone Marrow Transplantation

Mice were given sulfamethoxazole and trimethoprim oral suspension (0.2%; Hi-Tech Pharmacal Co) in their water, ad libitum, for 1 week before and 5 weeks after irradiation. Male recipient *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice (8 weeks old) were irradiated with a total of 13 Gy (2 doses of 650 rads 4 hours apart) using a Cs^{137} irradiator (JL Shepherd, San Fernando, CA). Irradiated mice were repopulated with bone marrow harvested from *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice donor mice via retro-orbitally injected cells (1×10^7 cells per mouse). Mice were allowed to recover for 5 weeks and then fed a Western diet for 12 weeks.

Plasma Collection and Processing of Heart and Aorta

Mice were sedated with 3% isoflurane, and blood was collected from the inferior vena cava into a 25 gauge×1″ needle pre-coated with 3.8% sodium citrate. The mice were then humanely euthanized. An aliquot of blood was analyzed for complete blood count using a Hemavet 950 LV veterinary multi-species hematology system (Drew Scientific). Blood was centrifuged at 4000*g* for 15 minutes to prepare platelet poor plasma and then stored at −80°C until use. The heart, aorta, and body were perfused with sterile saline injected into the left ventricle at physiological pressure. Hearts were separated from the aorta under a dissecting microscope and then placed in optimal cutting temperature, frozen, and stored at −80°C until processed. The majority of aortas were extracted and placed into formalin (10% wt/vol) for 48-hour fixation, before being switched to sterile saline and then stored until dissection and processing. Several aortic arches were processed, after diet, for mRNA and protein (described in later sections).

Plasma Lipid Analyses

Mouse plasma lipid concentrations were analyzed with the following commercially kits: total plasma cholesterol (Total Cholesterol E), triglycerides (L-Type TG [triglycerides] M), LDL-C (low-density lipoprotein cholesterol; L-Type LDL-C), and HDL-C (high-density lipoprotein cholesterol; L-Type HDL-C) from Wako Chemicals (Richmond, VA).

Aortic Sinus Atherosclerosis Quantification

Atherosclerotic lesions in the aortic sinus were cut and processed as previously described.[25] In brief, hearts were thawed from −80°C storage in optimal cutting temperature and cut parallel to the tricuspid valve ≈3 mm from the base of the ventricles. The aortic sinus was placed perpendicular to the bottom of a tissue mold and then covered in optimal cutting temperature and sectioned. After the appearance of the full tricuspid valve leaflets, the aortic sinus was serially sectioned in 10 µm increments over the course of at least 8 to 10 microscope slides with at least 9 sections per slide covering a distance of ≈720 µm from the aortic sinus extending into the ascending aorta. Slides were then stained with Oil red O, counterstained with hematoxylin/eosin, mounted, and lesion area quantified using National Institutes of Health (NIH) Image J Fiji software (NIH, Bethesda, MD). All quantification was verified by at least 2 blinded observers.

En Face Atherosclerosis Quantification

En face atherosclerosis was performed and quantified, as previously described. In brief, aortas were removed from storage in sterile saline

and cleaned free of all adventitia. The left subclavian artery was removed, and all but 1 mm of the innominate/brachiocephalic and left common carotid artery was removed for standardization purposes. The aorta was then cut open along the outer curvature along the innominate and left common carotid artery down to the subclavian artery. The inner curvature was then cut open from the ascending aorta down to the iliac bifurcation. The aorta was pinned flat with dissection pins onto a tray fitted with Sudan black-stained paraffin. Aortic arch was defined as the beginning of the ascending aortic arch to 3 mm distal the subclavian artery. Thoracic aorta was 3 mm distal the subclavian artery to the last intercostal artery. Abdominal aorta was defined as last intercostal artery to the iliac bifurcation. Images were taken with a Nikon SMZ800N dissecting microscope with 16MP camera. All images were taken in range of a ruler with standard millimeter hash marks at the same depth and magnification. Atherosclerotic lesions were quantified by percentage of atherosclerotic area versus the total area of the aorta being addressed using Image J Fiji software (NIH, Bethesda, MD). All atherosclerotic quantification was verified by at least 2 blinded users.

Immunohistochemistry and Histological Processing of Aortic Sinuses

Histological analysis was performed on fresh frozen aortic sinus sections using Picrosirius Red (PolyScientific, catalog number 24901-500). Images were captured using a polarizing microscope with ×10 objective (Nikon Optiphot Polarizing Microscope). Immunostaining was performed on frozen serial sections as described previously.[26] Human carotid atherosclerotic sections were obtained from Origene Technologies, Inc. In brief, sections were fixed with ice-cold ethanol and stained with SMC α-actin 1A4 Cy3 conjugated antibody (5 µg/mL; Sigma Aldrich, catalog number C6198). Human and mouse PAR2 staining was performed with mouse monoclonal anti-PAR2 Alexa Fluor 488 (1/50 dilution; clone SAM11; SC-13504 AF488; Santa Cruz Biotechnologies). Sections were mounted with prolong gold antifade mounting solution (Thermo Fisher) and images visualized on a fluorescent microscope (Olympus 1X71). Immunostaining of CD68 used a rat anti-CD68 antibody (FA-11; catalog number MCA1957, AbD Serotec; 1/50 dilution incubated overnight 4°C). Subsequent application of a goat anti-rat (mouse absorbed) biotinylated antibody (Vector, BA-9401; 1/500 dilution incubated 1-hour room temperature). Positive reactive areas were visualized via application of an ABC kit (15 minutes 37°C) and subsequent detection with AEC (3-amino-9-ethylcarbazole) chromogen (2 applications of 10 minutes room temperature; Vector). Several controls were used, including no primary antibody, no primary and secondary antibodies, and nonimmune IgG (not shown). Images were captured with a ×4, ×10, and ×20 objective lens using a Nikon Eclipse FN1 scope.

Mouse Aortic VSMC Isolation

Mouse aortic arch VSMCs were isolated as previously described with several modifications.[27] In brief, we only used the heavily atherosclerotic prone region of the aortic arch, extending from the start of the ascending aorta to 3 mm distal the subclavian artery (including the innominate and left common carotid arteries) for purposes of standardization. Aortic arches and associated vessels were removed from $Par2^{+/+}$ and $Par2^{-/-}$ mice (aged 6–7 weeks) and dissected free of all adventitia in sterile saline solution. Aortas were then allowed to incubate in a solution of 1 mg/mL collagenase type II (Worthington Chemical) for 15 minutes at 37°C with 5% CO_2. Aortas were then taken, dissected open, and endothelium removed with a sterile Q-tip. Aortas were cut into small pieces and placed in a solution of 1 mg/mL collagenase type II (Worthington Biochemical, catalog LS004174), 1 mg/mL soybean trypsin inhibitor (Worthington Biochemical LS003570), elastase (Worthington Biochemical LS002279, final concentration ≈1 U/mL), and 1% penicillin/streptomycin (Gibco) made in sterile 1× PBS for 1 hour, as previously described.[28] After 1 hour of incubation, aortas were triturated with a 21 gauge needle repeatedly until tissues were broken apart. VSMCs were then maintained in DMEM with fetal bovine serum (20% vol/vol; Omega Scientific) and penicillin/streptomycin (1% wt/vol; Gibco) in a cell culture incubator. VSMC phenotype was verified via staining and visualization of SMC α-actin clone 1A4 Cy3 labeled (Sigma Aldrich).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from the aortic arches of $Ldlr^{-/-}$ mice fed a normal chow or Western diet for 24 weeks as previously described (n=15 each group, 3 aortas combined into 1 sample for 5 individual samples) and described below.[27] RNA was also isolated from VSMCs using the TRIzol method (Invitrogen, catalog number 18091200) and reverse transcribed into cDNA using random hexamers in a SuperScript IV first-strand synthesis system kit (Invitrogen, Carlsbad, CA). Levels of different mRNAs were analyzed by real-time polymerase chain reaction using TaqMan Fast Advanced Master Mix and Stratagene Mx3005P (Agilent Technologies). We used TaqMan probe sets with the following catalog numbers: Human *PAR1* (Hs00608346_m1), Mouse *Par1* (Mm00433160_m1), Human *PAR2* (Hs00608346_m1), Mouse *Par2* (Mm00433160_m1), Mouse Ccl2 (Mm00441243_g1), and Mouse Cxcl1 (Mm04207460_m1). All human mRNA was extrapolated to 18s rRNA (Hs99999901_s1), and mouse mRNA was extrapolated to Hprt (hypoxanthine-guanine phosphoribosyltransferase; Mm00446968_m1).

Human Carotid Artery Atherosclerotic Samples

Human carotid artery atherosclerotic samples were obtained from Origene Technologies, Inc. In brief, frozen sections (n=5 patient samples, 25 sections per patient) were purchased, and protein (20 combined sections per patient) and mRNA (5 combined sections per patient) were obtained using a previously established method by Vrana et al[29] and an RNAqueous-Micro total RNA isolation kit (Thermo Fisher/Ambion; catalog number AM1931), respectively.

Boyden Chamber Assay

Monocyte migration and chemotaxis were measured with a 5-µm pore size chemotaxis assay in a 96-well plate (Cell Biolabs Inc, catalog number CBA-105) per company specifications. In brief, $Par2^{+/+}$ and $Par2^{-/-}$ aortic arch VSMCs were isolated, as described above and plated in a 96-well plate at a density of 1.0×10^3 cells per well. After 1 week of growth, cells were serum starved for 24 hours and treated with control LDL (50 µg/mL; Alfa Aesar catalog number BT-903), recombinant mouse IL-1β (10 ng/mL; catalog 401-ML/CF; R&D Systems, Inc), recombinant mouse TNF (tumor necrosis factor)-α (100 ng/mL; catalog 410-MT/CF; R&D Systems, Inc), or oxLDL (oxidized LDL; 50 µg/mL; Alfa Aesar catalog number BT-910) for 24 hours. Media was then harvested, centrifuged, and supernatant collected. Media was directly added to another 96-well plate with migration membranes, and THP-1 monocytes (ATCC [American-type culture collection]) were added to the top chamber. After 24 hours of incubation, monocyte migration through the membrane was detected via fluorescent staining, as per company instructions.

PAR2 ELISA

PAR2 protein in both mouse and human samples were measured using a sandwich ELISA.

Polyclonal rabbit anti-human PAR2 (PA5-33527; Invitrogen) was coated onto Nunc MaxiSorp flat-bottom 96-well plates (2 µg/mL antibody in 50 µL) overnight at 4°C. Wells were then washed 3× and 200 µL/well with 1× PBS/0.1% Triton X-100, and then blocked with 200 µL 3% BSA and 0.1% Triton X-100 in 1× PBS for 2 hours in a humidity chamber. Wells were then washed 3× and 200 µL/well with 1× PBS/0.1% Triton X-100. Using normal and atherosclerotic aortic arches (mice) and normal and atherosclerotic carotid arteries (human), we added 1 µg of protein diluted in 50 µL of 0.2% BSA/0.1% Triton X-100/1 µL protease inhibitor cocktail (Sigma P4830) to each well for 1 hour at 37°C in triplicate. PAR2 recombinant protein (Abcam; catalog number ab152372) ranging from 1 to 15.5 ng/mL serially diluted was used as a calibration curve. Wells were then washed 3× and 200 µL/well with 1× PBS/0.1% Triton X-100. Horse radish peroxidase (HRP)–conjugated monoclonal mouse anti-human PAR2 (SAM11; Santa Cruz, catalog number sc-13504 HRP) was added to each well

(1:1000 dilution in 1× PBS/0.2% BSA/0.1% Triton X-100, 50 µL/well) and incubated for 1 hour at 37°C. Wells were washed 3× with 200 µL/well of 1× PBS/0.1% Triton X-100 and then 50 µL of substrate solution (TMB [3,3′,5,5′-tetramethylbenzidine] Super Sensitive 1 Component HRP MIcrowell substrate (SUBS); Biomol, catalog number ICT-6329) for 30 minutes at room temperature. The reaction was then halted using 100 µL stop solution for TMB microwell substrate (STOPT; Biomol, catalog number ICT-6343). Absorbance was read within 10 minutes at 450 nm using a Cytation 3 (Bio-Tek). Absorbance values of the standards were extrapolated to a nonlinear third-degree polynomial equation. Protein from PAR2-deficient aortas and VSMC cultures was not detected using this ELISA (data not shown). Although a previous publication demonstrated that SAM11 was not specific for PAR2 in arterial lysates of PAR2-deficient mice, our studies use a polyclonal capture of PAR2 before detection with the SAM11 antibody, which confers specificity in an ELISA setting.[30]

Commercial ELISAs

The following commercially available kits were used for determining protein and supernatant concentrations of CCL2 and CXCL1: CCL2 mouse quantikine kit (R&D Systems, Inc; catalog number MJE00) and CXCL1/KC (keratinocyte chemoattractant)/Gro (human growth-related oncogene) mouse quantikine kit (R&D Systems, Inc; catalog number MKC00B).

Protein Assay

Where applicable, protein was quantified in cell or tissue samples using the DC protein assay (BioRad Inc) in the 96-well plate assay according to the product manual.

Research Statistics and Data Representation

All bar and line graphs were created with Sigma Plot v.13 (SPSS, Chicago, IL). All statistical analysis was performed using SigmaStat, now incorporated into Sigma Plot v.13. Data are represented as mean±SEM. For 2 group comparison of parametric data, a Student *t* test was performed, whereas nonparametric data were analyzed with a Mann–Whitney rank-sum test. Statistical significance between multiple groups was assessed by 1-way ANOVA on Ranks with a Dunn post hoc, 1-way ANOVA with Holm–Sidak post hoc, or 2-way ANOVA with Holm–Sidak Post Hoc, when appropriate. Statistical significance among groups performed temporally was assessed by either a 1-way repeated measures ANOVA (parametric) or repeated measures ANOVA on ranks (nonparametric), where appropriate. Values of $P<0.05$ were considered statistically significant.

Study Approvals

All mouse studies were performed with the approval of the University of North Carolina at Chapel Hill and the University of Cincinnati Institutional Animal Care and Use Committees.

Results

PAR1 and PAR2 Expression Is Increased in Mouse and Human Atherosclerotic Prone Regions

PAR2 expression was measured in normal and atherosclerotic specimens from both mice and humans. Male *Ldlr*$^{-/-}$ mice were fed either a control or high-fat and cholesterol Western diet for 24 weeks, and aortic arches were harvested. We also used human atherosclerotic carotid artery specimens and age-matched healthy human control arteries. We found that *Par2* mRNA and protein were significantly elevated in atherosclerotic versus normal vessels in both mouse and human arterial tissue (Figure 1A through 1D). Importantly, this increased expression is similar in mouse and human atherosclerotic

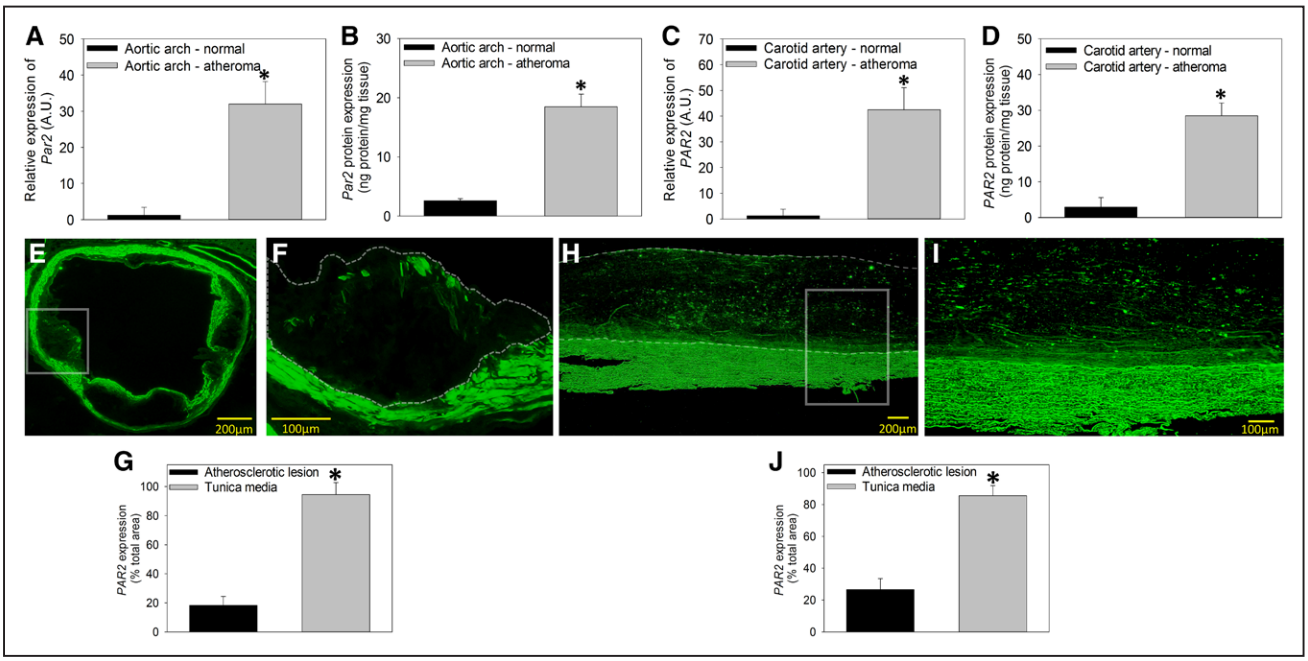

Figure 1. PAR2 (protease-activated receptor 2) mRNA and protein are increased in the atherosclerotic prone regions of mice and humans. Normal mouse aortic arch (low-density lipoprotein receptor–deficient [*Ldlr*$^{-/-}$] mice fed a chow diet for 24 wk) or atherosclerosis-containing aortic arches (*Ldlr*$^{-/-}$ mice fed a Western diet for 24 wk) were examined for (**A**) mRNA extrapolated to normal and (**B**) protein expression (n=5 individual samples per group). Normal or diseased human carotid arteries were isolated and examined for (**C**) mRNA extrapolated to normal and (**D**) protein expression (n=5 individual samples per group). Immunologic detection of PAR2 expression was also performed on mouse atherosclerotic aortic sinus (**E**, **F**) and human carotid atherosclerotic lesions (**H**, **I**; n=4–5 individual samples per species; ×4 magnification **E** and **H**; ×10 magnification **F** and **I**). **G**, **J**, Quantification of PAR2 staining in the lesion or media of atherosclerotic lesions. Solid white box is zoomed in area. Dashed white line is the area of the lesion. Histobars represent mean±SEM. *$P<0.05$ for comparisons to normal aorta/artery or lesion vs tunica media (2-tailed Student *t* test). A.U. indicates arbitrary units.

arteries (mRNA: 32- versus 42-fold; protein: 7.0- versus 9.5-fold, respectively). PAR2 protein expression was further assessed in the atherosclerotic aortic sinus (mice) and carotid artery (human) by immunofluorescence (Figure 1E through 1J; negative controls Figure I in the online-only Data Supplement). The majority of PAR2 protein expression is localized to the tunica media containing VSMCs compared with the atherosclerotic lesion (Figure 1G and 1J) in both species.

Similar to PAR2, *Par1* mRNA (Figure IIA and IIB in the online-only Data Supplement) was significantly elevated in the atherosclerotic versus normal vessels in both mouse and human arterial tissue. PAR2 deficiency had no effect on atherosclerotic-induced Par1 mRNA regulation in *Ldlr*$^{-/-}$ mice fed a Western diet for 12 or 24 weeks (Figure IIIC in the online-only Data Supplement).

Effect of PAR1 or PAR2 Deficiency Is Associated With Reduced Diet-Induced Atherosclerosis in Mice

To examine the contribution of PAR2 in atherosclerosis, we examined 8- to 10-week-old male and female *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice (n=minimal 16 each group) fed a Western diet for 12 weeks. Compared with proficient controls, PAR2 deficiency was associated with dramatically less atherosclerosis in both the aortic sinus and aortic arch independent of sex (Figure 2A through 2F). Histological examination of the aortic sinus showed significantly less macrophage accumulation (Figure 2G, 71% decrease), an increase in VSMC α-actin (Figure 2H, 152% increase), and an increase in Type I collagen (Figure 2I, 490% increase) in PAR2-deficient mice compared with controls.

PAR2 deficiency did not affect total plasma cholesterol concentrations or individual lipoproteins (Table). However, we did observe decreased weight gain, liver weight, and fat pad weights in PAR2-deficient mice versus normal controls (Figure III in the online-only Data Supplement). This observation is consistent with a previous study demonstrating a role for PAR2 in diet-induced obesity.[31] To eliminate an effect of changes in weight gain on atherosclerosis, we used a low-fat (4.3% fat wt/wt) modified AIN76A semisynthetic diet containing 0.15% cholesterol (high cholesterol diet) in a cohort of male *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice (n=9 each genotype) for 12 weeks. Similar increases in cholesterol to the Western diet were observed with this diet without the complications of PAR2-dependent changes in weight gain, hyperglycemia, insulin resistance, or hypertension.[24] The mice fed the high cholesterol diet gained minimal weight (not significantly different between the genotypes), and similar to Figure 2, PAR2 deficiency was associated with less atherosclerosis (Figure IV in the online-only Data Supplement).

To determine if PAR1 influences atherosclerosis, *Ldlr*$^{-/-}$/*Par1*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par1*$^{-/-}$ mice (n=9 each group) were fed a Western diet for 12 weeks. Atherosclerosis was quantified in the aortic sinus and aortic root of these mice. We found that PAR1 deficiency had no effect on diet-induced atherosclerosis compared with proficient controls (Figure VA, VB, VD, and VE in the online-only Data Supplement). Further, there was no difference in macrophage accumulation suggesting no differences in infiltration or cellularity (Figure VC, VF, and VG in the online-only Data Supplement). No differences

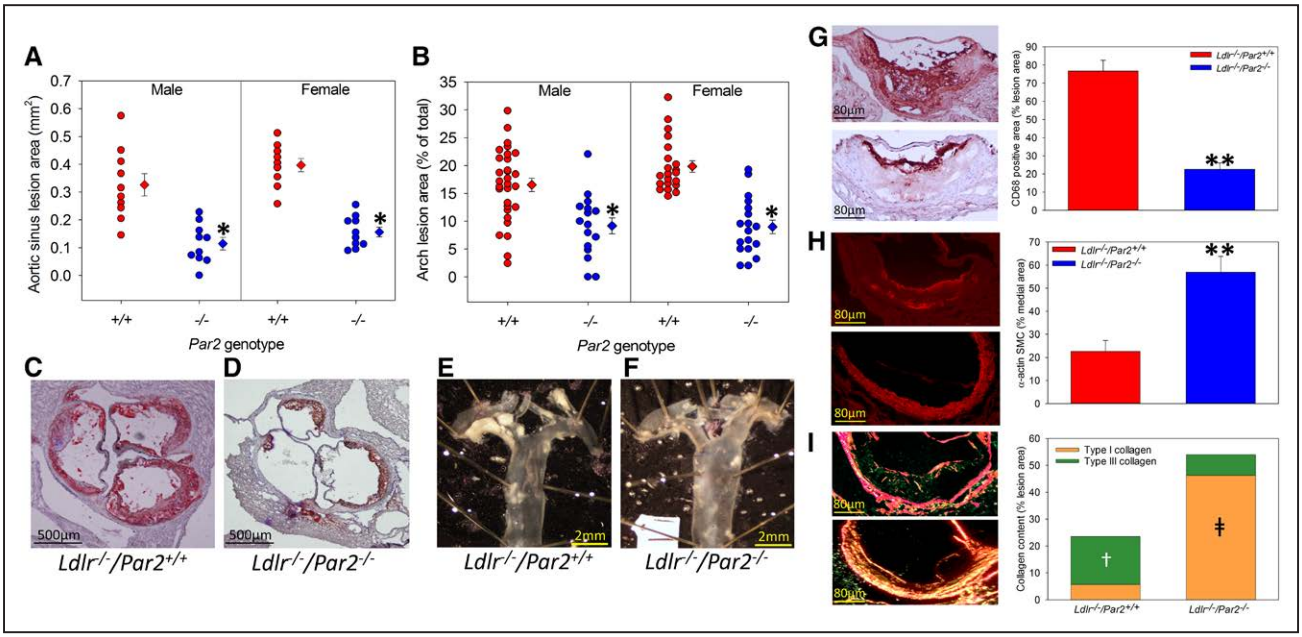

Figure 2. PAR2 (protease-activated receptor 2) deficiency reduces the formation of early atherosclerosis. Male and female low-density lipoprotein receptor–deficient (*Ldlr*$^{-/-}$) mice that were *Par2*$^{+/+}$ and *Par*$^{-/-}$ (n=16–30) were fed a high-fat/cholesterol diet for 12 wk. **A**, Percent lesion area of the aortic sinus and (**B**) en face area of the aortic arch where circles represent individual measurements, diamonds are means±SEM. **P*=0.001 for comparisons of –/– to +/+ (2-way ANOVA with Holm–Sidak post hoc). **C**, **D**, Representative images of Oil red O stained aortic sinus and (**E**, **F**) unstained en face images of the aortic arch. Representative images and quantification of (**G**) CD68, (**H**) smooth muscle cell (SMC) α-actin, and (**I**) picrosirius red–stained aortic sinus lesions (*Ldlr*$^{-/-}$/*Par2*$^{+/+}$—**top**; *Ldlr*$^{-/-}$/*Par2*$^{-/-}$—**bottom**). Histobars represent means±SEM. ***P*<0.005 for comparison of –/– to +/+ (2-tailed Student *t* test). ‡*P*<0.001 for comparison of –/– to +/+ and †*P*<0.05 for comparison of +/+ to –/– (1-way ANOVA on Ranks with Dunn post hoc).

Table. Metabolic and Lipid Parameters From Study Mice

Mouse Group	Sex	Mouse Number	Diet (wk)	Weight (g)	TPC (mg/dL)	HDL (mg/dL)	Trigs (mg/dL)
Ldlr$^{-/-}$/*Par2*$^{+/+}$	Female	21	12	37.8±1.4*	1138±73.1	201±21.8	349±42.8
Ldlr$^{-/-}$/*Par2*$^{-/-}$	Female	18	12	34.2±0.9	1122±52.8	191±12.8	336±23.4
Ldlr$^{-/-}$/*Par2*$^{+/+}$	Male	30	12	41.9±1.2*	1173±64.4	194±26.9	394±53.4
Ldlr$^{-/-}$/*Par2*$^{-/-}$	Male	16	12	37.3±1.3	1097±99.1	205±59.7	364±94.1
Ldlr$^{-/-}$/*Par2*$^{+/+}$	Male	12	24	43.2±1.1	1187±60.7	266±13.9	719±68.3
Ldlr$^{-/-}$/*Par2*$^{-/-}$	Male	12	24	41.3±1.1	1165±215.4	214±37.9	647±40.3
Ldlr$^{-/-}$/*Par2*$^{+/+}$ LFD	Male	9	12	26.5±1.3	1098±42.8	205±25.8	318±41.9
Ldlr$^{-/-}$/*Par2*$^{-/-}$ LFD	Male	9	12	26.7±1.2	1125±39.6	219±41.8	357±42.8
Ldlr$^{-/-}$/*Par1*$^{+/+}$	Male	15	12	42.1±1.7	1204±72.8	192±26.8	419±38.4
Ldlr$^{-/-}$/*Par1*$^{-/-}$	Male	16	12	41.7±1.4	1249±64.7	211±18.9	448±53.7
Par2$^{+/+}$ into *Par2*$^{+/+}$	Male	11	12	36.0±1.5†	1221±48.3	168±24.5	293±43.7
Par2$^{-/-}$ into *Par2*$^{+/+}$	Male	13	12	32.5±0.8	1131±52.9	173±21.1	250±17.4
Par2$^{+/+}$ into *Par2*$^{-/-}$	Male	13	12	31.8±1.1	1076±65.2	154±14.9	285±39.0
Par2$^{-/-}$ into *Par2*$^{-/-}$	Male	12	12	30.8±1.1	1124±51.4	159±31.2	300±15.5

All mice were fed a Western diet, except the LFD group, which was fed a semisynthetic diet. All weights are taken at the end of the study and plasma parameters measured from terminal blood collection. HDL indicates high-density lipoprotein; LFD, low-fat diet; TPC, total plasma cholesterol; and Trigs, triglycerides.

*P<0.01 vs Par2$^{-/-}$ mice; Mann–Whitney rank-sum U test.

†P<0.008 vs all other chimeric groups; ANOVA on Ranks.

were observed between genotypes on total plasma cholesterol, lipoprotein distribution, or weight. These results indicate that PAR1 does not play a role in the development of atherosclerosis in the *Ldlr*$^{-/-}$ model of atherosclerosis.

PAR2 on Nonhematopoietic Cells Contributes To Atherosclerosis

We used bone marrow transplantation to define the cellular source of PAR2 that contributes atherosclerosis. Irradiated

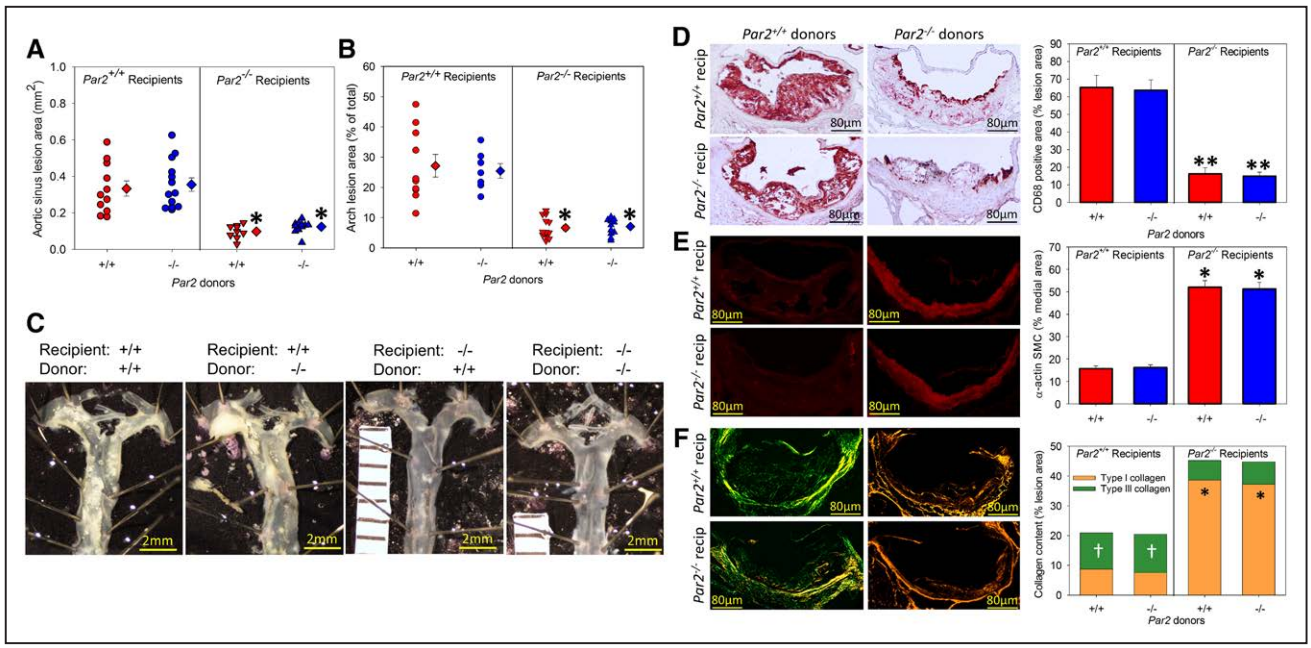

Figure 3. Nonhematopoietic PAR2 (protease-activated receptor 2) reduces atherosclerosis. Male *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice (8–10 wk old, n=10–12 each group) were irradiated (1300 rads split into 2 equal doses 4 h apart) and repopulated with *Par2*$^{+/+}$ or $^{-/-}$ bone marrow. Mice were allowed to recover for 5 wk and then fed a Western diet for 12 wk. **A**, Percent lesion area of the aortic sinus and (**B**) en face area of the aortic arch where circles represent individual measurements, diamonds are means±SEM. **C**, Representative images of unstained en face images of the aortic arch. Representative images and quantification of (**D**) CD68, (**E**) smooth muscle cell (SMC) α-actin, and (**F**) picrosirius red–stained aortic sinus lesions. Histobars represent means±SEM. *P=0.001; **P<0.05 for comparisons of –/– to +/+; †P<0.05 for comparison of +/+ to –/– (**A**, **B**, **D**: 2-way ANOVA with Holm–Sidak post hoc; **C**, **E**: 2-way ANOVA on Ranks Dunn post hoc). *Lldr*$^{-/-}$ indicates low-density lipoprotein receptor–deficient mice.

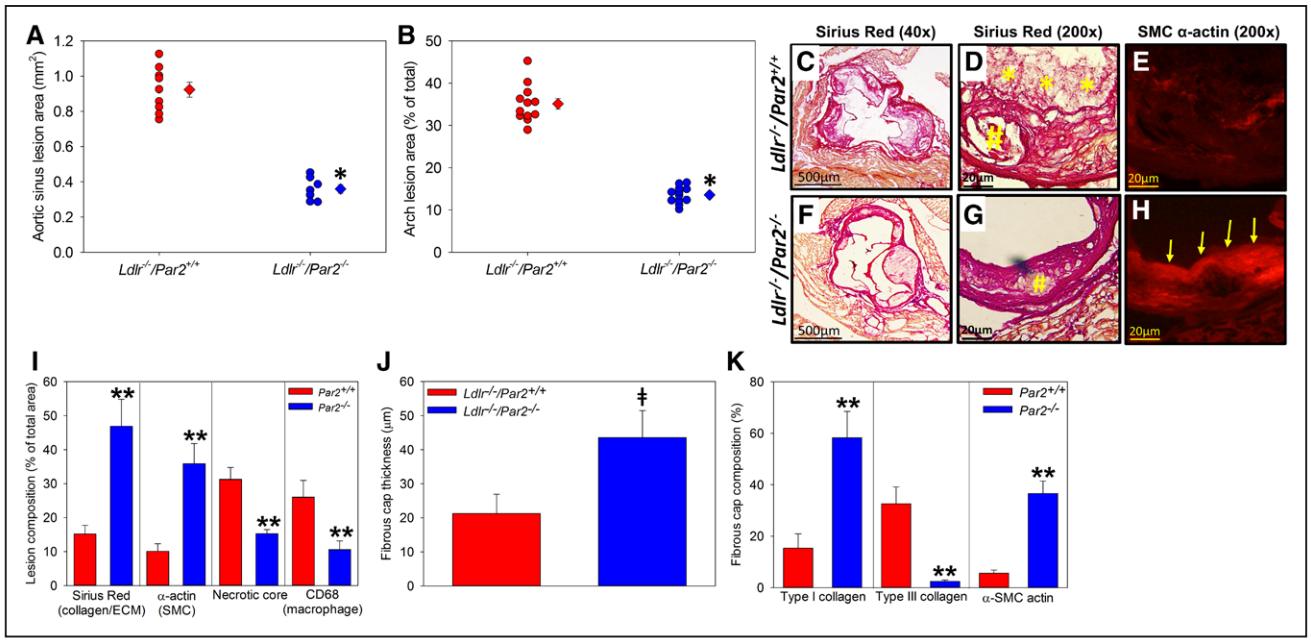

Figure 4. PAR2 (protease-activated receptor 2) deficiency attenuates the formation of advanced atherosclerosis and increases plaque stability. Male low-density lipoprotein receptor–deficient (*Ldlr*$^{-/-}$) mice (8–12 wk) that were *Par2*$^{+/+}$ and $^{-/-}$ (n=12) were fed a high-fat/cholesterol diet for 24 wk. **A**, Percent lesion area of the aortic sinus and (**B**) en face area of the aortic arch where circles represent individual measurements, diamonds are means±SEM. **C**, **D**, **F**, **G**, Sirius red stain for collagen and extracellular matrix (ECM) at ×40 magnification (**C**, **D**) and ×200 magnification (**D**, **G**). Yellow * and # represent secondary atherosclerotic lesions and necrotic core, respectively. **E**, **H**, Smooth muscle cell (SMC) α-actin immunofluorescence where yellow arrows highlight the SMC cap in the –/– mice. Quantification of (**I**) aortic sinus lesion collagen, SMC, necrotic core, and CD68; (**J**) fibrous cap thickness; and (**K**) fibrous cap composition of collagen and SMC α-actin). Histobars represent means±SEM. *P=0.001 (Mann–Whitney rank-sum test), ǂP<0.05 (Mann–Whitney U test), **P<0.05 (ANOVA on ranks Dunn post hoc) for comparisons of –/– to +/+.

male *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ (n=11–13) and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ (n=9–10) mice were transplanted with bone marrow from either *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ or *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice. Transplanted mice were given a 5-week rest period and then fed a Western diet for 12 weeks. Atherosclerosis in the aortic sinus and aortic arch was significantly less in all *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice independent of recipient genotype (Figure 3A through 3C). All groups with PAR2 deficiency (both hematopoietic and nonhematopoietic) gained significantly less weight than the PAR2 proficient control group (Table; Figure VI in the online-only Data Supplement). This weight difference did not affect the outcome of the atherosclerosis studies, as demonstrated by hematopoietic deficiency of PAR2 having similar weight to nonhematopoietic PAR2-deficient mice with a greatly increased burden of atherosclerosis. Deficiency of PAR2 in the nonhematopoietic lineage recapitulated the content of the aortic sinus observed in mice with a global deficiency in PAR2 with less macrophage accumulation (Figure 3D, 79% decrease), an increase in medial SMC α-actin (Figure 3E, 222% increase), and an increase in Type I collagen (Figure 3F, 443% increase) when compared with PAR2 deficiency in the hematopoietic compartment and control mice.

PAR2 Deficiency Results in a Protective Plaque Phenotype

To determine the role of PAR2 in a chronic progression model, we examined male *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice (8–10 weeks of age, n=12 mice per genotype) fed a Western diet for 24 weeks. PAR2 deficiency was associated with significantly less atherosclerotic burden in the aortic sinus and aortic root compared with proficient controls in an advanced model of atherosclerosis (Figure 4A and 4B). PAR2 deficiency was associated with more robust VSMC α-actin expression as a percentage of the total lesion (Figure 4D, 4E, 4G, and 4H—255% increase; P<0.001) and a thicker fibrous cap (Figure 4G, 4I, and 4J—105% increase; P<0.001). PAR2 deficiency also resulted in less necrotic core area as measured by acellular regions (represented by # in Figure 4C, 4F, and 4H). Finally, PAR2 deficiency attenuated secondary lesion formation on top of the fibrous cap (represented by * in Figure 4C) quantified as macrophage positive area (data not shown; +/+: 15±4.2%; –/–: 3.2±1.1%; P<0.001). No differences in total plasma cholesterol, triglycerides, or lipoprotein distribution were found between the different genotypes (Table). Similar to the acute experiments in Figure 2, PAR2 deficiency significantly reduced weight gain during chronic feeding (Table; Figure VI in the online-only Data Supplement). However, no significant difference was observed in weight at the penultimate 23- and 24-week time points demonstrating a catch-up effect at the end of the study.

PAR2 Deficiency Attenuates the Production of Chemokines

Previous studies have shown that PAR2 has a critical role in the production of proinflammatory cytokines and chemokines in vitro and in vivo.[14,15] Therefore, we examined the level of several atherosclerotic proinflammatory cytokines and chemokines in the plasma from our acute and chronic studies.

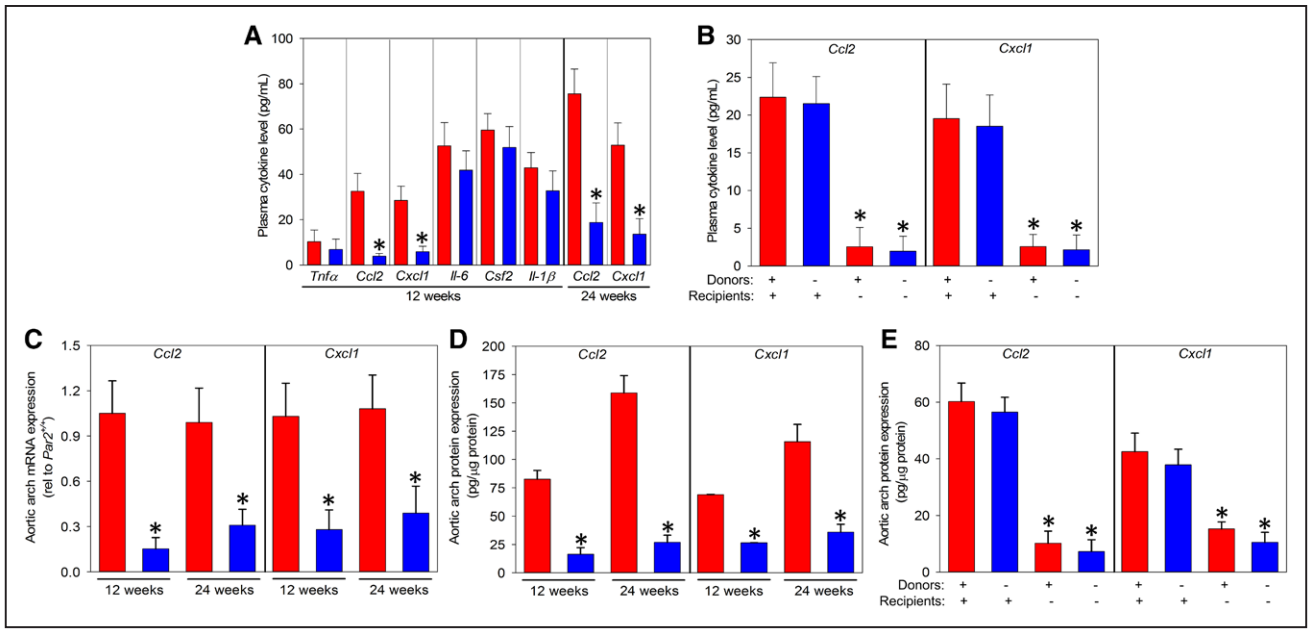

Figure 5. PAR2 (protease-activated receptor 2) deficiency attenuates *CCL2* (chemokine C-C motif ligand 2) and C*xcl1* in the plasma and atherosclerotic lesions. Plasma cytokines were analyzed from aforementioned (**A**) 12- and 24-wk Western diet–fed *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice and (**B**) *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ irradiated and repopulated (*Par2*$^{+/+}$ or $^{-/-}$ bone marrow) mice. In addition, *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice, and a cohort of similarly irradiated and repopulated mice, were fed a Western diet for 12 (normal and irradiated) and 24 wk (normal), and their aortic arches (from aortic sinus to end of the subclavian artery) were harvested and processed for (**C**) mRNA and (**D**, **E**) protein examining the chemokines *Ccl2* and *Cxcl1* (n=5 each time point and genotype). Histobars represent means±SEM. *P<0.01 for comparisons of –/– to +/+ and –/– recipients to +/+ recipients (ANOVA on ranks with Dunn post hoc analysis). Red histobars represent *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ (and +/+ donors) and blue histobars represent *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ (and –/– donors) mice. *Lldr*$^{-/-}$ indicates low-density lipoprotein receptor–deficient mice.

Although several cytokines were lower in PAR2-deficient mice, the chemokines CCL2 (also known as MCP-1; ≈80% less) and CXCL1 (also called KC or N51; ≈75% less) were significantly attenuated in PAR2-deficient mice versus normal controls at both 12- and 24-week time points (Figure 5A). These chemokines were also decreased in all PAR2-deficient recipients on analysis of the bone marrow transplantation experiments (Figure 5B). We also measured chemokine mRNA and protein isolated from the aortic arch (n=5 each genotype per time point) of 8- to 10-week-old male *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice were fed a 12- or 24-week Western diet, We found that PAR2 deficiency was associated with significantly less expression of *Ccl2* and *Cxcl1* mRNA (Figure 5C) and protein (Figure 5D and 5E) in the aortic arch of atherosclerotic mice.

PAR2 Mediates SMC-Induced Cytokine Expression and Monocyte Chemotaxis

VSMCs play a critical role in atherogenesis via phenotypic modulation to a synthetic state characterized by increased proliferation, migration, and loss of contractile proteins, such as α-actin.[32] Our data demonstrate that PAR2 deficiency attenuates the loss of α-actin expression in early lesions. In addition, PAR2 is a potent mediator of VSMC mitogenesis via the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and ERK1/2 (extracellular signal–regulated kinases 1/2).[15,33–37] Activation of PAR2 also increased expression of key proinflammatory cytokines IL-1β and TNFα,[38,39] which are critical mediators of CCL2.[40–42] Together with our nonhematopoietic chimeras, we examined the contribution of VSMC PAR2 to of CCL2 and CXCL1 expression. To assess if PAR2-deficient VSMCs have an effect on CCL2 or CXCL1 expression, we evaluated the response of *Par2*$^{+/+}$ and *Par2*$^{-/-}$ VSMCs to the proinflammatory cytokines to IL-1β (10 ng/mL), TNFα (100 ng/mL), and the atherogenic ligand oxLDL (50 μg/mL) compared with PAR2 agonist peptide (AP, SLIGRL-NH2, 100 μmol/L) and the placebo control LDL. *Ccl2 and Cxcl1* mRNA expression in VSMCs and secreted *Ccl2 and Cxcl1* were significantly attenuated in PAR2-deficient cells under all treatment conditions (Figure 6A through 6D). Interestingly, basal levels of *Ccl2* were significantly decreased in *Par2*$^{-/-}$ VSMCs compared with *Par2*$^{+/+}$ (Figure VII in the online-only Data Supplement; no change in *Cxcl1*). Finally, monocyte migration was attenuated in a Boyden chamber with *Par2*$^{-/-}$ VSMCs treated with IL-1β, TNFα, or oxLDL (Figure 6G) compared with *Par2*$^{+/+}$ controls. Pre-treatment with a CCL2/CXCL1 antibody cocktail significantly decreased IL-1β–induced, TNFα-induced, and oxLDL-induced monocyte migration (data not shown; P<0.001 all versus all agonists).

Discussion

We confirmed the presence of PAR1 and PAR2 in both mouse and human atherosclerotic lesions. Importantly, we found that PAR2 deficiency but not PAR1 deficiency is associated with decreased early- and late-stage atherosclerosis. In the PAR2-deficient mice, we observed less macrophage accumulation, more medial SMC α-actin expression, and reduced type I collagen deposition in

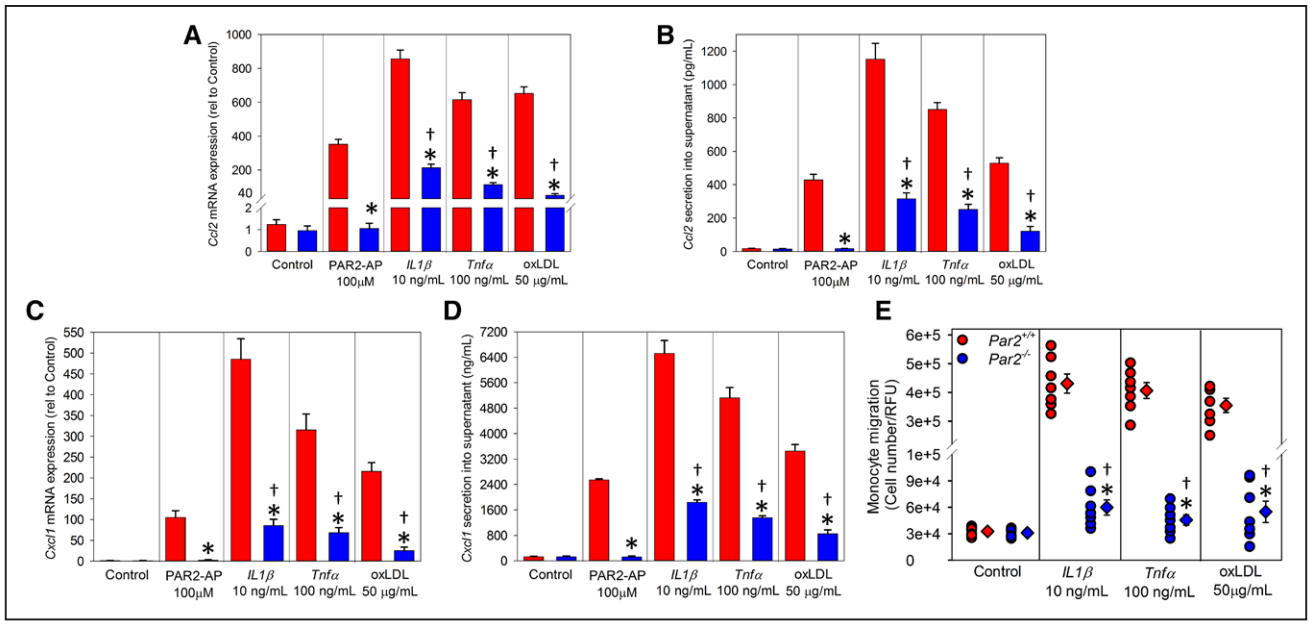

Figure 6. *Ccl2* (chemokine C-C motif ligand 2) and *Cxcl1* expression and secretion is reduced in PAR2 (protease-activated receptor 2)-deficient vascular smooth muscle cells (VSMCs). Mouse aortic arch VSMCs were treated for (**A**, **C**) 4 or (**B**, **D**) 24 h with PAR2-AP (100 μmol/L), *IL-1*β (10 ng/mL), *Tnf*α (100 ng/mL), or the atherogenic ligand oxLDL (oxidized low-density lipoprotein; 50 μg/mL) and mRNA extracted or protein supernatant quantified at respective time points. mRNA was extrapolated to placebo control (LDL, 50 μg/mL). **E**, *Par2*$^{+/+}$ and *Par2*$^{-/-}$ mouse VSMCs were treated with placebo control (LDL, 50 μg/mL), *IL-1*β (10 ng/mL), *Tnf*α (100 ng/mL), or oxLDL (50 μg/mL) for 24 h, and monocytes were added to a transwell Boyden chamber and infiltration/adhesion quantified (n=6 performed in triplicate). Circles represent individual measurements, diamonds are means±SEM. *P<0.001 for comparisons of –/– to +/+ treatment groups; †P<0.05 –/– treatment vs control (2-way ANOVA Tukey post hoc). Red histobars represent *Ldlr*$^{-/-}$/*Par2*$^{+/+}$ and blue histobars represent *Ldlr*$^{-/-}$/*Par2*$^{-/-}$ mice. *Lldr*$^{-/-}$ indicates low-density lipoprotein receptor–deficient mice.

acute and chronic atherosclerosis. Similar results were observed when PAR2 was deleted in nonhematopoietic cells. This effect was not dependent on changes in cholesterol and lipid profiles or its effects on body weight. After the completion of our study, another group reported that PAR2 deficiency was associated with reduced atherosclerosis in the *apoE*$^{-/-}$ model.[43]

PAR2 deficiency was associated with reduced levels of proatherosclerotic chemokines in both the plasma and atherosclerotic lesions. We further demonstrated that treatment of VSMCs with various ligands leads to CCL2 and CXCL1 expression, secretion, and resultant monocyte chemotaxis and that these responses were attenuated in PAR2-deficient cells. These results suggest that nonhematopoietic PAR2 contributes to the formation of atherosclerosis. This suggests that targeting PAR2 may reduce atherosclerosis and stabilize plaques.

Elevated levels of TF, and associated PAR2 activating proteases FVIIa and FXa, are observed in human atherosclerotic plaques and TF expression increases in conjunction with human atherosclerotic progression.[44–47] Importantly, TF is highly expressed in mouse models of atherosclerosis.[48,49] However, heterozygous TF mice (*apoE*$^{-/-}$/*TF*$^{+/-}$) and a deficiency of TF in bone marrow cells (transplanted into an *Ldlr*$^{-/-}$ model) did not attenuate atherosclerosis versus control mice.[50] The contribution of nonhematopoietic cell TF could not be analyzed because of premature death in whole body TF-deficient mice (apoE$^{-/-}$/low TF). However, similar to our study, Zhou et al[51] showed that FXa inhibition with rivaroxaban increased the thickness of protective fibrous caps and decreased aortic CCL2 in advanced lesions of *apoE*$^{-/-}$ mice compared with placebo controls. Moreover, Hara et al[16] found that rivaroxaban administration to *apoE*$^{-/-}$ mice for 20 weeks reduced atherosclerotic burden in the aortic sinus and the aortic root. Similar to our observations with PAR2 atherosclerotic lesions in PAR2-deficient mice, Hara et al[16] demonstrated rivaroxaban treatment resulted in significantly less lipid deposits, macrophage accumulation, more collagen deposition, and a trend toward increased SMC α-actin staining. Both of these publications with rivaroxaban hypothesized the actions of the drug occurred through inhibition of FXa signaling via either PAR2 or PAR1. Given the lack of protection with PAR1 deletion and the similarities between rivaroxaban and our data with PAR2 deletion, we postulate that the protective actions of rivaroxaban on atherosclerosis may occur through inhibition of FXa-PAR2 signaling.

Atherosclerosis progression induces VSMC phenotypic switching toward a proinflammatory phenotype resulting in dedifferentiation and loss of medial SMC α-actin and other selective VSMC-defining contractile markers and subsequent migration into the lesion.[52] We observe that PAR2 deficiency attenuated the loss of medial VSMC α-actin in early lesions while increasing fibrous cap thickness and strength (type I collagen) in advanced lesions. These results are also indicative of a vulnerable plaque phenotype characterized by the presence of large lipid-rich vacuoles covered by thin fibrous caps, decreased type I collagen content, and the accumulation of macrophages,[53] which are all reversed with PAR2 deficiency. When combined with our studies demonstrating that this effect is nonhematopoietic coupled with the role of PAR2 in VSMC mitogenesis, it is likely that PAR2 is mediating atherosclerosis via a VSMC-mediated mechanism. We further speculate that

VSMC PAR2 represents a primary source of CCL2, CXCL1, and resultant monocyte infiltration based on our chimeric and in vitro experiments. Paramount to this speculation is that PAR2 deficiency attenuates VSMC-induced monocyte chemotaxis via oxLDL and the CCL2 master regulators IL-1β and TNFα.[40–42] Future studies will determine the effects of VSMC PAR2 using cell-specific deletions.

We have previously demonstrated that oxLDL can induce the expression of TF via activation of a TLR (toll-like receptor)4–TLR6–CD36 heterotrimeric complex in hyperlipidemia resulting in the activation of coagulation.[54] Interestingly, we show that oxLDL stimulation of PAR2-deficient VSMCs express significantly less CCL2 and have reduced monocyte chemotaxis compared with proficient cells. Previous studies have shown that there is cross-talk between PAR2 and TLR4 where activation of PAR2 can enhance TLR4-dependent signaling.[55] Indeed, PAR2 interacts with TLR4 via cytoplasmic binding to MyD88 (myeloid differentiation factor 88) and MyD88-independent signaling via cytoplasmic binding to TRIF (toll-like receptor adaptor molecule 1) during lipopolysaccharide-induced endotoxemia.[55] Furthermore, TLR4-deficient mice have reduced aortic PAR2 expression and decreased responsiveness to PAR2-AP in macrophages.[55,56] In addition, oxLDL induction of CCL2 expression in VSMCs is reduced in TLR4-deficient cells, which is comparable to our results with PAR2.[57] Given these comparisons and the large literature on TLR4 and atherosclerosis, it is reasonable to speculate that PAR2 and TLR4 may work cooperatively in the formation and progression of atherosclerosis.

Recent studies have demonstrated that PAR2 deficiency (mice) and PAR2 inhibition (rats) lead to reduced weight gain and insulin resistance on a 60% high-fat diet and a metabolic syndrome high carbohydrate and fat diet, respectively.[31,58] Our results confirm that PAR2 deficiency results in attenuated weight gain during 12 weeks of Western diet feeding studies with a 42% high-fat diet. We also demonstrate that PAR2 deficiency attenuated fat accumulation and liver weight in our 12-week study, similar to Badeanlou et al.[31] However, our results suggest that PAR2 expression in both hematopoietic and nonhematopoietic cells may contribute to weight gain, whereas previous studies suggest that only nonhematopoietic PAR2 is involved. Further, we did not detect any differences in glucose metabolism or insulin resistance after 12 weeks of diet. As a result, we did not examine differences during the 24-week time point. The different results from these studies may be because of a variety of factors ranging from different strains of PAR2-deficient mice, variations in dietary fat, room temperatures, and gut microbes present in different mouse facilities. We further demonstrate that these changes in weight had no inadvertent effects on our atherosclerotic outcomes by using a synthetic diet and demonstrating no change in atherosclerosis (despite changes in weight) between the *Par2*$^{+/+}$ recipient groups in our bone marrow transplantation study.

In summary, our study demonstrates that PAR2 but not PAR1 plays a role in atherosclerosis in the *Ldlr*$^{-/-}$ model. Further, atherosclerotic plaques formed in PAR2-deficient mice had a more stable morphology. Mechanistic studies suggest that PAR2 on VSMCs enhances the expression of CCL2, CXCL1, and monocyte recruitment. These findings suggest that PAR2 may be a novel therapeutic target to reduce atherosclerosis and stabilize plaques.

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Disclosures

None.

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Highlights

- Deficiency of PAR2 (protease-activated receptor 2) attenuates the development of acute and chronic atherosclerosis.
- PAR2 deficiency confers a protective plaque phenotype.
- PAR2 deficiency in nonhematopoietic cells is atheroprotective.
- PAR2 regulates the production of MCP-1 (monocyte chemoattractant protein 1) and promotes monocyte chemotaxis.