G Protein–Coupled Receptor Kinase-5 Attenuates Atherosclerosis by Regulating Receptor Tyrosine Kinases and 7-Transmembrane Receptors


Objective—G protein–coupled receptor kinase-5 (GRK5) is a widely expressed Ser/Thr kinase that regulates several atherogenic receptors and may activate or inhibit nuclear factor-κB (NF-κB). This study sought to determine whether and by what mechanisms GRK5 affects atherosclerosis.

Methods and Results—Grk5−/−/Apoe−/− mice developed 50% greater aortic atherosclerosis than Apoe−/− mice and demonstrated greater proliferation of macrophages and smooth muscle cells (SMCs) in atherosclerotic lesions. In Apoe−/− mice, carotid interposition grafts from Grk5−/− mice demonstrated greater upregulation of cell adhesion molecules than grafts from wild-type mice and, subsequently, more atherosclerosis. By comparing Grk5−/− with wild-type cells, we found that GRK5 desensitized 2 key atherogenic receptor tyrosine kinases: the platelet-derived growth factor receptor-β (PDGFR-β) in SMCs, by augmenting ubiquitination/degradation; and the colony-stimulating factor-1 receptor (CSF-1R) in macrophages, by reducing CSF-1-induced tyrosyl phosphorylation. GRK5 activity in monocytes also reduced migration promoted by the 7-transmembrane receptor for monocyte chemoattractant protein-1 CC chemokine receptor-2. Whereas GRK5 diminished NF-κB-dependent gene expression in SMCs and endothelial cells, it had no effect on NF-κB activity in macrophages.

Conclusion—GRK5 attenuates atherosclerosis through multiple cell type-specific mechanisms, including reduction of SMC and endothelial cell NF-κB activity and desensitization of receptor-specific signaling through the monocyte CC chemokine receptor-2, macrophage CSF-1R, and the SMC platelet-derived growth factor receptor-β. (Arterioscler Thromb Vasc Biol. 2012;32:308-316.)

Key Words: cytokines • growth factors • receptors • signal transduction
congenic Grk5−/− mice, we investigated cell type-specific effects of GRK5 on proatherogenic signaling and cellular activities.

Methods

For complete Methods, please see supplemental materials, available online at http://atvb.ahajournals.org.

Mice

All mice were congenic on the C57BL/6 background. Our Grk5−/− mice17 were crossed with Apoe−/− mice (The Jackson Laboratory) to obtain sibling Apoe−/−/Grk5−/− and Apoe−/−/Grk5+/− mice used for generating congenic lines. Primary cell lines were obtained from Grk5−/− and WT mice.

Atherosclerosis Experiments

Male Apoe−/− and Apoe−/−/Grk5−/− mice were fed a Western diet for 12 weeks from age 5 weeks; aortic harvest and atherosclerosis quantitation were performed as described.19,20 To quantitate proliferating cells, we injected mice intraperitoneally 24 hours before euthanization with 5-ethyl-2′-deoxyuridine.21 Orthotopic interposition grafting of the common carotid artery was performed as described.20,22 Carotid endothelial denudation was performed as described.19 Quantitation was performed by observers blinded to specimen identity.

Cell Culture and Assays

Mouse aortic SMCs and ECs were isolated by enzymatic digestion of aortas and passaged as described.18,19 Mδs were derived from bone marrow precursors.23 Monocytes were isolated by negative selection from bone marrow of WT and Grk5−/− mice matched for age and gender, using the EasySep Monocyte Enrichment Kit (StemCell Technologies, Inc). Proliferation, migration, and immunoprecipitation studies were performed as described.19

Results

GRK5 Attenuates Atherosclerosis

To determine the effect of GRK5 activity on atherosclerosis, we first compared aortic atherosclerosis between congenic male Apoe−/− mice that were +/+ or −/− at the Grk5 locus. These mice had equivalent systolic blood pressures and heart rates: 117±7 versus 116±8 mm Hg and 650±40 versus 640±40 beats per minute, respectively (n=10/genotype). After consuming a Western diet for 12 weeks, these mice also had equivalent weights (30±3 and 31±4 g) and serum levels of total cholesterol, high-density lipoprotein cholesterol, and triglycerides: 36±3 versus 39±2, 1.2±0.2 versus 1.0±0.2, and 2.7±0.2 versus 2.7±0.1 mmol/L, respectively, for Grk5+/+/Apoe−/− and Grk5−/−/Apoe−/− mice (n=18/genotype). Nonetheless, cholesteryl ester-rich Sudanophilic lesions occupied 50% more of the aortic intimal surface area in Grk5−/−/Apoe−/− than in Grk5+/+/Apoe−/− mice (P<0.002, Figure 1A). Thus, systemic expression of GRK5 reduced atherosclerosis through mechanisms that appear independent of hemodynamic and lipid parameters. Furthermore, the reduction of atherosclerosis in GRK5-expressing mice correlated with reduction of cell proliferation in the aortic media and atherosclerotic lesions, in both SMC-rich and Mδ-rich regions of the plaques (Figure 1B).

We reasoned that GRK5 would likely mediate antiatherogenic activity in arterial wall cells, because GRK5 alters SMC signaling triggered by the atherogenic SMC PDGFRβ17,24 and because overexpression of GRK5 in ECs can reduce serum-induced 1xBo degradation and NF-κB activity.13 To test this possibility, we performed orthotopic (interposition) grafting of carotid arteries from congenic WT and Grk5−/− mice into congenic Apoe−/− mice. This model of accelerated atherosclerosis produces complex plaques comprising necrotic cores, cholesterol clefts, and SMC-rich fibrous caps and responds to genetic manipulation in a manner concordant with aortic atherosclerosis.20 We first harvested these carotid grafts 2 weeks postoperatively, before Mδs infiltrated the intima, to observe preatherosclerotic changes in SMC and EC protein expression.20 In these 2-week carotid grafts, GRK5 protein expression increased 2.3±0.3-fold compared with cognate unoperated WT carotid arteries (Supplemental Figure III), consistent with the concept that GRK5 activity is antiatherogenic in SMCs and ECs. Moreover, several atherogenic parameters were augmented in Grk5−/− as compared with WT preatherosclerotic grafts. PDGFβR activation was 2-fold greater in Grk5−/− carotids, as assessed by immunofluorescence for the phospho-Tyr1021 PDGFβ (Figure 2)—even though total PDGFβ levels were equivalent to WT (Supplemental Figure III). Greater NF-κB activity manifested in Grk5−/− than in WT carotids, as judged by
increases in intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemotactic protein-1 protein expression (Figure 2). This augmented atherogenic signaling and inflammatory gene expression in Grk5−/− carotids evolved into more extensive atherosclerosis than observed in WT carotid grafts: 6 weeks postoperatively, Grk5−/− carotids had 2-fold larger atheromata than WT carotid grafts (Figure 2). Thus, even when expressed just in arterial wall cells, GRK5 reduced atherogenesis.

**GRK5 Reduces Nonatherosclerotic Neointimal Hyperplasia**

Because GRK5 activity reduced arterial wall contributions to atherosclerosis, we expected that GRK5 would reduce SMC migration and proliferation evoked by inflammatory stimuli in a nonatherogenic setting, too.15 To test this hypothesis, we denuded the endothelium of the common carotid artery in WT and Grk5−/− mice. This procedure produces neointimal hyperplasia comprising only SMCs from the artery itself, as we have shown with green fluorescent protein–transgenic mice.19 Although carotid arteries of carotid grafts had 2-fold larger atheromata than WT carotid grafts: 6 weeks postoperatively, Grk5−/− carotids had 2-fold larger atheromata than WT carotid grafts (Figure 2). Thus, even when expressed just in arterial wall cells, GRK5 reduced atherogenesis.

**Figure 2.** Arterial wall–specific G protein–coupled receptor kinase-5 (GRK5) deficiency increases platelet-derived growth factor receptor-β (PDGFRβ) activation, nuclear factor-κB (NF-κB)–dependent gene expression, and atherosclerosis. Carotid arteries from congenic wild-type (WT) or Grk5−/− mice were placed as orthotopic interposition grafts into congenic Apoe−/− mice. A, Grafts were harvested 2 weeks postoperatively, before intimal macrophages (Mδs) could be identified. Serial frozen sections were stained with hematoxylin/eosin (H&E) or fluorescently immunostained for the indicated proteins and Hoechst 33342 (DNA) (PDGFRβ-pY1021 indicates PDGFRβ autophosphorylated on Tyr1021). B, Protein fluorescence intensity was quantitated as described in Methods, and normalized within each staining group to WT samples to obtain fold greater than control. The means±SE of 4 specimens in each genotype are plotted. Scale bars=50 μm. Compared with control: *P<0.01. C, Sections from carotid grafts harvested 6 weeks postoperatively were stained with a modified connective tissue stain. Scale bars=100 μm. D, Neointimal, medial, and cross sectional area are plotted as means±SE from ≥8 specimens of each genotype. Compared with WT grafts: *P<0.03.

**GRK5 Diminishes NF-κB-Driven Gene Expression in ECs**

Data from our carotid grafts suggest that physiologically expressed GRK5 diminishes the expression of NF-κB–dependent cell adhesion molecules in arterial wall cells (Figure 2). To ascertain the effects of GRK5 on NF-κB activation specifically in ECs, we stimulated Grk5−/− and WT ECs with TNFα and assayed protein from the combined cytoplasmic plus nuclear EC compartments.15 Over a 2-hour time course, IκBα levels were 35±9% lower in Grk5−/− than in WT ECs (P<0.02, Figure 3A), whereas phospho-Ser32/36-IκBα levels (normalized to total IκBα levels) were equivalent in Grk5−/− and WT ECs (Figure 3A). Congruently, after 24 hours, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression were 1.6- to 2.2-fold higher in Grk5−/− than in WT ECs (Figure 3B and 3C)—even though TNFR1 and NF-κB p65 levels were equivalent in Grk5−/− and WT ECs (Figure 3D). Thus, these data support the idea that physiological GRK5 activity reduces cytokine-promoted NF-κB activity in ECs, just as GRK5 overexpression does.15
GRK5 Regulates SMC PDGFRβs by Enhancing Ubiquitination

Our carotid graft and carotid endothelial denudation studies implicated GRK5 in reducing atherogenic SMC activation. To determine more directly whether GRK5 activity reduces atherogenic SMC activation, we assayed primary SMCs from WT and congenic Grk5 mice for migration and proliferation (≥3 lines from each genotype). Proliferation in response to PDGF was 80±30% greater in Grk5−/− than in WT SMCs (Figure 4A). Furthermore, migration in Grk5−/− SMCs was 1.7- to 3-fold greater than that in WT SMCs, in response to platelet-derived growth factor, serum, or the multiple cytokines present in conditioned medium from activated Mφs (Figure 4B). Thus, SMC GRK5 reduces SMC migration and proliferation evoked by atherogenic stimuli, both in vitro (Figure 4) and in vivo (Supplemental Figure IV). Despite this apparent antiproliferative role for GRK5 in SMCs, the proliferation of Grk5−/− and WT ECs was indistinguishable (data not shown).

To discern mechanisms for long-term GRK5-mediated PDGFRβ desensitization pertinent to SMC proliferation and migration, we tested whether GRK5 could augment PDGFRβ ubiquitination, a process that triggers PDGFRβ degradation in the lysosome.28 As Figure 4C demonstrates, WT and Grk5−/− SMCs had indistinguishable levels of ubiquitin and PDGFRβ ubiquitin E3 ligase Cbl. Nonetheless, ubiquitination of the PDGFRβ was 2.6±0.6-fold greater in WT than in Grk5−/− SMCs (P<0.02), even though ubiquitination of the epidermal growth factor receptor was equivalent in the 2 SMC types. Concordantly with these ubiquitination results, degradation of the PDGFRβ occurred more rapidly in WT than in Grk5−/− SMCs (Figure 4C). Thus, in a receptor-specific manner, GRK5 appears to enhance PDGFRβ ubiquitination and degradation in SMCs.

Regulation of Monocyte Migration by GRK5

Because monocyte migration is integral to atherogenesis and because GRK5 does not desensitize CXCR4-dependent lymphocyte migration,2 we tested whether GRK5 affects monocyte migration. Monocytes isolated from the bone marrow of Grk5−/− and WT mice (≥5 each) showed equivalent expression levels of important monocyte chemotactic receptors: the colony-stimulating factor-1 receptor (CSF-1R), a receptor tyrosine kinase, and the CC chemokine receptor-2, the 7 transmembrane receptor for monocyte chemoattractant protein-1 (Figure 5). In response to the protein kinase C activator phorbol ester, Grk5−/− and WT monocytes demonstrated equivalent migration (Figure 5C). However, in response to CSF-1, monocyte chemoattractant protein-1, or the ≥28 cytokines present in conditioned medium from our activated Mφs,20 migration was 2.0±0.3-fold greater in Grk5−/− than in WT monocytes (Figure 5C). Thus, GRK5 desensitizes the monocyte migratory response triggered by the CSF-1R and CCR2 in a receptor-specific fashion. Nevertheless, the prevalence of monocytes among peripheral blood leukocytes was equivalent in Grk5−/−/Apoe−/− and Grk5+/+/Apoe−/− mice consuming a Western diet for 4 weeks (14±4%, n=4/genotype).
Pathway-Specific Regulation of Mφ TLR4 and TNFRI by GRK5

Thus far, we have seen pleiotropic antiatherogenic activities of GRK5 in SMCs, ECs, and monocytes. However, GRK5 has been reported to mediate potentially proatherogenic signaling in Mφs by augmenting TLR4- or TNFRI-promoted NF-κB activity.1,14 To investigate this further, we compared NF-κB activation between 2 types of WT and Grk5 KO Mφs: bone marrow–derived (5 lines/genotype) and thioglycollate-elicited (from the peritoneum, 2 lines/genotype); both Mφs demonstrated equivalent NF-κB activity, even though GRK5 decreased NF-κB activity, even though GRK5 decreased NF-κB activity in SMCs and ECs (Figures 2 and 3).

Although NF-κB signaling was equivalent in WT and Grk5 KO Mφs, extracellular signal-regulated kinase (ERK) signaling was not. Mφ ERK1/2 signaling elicited via TLR4 or TNFRI was ~50% greater in Grk5 KO than in WT Mφs (P<0.05), even though Mφ ERK1/2 signaling elicited by phorbol ester was equivalent in Grk5 KO and WT Mφs (Supplemental Figures VII and VIII). Thus, Mφ Grk5 reduced TLR4- and TNFRI-induced ERK1/2 signaling in a receptor-specific manner.

GRK5 Reduces Mφ CSF-1R Activation

Because Mφ proliferation is augmented in Grk5 KO atheroma (Figure 1) and because signaling through the CSF-1R is...
critical for Mφ proliferation,\textsuperscript{31} we tested whether GRK5 affected CSF-1R activation. Although CSF-1R density was equivalent in WT and Grk5\textsuperscript{-/-} Mφs, agonist-induced auto-phosphorylation of the CSF-1R was 50\% greater in Grk5\textsuperscript{-/-} than in WT Mφs (P<0.05, Figure 6 and data not shown). Congruently, CSF-1R-1R-activated Akt activation was 50\% greater (n=5, P<0.03) and ERK activation was 34\% greater (n=4, P<0.05) in Grk5\textsuperscript{-/-} than in WT Mφs, even though ERK activation evoked by phorbol ester was equivalent among WT and Grk5\textsuperscript{-/-} Mφs (Figure 6C and Supplemental Figure VIIIC). Concordant data among 5 pairs of independently isolated WT and Grk5\textsuperscript{-/-} Mφ lines strongly supported the inference that changes in CSF-1R activity were GRK5-dependent.

GRK5 activity reduces receptor tyrosine kinase autophosphorylation of both the CSF-1R (Figure 6) and the GRK5-dependent. However, GRK5 appeared to achieve different effects on agonist-induced ubiquitination of these 2 receptors, even though the ubiquitin E3 ligase Cbl plays a major role in ubiquitination of both receptors.\textsuperscript{32,33} Whereas GRK5 enhanced PDGFRβ ubiquitination in SMCs (Figure 4C), it did not affect CSF-1R ubiquitination in Mφs (Figure 6A). Despite failing to augment CSF-1R ubiquitination, GRK5 appeared to mediate long-term Mφ CSF-1R desensitization: CSF-1-induced proliferation was 31\% greater in Grk5\textsuperscript{-/-} than in WT Mφs over 3 days (P<0.02, Figure 6D), congruent with the 67\% increased prevalence of S-phase Mφs observed in Grk5\textsuperscript{-/-}/apo-e\textsuperscript{-/-} atheromata (Figure 1). Thus, GRK5 appears to use overlapping but distinct mechanisms of regulation for the related receptor tyrosine kinases PDGFRβ and CSF-1R.

Discussion

Our data demonstrate the novel finding that GRK5 activity attenuates atherosclerosis, and that it does so through distinct antiatherogenic mechanisms in ECs, SMCs, monocytes, and Mφs. These cell-specific mechanisms encompass diverse signaling systems, including the receptor tyrosine kinases CSF-1R and PDGFRβ, the 7-transmembrane receptor CCR2, the innate immunity receptors TLR4 and TNFR1, and the transcription factor NF-κB. Thus, despite data from overexpression and model cell systems suggesting the possibility that GRK5 could mediate proatherogenic activities,\textsuperscript{9,11,13,14} net physiological GRK5 activity clearly appears to be antiatherogenic.

Because GRK5-mediated phosphorylation of 7-transmembrane receptors promotes receptor/β-arrestin association,\textsuperscript{1} it may seem paradoxical that whereas GRK5 activity is antiatherogenic, β-arrestin2 activity is proatherogenic.\textsuperscript{19} However, at least 2 possibilities may help reconcile these findings. First, GRK5-mediated receptor phosphorylation may promote the association of receptors with β-arrestin1 more so than with β-arrestin2—and because β-arrestin1 can exert effects antagonistic to β-arrestin2 (particularly in SMCs),\textsuperscript{19} the global effect of GRK5-mediated phosphorylation may result from a balance of β-arrestin isoform activity that favors β-arrestin1. Second, even if it does promote atherogenic β-arrestin2 activity, SMC GRK5 may still achieve overall antiatherogenic effects by diminishing atherogenic PDGFRβ signaling,\textsuperscript{17,18} which is not affected by β-arrestin2 in SMCs\textsuperscript{19} even though the PDGFRβ is phosphorylated by GRK5 in SMCs.\textsuperscript{17} Our study reveals a novel mechanism by which GRK5 alters PDGFRβ signaling: enhancing PDGFRβ ubiquitination, which enhances PDGFRβ degradation. GRK5-mediated ser1 phosphorylation of the PDGFRβ may trigger ubiquitin E3 ligase activity in a manner analogous to the Ser/Thr kinase 1xB β-kinase-β, with which GRK5 (under specific conditions) appears to share the substrate 1xBα.\textsuperscript{13}

Our work is the first to demonstrate that GRK5 regulates the CSF-1R, a receptor tyrosine kinase belonging to the PDGFR subfamily,\textsuperscript{31} through mechanisms distinct, in part,
Figure 6. G protein–coupled receptor kinase-5 (GRK5) activity in macrophages (Mφs) reduces colony-stimulating factor-1 receptor (CSF-1R)–induced signaling and proliferation. A to C, Quiescent bone marrow–derived Mφs were exposed to serum-free medium lacking (−) or containing (+) 1.7 nmol/L CSF-1 for 10 minutes (37°C) and lysed. A, Mφ lysates were subjected to immunoprecipitation (IP) with anti-CSF-1R or nonimmune (−) IgG; immunoprecipitates were subjected to parallel SDS-PAGE and serial immunoblotting (IB) for CSF-1R and then either phospho-Tyr (pY) or ubiquitin (Ubiq). B, Ubiquitin and pY band densities were normalized to cognate CSF-1R band densities; these ratios were normalized to CSF-1-stimulated wild-type (WT) Mφs in each experiment, to obtain percentage of WT, plotted as means ± SE of 4 experiments with independently isolated pairs of WT and Grk5−/− Mφ lines. Compared with WT; *P < 0.05. C, Mφ lysates (40 μg protein/lane) were immunoblotted for the indicated proteins. Bands for pAkt and pERK1/2 were normalized to the Grk2 loading control within each experiment; ratios were normalized to CSF-1-stimulated WT Mφs for quantitation. Results from a single experiment represent 3 performed with independently isolated pairs of WT and Grk5−/− Mφ lines. Blots probed with isotype control primary IgG yielded no corresponding bands (not shown). D, Bone marrow–derived Mφs from WT and Grk5−/− mice were cultured in the presence of CSF-1, and the number of Mφs per well were determined at the indicated times. Shown are the means ± SE of 4 experiments performed with 4 independently isolated pairs of WT and Grk5−/− Mφ lines. Compared with WT growth curve: *P < 0.02. ERK indicates extracellular signal-regulated kinase.

from those that obtain with the PDGFRβ. Whereas GRK5 reduces Tyr autophosphorylation of both receptors and signaling downstream of both receptors, it enhances ubiquitination of only the PDGFRβ (Figures 4 and 6).17 This receptor-specific difference in agonist-promoted ubiquitination is particularly notable because Cbl is believed to be a major E3 ubiquitin ligase acting on both receptors.32,33 Whereas GRK5 reduces SMC and Mφ proliferation promoted through the PDGFRβ and CSF-1R, respectively, it reduces ERK signaling evoked only by the CSF-1R (Figures 4 and 6).17 The CSF-1R is known to be phosphorylated on Ser residues,31 but it remains to be established whether GRK5 mediates this phosphorylation.

Our carotid transplant data support the view that GRK5 activity in SMCs and ECs reduces atherogenesis. That SMC proteins can exert antiatherogenic activity has been demonstrated in only a limited number of instances. SMC-specific deletion of peroxisome proliferator–activated receptor-γ also augments atherosclerosis in Ldlr−/− mice.34 In a similar fashion, our surgical chimeric mouse carotid graft model shows that SMC GRK5 reduces not only PDGFRβ activation but also NF-κB activation (Figure 2). The mechanism of GRK5–mediated inhibition of NF-κB activity may be the ability of GRK5 to bind to IkBα and prevent its degradation, as demonstrated in overexpression systems.15

Although we found that GRK5 reduces NF-κB activity in ECs and SMCs, we found that GRK5 has no effect on NF-κB activity in Mφs. Our Mφ findings contrast with those obtained by others,14 who found that GRK5 increased Mφ NF-κB activity—but in Mφs from Grk5−/− and WT mice that were backcrossed only 5 generations to C57BL/6 and used as nonlittermates14 (instead of our C57BL/6-congenic mice). Thus, interstrain differences in loci other than Grk5 could well have confounded these previous studies. In purified protein preparations, GRK5 can phosphorylate IκBα on the Ser32/Ser36 residues targeted by IκB kinase-β11; however, phospholipids that normally enhance GRK5 activity1
such a possibility is suggested by studies of the human increased GRK5 activity reduce atherosclerosis in humans? have found that endogenous GRK5 upregulates in SMCs and appear to do so in the context of atherogenesis. GRK5 may contribute to NF-κB phosphorylation of GRK5-mediated desensitization of the β1-adrenergic receptor than GRK5-Gln41 variant, the GRK5-Gln41 variant has been associated with reduced mortality in humans with chronic heart failure and with reduced adverse cardiovascular events in a case-control study of hypertensive humans with coronary artery disease. Whether these associations reflect a causal inverse relationship between GRK5 activity and mortality remains uncertain. However, if a causal relationship does exist, the most plausible mechanism would involve GRK5-mediated desensitization of the β1-adrenergic receptor, hyperstimulation of which can engender sudden cardiac death in subjects who experience chronic heart failure or acute myocardial infarction. It remains to be determined whether enhancing GRK5 expression or activity to mitigate atherosclerosis will prove practicable.

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Disclosures

None.

References


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Expanded Methods

Mice
All mice were congenic (≥10 generations back-crossed) on the C57BL/6 background. Our previously described Grk5−/− mice1 were crossed with Apoe−/− mice (Jackson Labs, stock #002052), ultimately to obtain sibling Apoe−/−/Grk5−/− and Apoe−/−/Grk5+/+ (“Apoe−/−”) mice used for generating congenic mouse lines. Primary cell lines were obtained from Grk5−/− and WT mice. All mutant mice were back-crossed to C57BL/6J WT (Jackson Labs, stock #000664) every 5 generations, to minimize genetic drift. All animal experiments complied with Duke University Institutional Animal Care and Use Committee guidelines.

Blood pressure was measured by tail cuff plethysmography with a MC4000 Blood Pressure Analysis System (Hatteras Instruments).

Because Grk5−/− mice were made originally by mating CMV-Cre mice with mice in which exons 7 and 8 of Grk5 had been flanked by loxP sites,2 it has been considered possible that exons 1-6 could produce a transcript encoding GRK5 amino acids 1-178 followed by an out-of-frame splice of exon 9 that would add 16 missense amino acids to GRK5(1-178) before termination. To test this possibility, we immunoblotted extracts of SMCs from WT and Grk5−/− mice with rabbit polyclonal IgG raised against GRK5 amino acids 94-157 (Santa Cruz Biotechnology, H-64). As seen in Supplemental Figure I, no protein is detected in the molecular weight range expected for GRK5(1-178), with or without an additional 16 amino acids (20.8-22.7 kDa). Confirming the absence of this theoretical GRK5 N-terminal peptide is important because of the IkBα-stabilizing properties described for the GRK5 N-terminal domain (amino acids 50-176).3

Atherosclerosis Experiments
With male Apoe−/− and Apoe−/−/Grk5−/− mice, we accelerated aortic atherosclerosis by administering a Western diet for 12 wk from age 5 wk. To facilitate examination of S phase cells in aortas, mice were injected intraperitoneally 24 h prior to sacrifice with 100 μg of the thymidine analogue 5-ethynyl-2′-deoxyuridine (EdU).4 Mice were sacrificed by CO2 asphyxia, perfused with PBS via the left ventricle (after right atrial incision), fixed for 3 min by perfusion with 10% formalin in PBS, and then stained by perfusion with Sudan IV (0.5% w/v in 15% H2O/35% ethanol/50% acetone).5 Aortas were then excised from where they emerged from the aorta to the iliac bifurcation, and pinned for en face evaluation as described.5,6 Pinned aortas were photographed at 20× with a Nikon SMZ 800 stereomicroscope and a DS-Fi1 camera, using Nikon image-concatenating software (NIS Elements™). The percentage of aortic intimal area occupied by Sudan IV-positive lesions was determined by pixel thresholding with NIH Image J, by observers blinded to specimen identity;5 aortic tracings excluded the branches off the arch.

Aortic root samples were embedded in OCT and frozen along with atrial and basal ventricular myocardium; 5-μm sections were taken proximally to distally, at 100-μm intervals from the appearance of the valve leaflets.5,6 Grk5−/− and Grk5+/+ specimens were analyzed at comparable distances from the appearance of the valve leaflets.5,6

Orthotopic interposition grafting of the common carotid artery was also performed as we have described5,8: WT and Grk5−/− mice matched for age (9-18 wk) and gender served as donors of carotid arteries transplanted into Apoe−/− hosts matched for age (11 ± 2 wk) and gender. Anesthesia was achieved with pentobarbital (50 mg/kg, i.p.). Right common carotid arteries (~8 mm long) of donor mice were harvested, flushed with and stored in lactated Ringer’s solution containing heparin (50 U/ml). The graft recipient mouse was injected with 10 units of heparin s.c. after anesthesia was induced. The right side of the neck was cleansed and shaved, then incised and dissected to expose the common carotid artery, vagus nerve, and jugular vein. The carotid artery was clamped proximally and distally, and the carotid graft was
anastomosed (end to side) with 11-0 nylon suture to the proximal and distal extents of the common carotid, at the sites of arteriotomies created with a 27G needle. We used 10-0 nylon suture to ligate the graft recipient’s common carotid artery segment that intervened between the proximal and distal graft anastomoses, and then cut this segment in its midportion; consequently, all carotid blood flow would traverse the (interposition) carotid graft. Next, proximal and distal clamps were released. After ensuring good hemostasis, the surgical field was closed in a single layer of 5-0 nylon suture. All mice were fed low-fat Purina Rodent Chow 5058, and were sacrificed 2 or 6 wk post-operatively. Grafts were harvested after perfusion with PBS to clear erythrocytes, without (OCT-embedded specimens) or with (paraffin-embedded specimens) additional perfusion-fixation with PBS/3.7% formaldehyde at 100 mm Hg.7 Sections for analyses (5-µm) were taken 2 mm proximal to the suture line for the distal graft anastomosis.

The carotid graft model produces atherosclerosis that is accelerated compared with aortic atherosclerosis, but nonetheless recapitulates the sequence of molecular and cellular changes observed in the Apoe−/− mouse brachiocephalic artery.7 Among the atherogenic commonalities between the native brachiocephalic artery and our carotid grafts is that atherosclerotic lesion fibrous cap SMCs originate only from the artery wall.9, 10 To demonstrate this characteristic of carotid graft atherosclerosis, we used apoE as a marker for (Apoe+/+) carotid-graft derived cells, and co-localized apoE immunofluorescence with SMC α-actin immunofluorescence (Supplemental Figure II).

Quantitation of carotid lesion cross sections was performed by observers blinded to specimen identity, using planimetry with NIH Image J as we have reported.7

Serum Lipid Measurements
Right ventricular blood was removed from each mouse after CO2 asphyxiation, allowed to coagulate at room temperature (20 min), and centrifuged (12,000 × g, 10 min); serum was stored at -80 °C until all samples could be run together. Total serum cholesterol was measured using the Cholesterol E Assay Kit (Wako Chemicals). Triglyceride levels were determined with a kit from Stanbio, Inc., according to the manufacturer’s protocol. High density lipoprotein (HDL) cholesterol was determined with the L-Type HDL-cholesterol Assay (Wako Chemicals, http://www.wakodiagnostics.com/r_hdl_cholesterol.html), according to the manufacturer’s protocol.

Histology/Immunofluorescence Microscopy
Perfusion-fixed specimens were paraffin-embedded, sliced and stained as we previously described.7 For immunofluorescence studies, carotid grafts were embedded in OCT compound and sliced at 5 µm. The sources of IgGs against the following antigens were obtained from the following sources1, 7: SMC actin (1A4, Cy3-conjugated mouse IgG), from Sigma; PDGFRβ (rabbit IgG, sc-432), PDGFRβ phosphorylated on Tyr-1021 (goat IgG, sc-12909), ICAM-1 (rabbit IgG, sc-7869), VCAM-1 (rabbit IgG, sc-8304), GRK5 (rabbit IgG, sc-565), and apolipoprotein E (goat IgG, sc-6385), from Santa Cruz Biotechnology, Inc; MCP-1 (rabbit IgG, #500-P113), from PeproTech, Inc. EdU incorporated into DNA of proliferating cells was detected with Alexa® 448-azide (Invitrogen), according to the manufacturer’s instructions.

Immunofluorescence was performed and quantitated by observers blinded to specimen identity, as we have described,5, 11, 12 using IgGs cited above. Nuclei were counterstained with Hoechst 33342 (10 µg/ml) during secondary antibody incubation. Specimens stained with single fluorophores yielded fluorescence only with the appropriate narrow band-pass fluorescence filter cube (Chroma, Inc.); that is, there was no “bleed-through” into other fluorescence channels. Images with individual fluorophores were merged with SPOT™ software.

To quantitate protein expression within arterial tissue, specimens from WT and Grk5−/− groups were stained and imaged simultaneously, batch-wise. Identical exposure times and incident light intensities were used to visualize each specimen, with a CCD camera. Specific staining was obtained by adjusting image intensities so that negative control specimens yielded no
immunofluorescence, or by subtracting mean fluorescence values of negative control specimens from those incubated with the relevant first antibody, with NIH Image J.  

Intensities thus derived were normalized to cognate fluorescence intensities obtained from nuclear fluorescent (Hoechst) staining of the same arterial section (as a read-out for cellularity). These ratios were averaged among all specimens within each staining group. Thus, GRK5, PDGFRβ, or phospho-PDGFRβ immunofluorescence was compared across groups in a manner that accounted for the cellularity of each specimen.

Confocal microscopy was performed as we described. Paraffin sections of 4 μm were visualized on a Zeiss LSM 510 META confocal microscope, after staining with goat anti-ApoE or non-immune goat IgG, followed by anti-goat IgG/Alexa Fluor® 488 (Invitrogen) along with Cy3-conjugated anti-SMC-α-Actin (1A4, Sigma) and DRAQ5™ (to stain nuclear DNA [Alexis Biochemicals]). Confocal images were acquired such that each channel was scanned successively using filter sets for multitrack sequential excitation (488, 568, and 633 nm) and emission (515-540 nm, Alexa® 488; 585-615 nm, Cy3; 650 nm, DRAQ5). To facilitate reliable co-localization studies, a 40× oil objective was used, and a pin-hole for each channel was matched for airy units to set optical slice thickness at 1 μm. Final processing of images was performed with Adobe Photoshop CS2 software; the brightness and contrast were adjusted for entire images and to the same extent for ApoE and control IgG images. Co-localization was examined as reported previously.

**Carotid Endothelial Denudation**

These studies were performed as we described previously, on mice anesthetized with pentobarbital (50 mg/kg), with a 0.36-mm-diameter coronary guidewire (Cordis) using a modification of the method of Lindner et al. We created a midline neck incision extending from the lower mandible to the sternum, and then loosely tied 10-0 “control sutures” at the proximal portion of the common and internal carotid arteries and at the mid-portion of the external carotid artery, to prevent blood flow without engendering arterial wall damage. We next extended a ~0.5 mm external carotid arteriotomy from the control suture to the carotid artery bifurcation, and flushed the common carotid with PBS. To denude the endothelium, we traversed the length of the common carotid three times with a 0.36-mm flexible angioplasty guidewire (Johnson and Johnson). After removing the wire, we ligated the external carotid artery proximally after flushing the common carotid with blood, and then completely released the control sutures.

At 2-4 weeks postoperatively, mice were anesthetized, as above, for harvest of the injured artery. The carotid artery was exposed through the previous surgical incision, and the thoracic cavity was opened. The right atrium was incised, and the mice were perfused with PBS via the left ventricle. For 4-wk specimens, the vasculature was subsequently perfusion-fixed in situ with 10% formalin in PBS for 20 minutes at a constant pressure of 100 mm Hg. The 2-wk specimens were incubated in 30% sucrose/PBS overnight and embedded in OCT compound. To facilitate orientation of the carotid artery during embedding, we excised the aortic arch along with both carotids extending distally to the carotid bifurcations. After excision, specimens for morphometry were incubated in 10% formalin/PBS for 24 hours, and then embedded in paraffin. Remnants of the suture used for ligation of the external carotid were employed to orient the specimen at the time of embedding, and as a landmark for sectioning. Carotid artery sections for morphometric analysis (paraffin-embedded) or immunofluorescence (OCT-embedded) were obtained 1 mm proximal to the suture used to ligate the external carotid.

**Cell Culture**

Mouse aortic SMCs were isolated by enzymatic digestion of aortas and passaged as we have described. Mouse aortas were stripped of adventitia and endothelial cells, and then digested at 37 °C for 1.5 h in PBS containing the following reagents (all from Sigma): collagenase II (1 mg/ml), elastase type III (0.125 mg/ml), soybean trypsin inhibitor (0.375 mg/ml), and bovine serum albumin (fraction V, 2 mg/ml) in DMEM. Released SMCs were cultured in DMEM with 20% FBS, 1% anti-mycotic/antibiotic (Invitrogen) and 1% Mycoplasma Removal Agent (MP Biomedicals) for one wk, and then in DMEM.
with 10% FBS, 1% penicillin/streptomycin thereafter. SMCs were split at 1:4, and used during passages 3–7. These primary SMCs demonstrated >95% prevalence of SMC α-actin expression by immunofluorescence.

Mouse aortic endothelial cells (ECs) were also isolated by enzymatic digestion and passaged as we described. Mouse thoracic aortas were isolated and perfused in situ with PBS containing 1000 U/ml heparin. Subsequently, the lumen of the aorta was flushed with 5 ml of 20% FBS/DMEM, and ECs were pelleted at 200 × g. ECs were then plated onto collagen type I-coated dishes and incubated at 37 °C in 20% FBS/DMEM for 2 h, after which non-adherent cells were removed. The adherent ECs were subsequently cultured in “EC growth medium”: DMEM containing 20% FBS (heat-inactivated), 1× L-glutamine, 1× non-essential amino acids, 1× sodium pyruvate (Invitrogen), 25 mmol/L Hepes (pH 7.4), 100 µg/ml heparin, 100 µg/ml EC growth supplement (Sigma), 1× antibiotic/antimycotic (Invitrogen), and 10 µg/ml ciprofloxacin (Sigma). Amphotericin and ciprofloxacin were removed from the medium 1 wk after EC isolation. The purity of EC preparations was assessed by the prevalence of factor VIII immunofluorescence, which was ≥ 90%.

Macrophages (Mφs) were derived from bone marrow precursors, from the peritoneum of mice injected 4 days earlier with 1 ml of 4% thioglycollate or (for studies of activated Mφs) from the peritoneum of mice injected 3 days earlier with 100 µg of concanavalin A in 0.5 ml PBS. To obtain bone marrow-derived Mφs, bone marrow was flushed from femurs and tibiae of Grk5-/- and WT mice matched for age and gender, and filtered through 70-µm mesh. Bone marrow-derived cells were then plated on petri dishes that were not tissue-culture-treated, in “Mφ growth medium”: RPMI 1640 with 10% heat-inactivated FBS, 15% (vol/vol) conditioned medium from L929 cells (ATCC), 100 U/ml penicillin and 100 µg/ml streptomycin. Bone marrow precursor cells that adhered within 3 days were cultured an additional 3 days in Mφ growth medium, and assayed as Mφs (see below). Bone marrow precursor cells that failed to adhere after the initial 3 days were transferred to new petri dishes, cultured in Mφ growth medium for 3 additional days, and then assayed as Mφs. Bone marrow precursor cells that failed to adhere after a total of 6 days in Mφ growth medium were discarded. Mφ growth medium was changed every 3 days, and Mφs were plated for assays after 7 days in culture. By immunofluorescence microscopy, all Mφs (but no SMCs) stained for the Mφ-specific antigen Mac3. The day before assays, Mφs adherent to petri dishes were detached by Cellstripper™ non-enzymatic cell dissociation solution (Mediatech, Inc.) and plated into tissue-culture-treated plastic dishes. Mφs were discarded after the third passage of 1:2. Thioglycollate-elicited peritoneal Mφs were cultured overnight and assayed the next day.

To obtain Mφ-conditioned medium from activated peritoneal Mφs, we washed confluent Mφs twice with serum-free medium, incubated Mφs in serum-free medium for 18 hr, and then filtered this “conditioned” medium (0.2 µm pore); the conditioned medium was frozen in aliquots at -80 °C before use in experiments.

**Endothelial Cell Assays**

For NFκB activity assays, ECs were serum-starved overnight (16 h) and then challenged ±TNF and ±LPS in serum-free medium for 10 min-24 h, after which EC lysates were prepared with 1× SDS sample buffer, so as to solubilize both cytoplasmic and nuclear proteins. Immunoblotting for the indicated proteins was performed as described, with the following additional IgGs (specified by antigen): TNFR1 (sc-7895), tubulin (sc-9104), p65 (sc-109), rabbit polyclonal IgG from Santa Cruz Biotechnology; IκBα (rabbit polyclonal) and phospho-Ser32/36-IκBα (#9246S, mouse mAb) from Cell Signaling.

For proliferation assays, ECs were plated on “day 0” at 5×10^3 cell/well in 96-well plates coated with collagen I, and allowed to attach overnight in EC growth medium: DMEM containing 20% heat-inactivated FBS, 1× L-glutamine, 1× non-essential amino acids, 1× sodium pyruvate, 25 mM Hepes (pH 7.4), 100 µg/ml heparin, 100 µg/ml EC growth supplement and 1% penicillin/streptomycin (all from...
Invitrogen). Incubations were terminated 2, 4, and 8 days after serum deprivation; medium was aspirated, ECs were washed with PBS, and then ECs were stained with 0.5% (w/v) crystal violet/5% (v/v) ethanol for 60 min. After extensive washing, ECs were dried completely, and EC-adsorbed crystal violet was dissolved in 40% acetic acid. The absorbance of each well was then read at 562 nm in sextuplicate, and corrected for the absorbance obtained in wells in which no ECs had been plated. EC protein was quantitated with a standard curve created from serially diluted, genotype-specific ECs plated in parallel. The relationship between OD562 and EC number was linear from 0 to 5×10³ ECs/well (data not shown).

SMC Assays
Proliferation and migration assays were performed as we described previously. PDGFRβ and EGFR immunoprecipitation studies (for PDGFRβ ubiquitination studies) were performed on serum-starved SMCs, as we have described, with the following IgGs from Santa Cruz Biotechnology, Inc.: anti-PDGFRβ IgG from goat (sc-1627) and rabbit (sc-432); anti-EGFR IgG from goat (sc-03G) and rabbit (sc-03R); anti-ubiquitin mouse IgG (sc-8017); anti-Cbl rabbit IgG (sc-170).

Monocyte Assays
Monocytes were isolated from bone marrow of WT and Grk5−/− mice matched for age and gender. Bone marrow cells were collected in PBS/2% FBS/1 mM EDTA (pH 7.4) and washed; erythrocytes were lysed in 0.15 M NH₄Cl/0.1 mM EDTA buffered with KHCO₃ to a final pH of 7.4. Non-monocytes were removed with the EasySep Monocyte Enrichment Kit (StemCell Technologies, Inc) with magnetic beads, according to the manufacturer’s instructions, and monocytes were decanted. Monocyte phenotype was assessed by cell surface immunofluorescence and flow cytometry, with the following IgGs (BioLegend, Inc.): allophycocyanin (APC)-conjugated anti-CD90.2 (clone 30-H12), anti-B220 (clone RA3-6B2), anti-CD49b (DX5), anti-NK1.1 (PK136), and anti-Ly-6G (clone 1A8); phycoerythrin (PE)-conjugated anti-CD11b (M1/70). All IgGs were used at a concentration of 2 μg/ml. Monocytes were defined as CD11b+, CD90.2lo, B220lo, CD49blo, NK1.1lo, and Ly-GGlo. Purity of monocytes collected in this manner was 84% ± 4%.

Purification of monocytes was performed using Igs from Santa Cruz Biotechnology, Inc. for CSF-1R (rabbit anti-CSF-1R, sc-692) and for CCR2 (goat anti-CCR2, sc-31564). Cognate non-immune IgG was used in parallel immunoblots.

Monocyte migration was assayed in a 96-well modified Boyden chamber with 5-μm pores (Neuroprobe, Inc.), according to the manufacturer’s instructions, with serum-free medium containing either vehicle or one of the following: 1.4 nmol/L CSF-1 (Millipore), 10 nmol/L MCP-1 (R & D Systems), 100 nmol/L tetradecanoyl phorbol acetate, or Mφ-conditioned serum-free medium. Monocytes (25 μl at 1×10⁶ per ml) were added to the top of each filter, and allowed to migrate for 2.5 h (37 °C). In parallel, serially diluted monocyte suspensions were placed in separate wells, for the purpose of creating genotype-specific standard curves for quantitation (below). After migration, 15-μl aliquots of medium from each bottom chamber were transferred to a white 96-well plate (Corning, Inc.), along with 10 μL of 25% FBS in RPMI, and incubated for 2 minutes (RT). To quantitate monocytes in each well, 25 μl of CellTiter Glo reagent (Promega) was added to each well; plates were shaken (RT) for 2 minutes, and allowed to equilibrate for 10 minutes (RT). Luminescence was read on a Mithras LB 940 BRET reader (Berthold Technologies). Luminescence was converted into monocytes/well by using data from genotype-specific monocyte standard curves. All data were reported as stimulated/unstimulated (fold/basal). Assays were performed in quintuplicate.

To determine peripheral blood monocyte Ly6C expression levels, APOE−/− and APOE−/−/Grk5−/− mice were fed a Western diet for 30 days, and then sacrificed for harvest of blood and bone marrow. Blood buffy coat and bone marrow cells were depleted of erythrocytes by incubation in 0.15 mol/L NH₄Cl, with subsequent pelleting and washing. Leukocytes were labeled with IgGs from Biolegend, Inc.: APC-labeled “lineage” IgGs (anti-CD90, anti-B220, anti-NK1.1, anti-CD49b, anti-Ly-6G), as well as PE-labeled anti-CD11b and FITC-labeled anti-Ly-6C. Cells incubated with single fluorophore-labeled IgGs
were used to determine nonspecific fluorescence, which was subtracted from total fluorescence in each fluorophore channel. The immunofluorescence of Ly6C was analyzed in a binary fashion, as “high” and “low,” only on cells that scored CD11b<sup>hi</sup>Lin<sup>lo</sup> (i.e., monocytes<sup>22</sup>). The prevalence of monocytes among peripheral blood leukocytes was equivalent in Grk<sup>5<sup>-/-</sup></sup>/Apoe<sup>-/-</sup> and Grk<sup>5<sup>+/+</sup></sup>/Apoe<sup>-/-</sup> mice (14±4%). The prevalence of Ly6C<sup>hi</sup> monocytes in bone marrow samples was 95±2% (n = 4), as expected<sup>23, 24</sup>—in both Grk<sup>5<sup>-/-</sup></sup> and Grk<sup>5<sup>+/+</sup></sup> mice.

Peripheral blood monocytes differed between Western diet-fed Grk<sup>5<sup>-/-</sup></sup>/Apoe<sup>-/-</sup> and Grk<sup>5<sup>+/+</sup></sup>/Apoe<sup>-/-</sup> mice: Ly6C<sup>hi</sup> monocytes constituted 61±10% and 56±10% of total peripheral blood monocytes in Grk<sup>5<sup>-/-</sup></sup> and Grk<sup>5<sup>+/+</sup></sup> mice, respectively (p < 0.05, paired analysis; n = 4 mice/genotype).

MΦ Assays
Prior to signaling assays, bone marrow-derived MΦs were serum-starved for 16 h. Immediately after harvest, thioglycollate-elicited peritoneal macrophages were cultured overnight in MΦ growth medium, then serum-starved for 4 hr prior to stimulation, following the protocol of Patial et al.<sup>20</sup> MΦs were stimulated for the indicated times with lipopolysaccharide (LPS) from <i>Salmonella enteritica</i> (Sigma L5886) or <i>E. coli</i> 0111:B4 (Sigma L2630<sup>20</sup>), both of which yielded equivalent results at concentrations of 1-10 μg/ml (not shown). In parallel, MΦs were stimulated with murine TNF (Millipore) for the indicated times. MΦs were then fixed/permeabilized and immunostained for NFκB p65 (see Figure 3).

Alternatively, MΦs were solubilized and immunoblotted, as we have reported;<sup>25</sup> total ERK1/2 and phospho-ERK1/2 (rabbit IgGs, Cell Signaling #9102 and #9101, respectively) were immunoblotted on parallel gels/membranes, because our anti-phospho-ERK1/2 cannot be efficiently stripped from the nitrocellulose.<sup>25</sup> Immunoblotting for other proteins used IgGs from the following sources: MIP-2 (sc-1388), rabbit polyclonal IgG from Santa Cruz Biotechnology; IKKβ and TLR4, rabbit poly- or monoclonal IgG from Cell Signaling.

Human CSF-1 was from Millipore. Anti-CSF-1-R IgGs from rabbit (immunoprecipitation) and goat (immunoblotting) were from Santa Cruz Biotechnology. CSF-1R immunoprecipitation assays were performed as we previously described for the PDGFR<sup>β</sup>.<sup>1</sup> Rabbit IgGs against total and phospho-Akt (Ser473) were from Cell Signaling Technology.

For proliferation assays, MΦs were plated at 2.5×10<sup>4</sup>/well in 96-well plates, and quantitated at the indicated time points by staining with 0.5% (w/v) crystal violet in 5% (v/v) ethanol, washing with PBS and then desorbing stain with 40% acetic acid. The absorbance of crystal violet in acetic acid was measured at 562 nm. Assays were performed in sextuplicate, and serially diluted, genotype-matched MΦ suspensions were plated and quantitated in parallel, to create standard curves of absorbance versus MΦ number (which were linear from 0 to 4×10<sup>5</sup> MΦs/well).

Cytokine secretion assays were performed with thioglycollate-elicited MΦs (2 independently isolated groups from B6-congenic WT and Grk<sup>5<sup>-/-</sup></sup> mice). MΦs were serum-starved for 4 h and then stimulated with LPS (1 μg/ml) for 12 hr (in 1 ml of serum-free medium per 35-mm dish). Cytokines secreted into the conditioned media from these MΦs were assayed with a Mouse Inflammatory Cytokines Multi-Analyte ELISA<sup>™</sup> Kit (MEM-004A, SABiosciences). This 12-cytokine ELISA measured interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IFNγ, TNF, CSF-1, and GM-CSF. ELISA-generated absorbance values were normalized to the total mass of MΦ protein present in the cognate 35-mm dish. Signals for IL-1B, IL-2, IL-4, IL-17A, IFNγ, and GM-CSF were below our limit of detection for both WT and Grk<sup>5<sup>-/-</sup></sup> MΦs (not shown). Signals for other cytokines were 5- to 50-fold/basal, and 10-200% of positive control values. Conditioned media from Grk<sup>5<sup>-/-</sup></sup> and WT MΦs yielded equivalent signals for the NFκB-dependent<sup>26-29</sup> cytokines presented in Results (IL-1α, IL-6, IL-12 and TNF), as well as for IL-10 and CSF-1.

Immunoblotting
Immunoblotting was performed by enhanced chemiluminescence and quantitated as we have reported.<sup>1</sup>
Statistical Analyses
Data are presented as mean ± S.D. in the text and as mean ± S.E. in the figures. Data from experiments with only two independent means were analyzed by $t$ tests. Data from experiments with multiple groups were compared by one-way ANOVA with Tukey’s post-hoc test for multiple comparisons. Time course analyses were performed by two-way ANOVA, and monocyte chemotaxis data were analyzed by repeated measures two-way ANOVA with a Bonferroni post-hoc test. Statistical software was Prism® 5 (GraphPad, Inc.).
Supplemental References


Supplemental Figure I. *Grk5*<sup>−/−</sup> mouse cells do not express the GRK5 N-terminal domain encoded by GRK5 exons 1-6. SMCs from Grk5<sup>−/−</sup> and WT mice were subjected to immunoblotting with rabbit IgG that recognizes GRK5 amino acids 94-157, or an equivalent concentration of non-immune control rabbit IgG. Shown is an immunoblot representative of 3 performed with equivalent results. The arrow designates the expected position of migration for a GRK5 peptide that theoretically could be produced from GRK5 exons 1-6 (which still reside in the genome of the *Grk5*<sup>−/−</sup> mouse).
Supplemental Figure II. Carotid graft atheroma fibrous caps contain SMCs derived only from the graft itself. Carotid grafts from Figure 2 were immunostained with IgG targeting SMC α-actin (red) and either apolipoprotein E (green, left) or no particular protein (right). (ApoE is expressed only in cells originating from the carotid grafts.) Photomicrographs presented are from a single staining experiment, representative of 4 experiments performed with equivalent results. L, lumen. Scale bars = 50 μm.
**Supplemental Figure III.** GRK5 and the PDGFRβ up-regulate in arterial wall cells during early stages of atherogenesis. Carotid artery grafts of the indicated genotype were harvested 2 wk post-operatively (as in Figure 2), when no intimal Mφs could be detected. Native carotid arteries were processed in parallel. Serial sections were immunostained for either GRK5 or the PDGFRβ, and counterstained with Hoechst 33342 (DNA). Specimens shown are from a single staining experiment, representative of 4 experiments with independent samples yielding equivalent results. Scale bars = 50 μm (original magnification ×220 or ×440).
**Supplemental Figure IV.** GRK5 deficiency increases neointimal hyperplasia induced by endothelial denudation. Congenic mice of the indicated genotype were sacrificed before (pre-injury) or 4 weeks after carotid artery de-endothelialization. A, Perfusion-fixed carotids were stained with a modified connective tissue stain. Samples shown represent ≥6 of each genotype. Scale bars = 100 μm. B, For each artery, the neointimal area was normalized to the medial area, and the luminal area was normalized to the total arterial cross sectional area. These ratios are plotted are means ± S.E. of ≥6 of each genotype. Compared with WT: *, p < 0.02. Uninjured arteries from WT and Grk5 KO mice demonstrated equivalent luminal and medial areas.
**Supplemental Figure V.** GRK5 reduces PDGFRβ activation in response to carotid injury. Injured carotids from Supplemental Figure III were immunostained for the PDGFRβ pY1021 (red) and counterstained for DNA (blue); specimens stained with isotype control IgG gave no immunofluorescence (control). Top: Scale bars = 50 μm. Bottom: Protein-specific immunofluorescence was divided by DNA fluorescence within the same microscopic field, for 4 specimens/group; these ratios were normalized to those obtained for WT specimens, to obtain “% of WT control” displayed as means ± S.E. Compared with WT: *, p < 0.03.
Supplemental Figure VI. SMCs, Mφs, and endothelial cells express comparable levels of GRK5. SMCs, Mφs, and endothelial cells (ECs) at confluency were solubilized in detergent buffer, and 40 µg of each cell line extract was subjected to SDS-PAGE and sequential IB for GRK5, GRK2, and tubulin. Shown are results from a single experiment, representative of 3 experiments with independently isolated cell lines.
Supplemental Figure VII. GRK5 activity in Mφs does not affect NFκB activation, but diminishes ERK activation induced by LPS. Bone marrow-derived Mφs from WT and congenic Grk5−/− mice were exposed to serum-free medium without (basal) or with LPS (10 μg/ml) for the indicated times. Results shown are from single experiments, representative of 3-4 independent experiments with independently isolated pairs of WT and Grk5−/− Mφ lines. A, Mφs plated on chamber slides (top) were challenged ±LPS for 30 min, fixed and permeabilized, and immunostained for the p65 subunit of NFκB. Shown are images from WT Mφs; equivalent findings obtained with Grk5−/− Mφs. Mφs in 6-well dishes (lower panel) were solubilized at the indicated time points, and immunoblotted for the indicated proteins. Isotype control primary IgG yielded neither fluorescence (top) nor corresponding bands (bottom). Equivalent results were obtained with thioglycollate-elicited peritoneal Mφs (not shown). B, Densities of IB bands were normalized to GRK2 (loading control) bands; these ratios were divided by those for unstimulated WT Mφs, to obtain “% of control,” plotted as mean ± S.E. from 3 independent experiments. C, Aliquots of Mφs from panel A were incubated without (“0”) or with LPS for 24 hr, and then solubilized and immunoblotted for the NFκB target gene product MIP2. D, Mφ lysates from panel A were IB’d serially for phospho-ERK1/2 (pERK) and GRK2 (loading control). Densities of pERK bands were normalized to cognate GRK2 bands on the same nitrocellulose, and these ratios were divided by corresponding ratios from WT Mφs stimulated 30 min, to obtain “% of control;” the means S.E. from 3 experiments are plotted. Compared with the WT time course: *, p < 0.05. Total ERK1/2 content was equivalent in Grk5−/− and WT Ms (parallel IB data not shown).
Supplemental Figure VIII. GRK5 activity in Mφs does not affect NFκB activation, but diminishes ERK activation induced by TNF. Bone marrow-derived Mφs from WT and congenic Grk5 KO mice were exposed to serum-free medium without (basal) or with tumor necrosis factor (TNF, 25 ng/ml), phorbol ester (TPA, 100 nmol/L), or LPS (10 μg/ml) for the indicated times. In IB panels, results shown are from single experiments, representative of 3-4 independent experiments with independently isolated WT and Grk5 KO Mφs, showing equivalent results. A, Mφs were solubilized at the indicated time points, and IB’d for the indicated proteins (p-IκB, phospho-IκBα; IKKβ, IκB kinase β). B, Densities of IB bands were normalized to GRK2 (loading control) bands; these ratios were divided by those for unstimulated WT Mφs, to obtain “% of control,” plotted as the means from 3 independent experiments. C, Mφs were exposed to the indicated stimulus for 15 min (37 °C), solubilized and IB’d serially for phospho-ERK1/2 (pERK) and GRK2. Densities of IB bands were normalized to GRK2 bands; these ratios were divided by those for unstimulated WT Mφs, to obtain “% of control,” with results reported in the text.