Coupling of Fcγ Receptor I to Fcγ Receptor IIb by Src Kinase Mediates C-Reactive Protein Impairment of Endothelial Function

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Rationale: Elevations in C-reactive protein (CRP) are associated with increased cardiovascular disease risk and endothelial dysfunction. CRP antagonizes endothelial nitric oxide synthase (eNOS) through processes mediated by the IgG receptor Fcγ receptor IIB (FcγRIIB), its immunoreceptor tyrosine-based inhibitory motif, and SH2 domain-containing inositol 5′-phosphatase 1. In mice, CRP actions on eNOS blunt carotid artery re-endothelialization.

Objective: How CRP activates FcγRIIb in endothelium is not known. We determined the role of Fcγ receptor I (FcγRI) and the basis for coupling of FcγRI to FcγRIIb in endothelium.

Methods and Results: In cultured endothelial cells, FcγRI-blocking antibodies prevented CRP antagonism of eNOS, and CRP activated Src via FcγRI. CRP-induced increases in FcγRIIb immunoreceptor tyrosine-based inhibitory motif phosphorylation and SH2 domain-containing inositol 5′-phosphatase 1 activation were Src-dependent, and Src inhibition prevented eNOS antagonism by CRP. Similar processes mediated eNOS antagonism by aggregated IgG used to mimic immune complex. Carotid artery re-endothelialization was evaluated in offspring from crosses of CRP transgenic mice (TG-CRP) with either mice lacking the γ subunit of FcγRI (FcγRI−/−) or FcγRIIb−/− mice. Whereas re-endothelialization was impaired in TG-CRP vs wild-type, it was normal in both FcγRI−/−; TG-CRP and FcγRIIb−/−; TG-CRP mice.

Conclusions: CRP antagonism of eNOS is mediated by the coupling of FcγRI to FcγRIIb by Src kinase and resulting activation of SH2 domain-containing inositol 5′-phosphatase 1, and consistent with this mechanism, both FcγRI and FcγRIIb are required for CRP to blunt endothelial repair in vivo. Similar mechanisms underlie eNOS antagonism by immune complex. FcγRI and FcγRIIb may be novel therapeutic targets for preventing endothelial dysfunction in inflammatory or immune complex-mediated conditions. (Circ Res. 2011;109:1132-1140.)

Key Words: C-reactive protein ■ endothelial nitric oxide synthase ■ Fc receptor

C-reactive protein (CRP) is an acute phase reactant and a predictor of increased risk for cardiovascular disorders, including myocardial infarction, sudden cardiac death, stroke, peripheral vascular disease, and hypertension.1–3 Studies in cell culture and in mice indicate that CRP antagonizes endothelial nitric oxide synthase (eNOS), resulting in the inhibition of endothelial cell migration, the promotion of endothelial cell–monocyte adhesion, impaired vascular wound repair, impaired vasorelaxation, and hypertension.4–6

CRP and immune complexes bind to cell plasma membranes via IgG Fcγ receptors (FcγRs).7–9 We previously demonstrated that not only CRP but also immune complexes potently antagonize eNOS in cultured endothelial cells.4 FcγR are categorized into activation receptors such as FcγRI, which contain a cytoplasmic immunoreceptor tyrosine-based activation motif located in its γ-chain subunit (Fcγγ), and inhibitory receptors such as FcγRIIb, which has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain.9 We previously showed that CRP inhibition of eNOS requires FcγRIIb, the phosphorylation of its ITIM, and the resulting recruitment and activation of SH2 domain-containing inositol 5′-phosphatase 1 (SHIP-1) via phosphorylation of Tyr1020. Activated SHIP-1 attenuates signaling downstream of PI3 kinase, blunting Akt activation and eNOS Ser1179 phosphorylation by Akt, thereby decreasing eNOS enzyme activation.5,10 However, how CRP activates FcγRIIb is not known.

To better understand the basis for CRP actions on endothelium, the present study was designed to delineate the mechanisms by which CRP activates FcγRIIb. In immune response cells such as lymphocytes, activating FcγR and inhibitory FcγR are frequently functionally coupled with the
actions of activating FcγR opposing inhibitory FcγR function in some instances, and the actions of activating FcγR being required to initiate inhibitory FcγR function in others.\(^9\,\,11\) Recognizing that FcγRI is coexpressed with FcγRIIB in endothelial cells,\(^4\,\,12\) we raised the hypothesis that FcγRI is required for FcγRIIB-mediated actions of CRP on endothelium. In addition to testing this hypothesis, studies were performed to determine how FcγRI is coupled to FcγRIIB in endothelium. We also investigated whether immune complex-induced eNOS antagonism entails similar FcγRI-FcγRIIB functional linkage. Furthermore, to determine if these processes are operative in vivo, studies of carotid artery re-endothelialization were performed in mice derived from crosses of transgenic CRP mice (TG-CRP) with FcγRI chain-null or FcγRIIB-null mice.

**Methods**

**Cell Culture and Transfection**

Using previously described approaches, primary bovine aortic endothelial cells (BAECs) were harvested, propagated, and used within nine passages.\(^10\) In selected experiments, BAEC were transfected with cDNA encoding hemagglutinin-tagged human FcγRIIB as described previously.\(^10\) In other experiments, primary aortic endothelial cells were harvested from FcγRIIB\(^+/−\) and FcγRIIB\(^−/−\) C57BL/6 mice.\(^13\) Briefly, mouse thoracic aortas were removed, placed in DMEM with 20% fetal bovine serum and 1000 U/mL heparin, washed with DMEM, and filled with DMEM containing 2 mg/mL collagenase type I (Worthington) for 45 minutes at 37°C. Endothelial cells were then collected by flushing with 5 mL of DMEM with 20% fetal bovine serum, pelleted, and plated onto a 35-mm dish coated with collagen type I. After a 2-hour incubation, cells were washed twice with phosphate-buffered saline and placed in DMEM with 20% fetal bovine serum, penicillin-streptomycin, 2 mmol/L L-glutamine, 1× nonessential amino acids, 1× sodium pyruvate, 25 mmol/L HEPES, 100 μg/mL heparin, and 100 μg/mL endothelial cell growth supplement (Sigma-Aldrich). Mouse primary endothelial cells were used within three passages.

**eNOS Activation Assays**

eNOS activation was assessed in whole cells by measuring \(^14\)C-L-arginine conversion to \(^14\)C-L-citrulline during 15-minute incubations as previously described.\(^5\) eNOS agonists used were insulin (500 nmol/L) and acetylcholine (Ach; 10 μmol/L). In select experiments, incubations were performed in the presence of human recombinant CRP (5 or 25 μg/mL), heat-aggregated IgG (100 μg/mL), PP2 (10 μmol/L; Calbiochem, Gibbstown, NJ), piceatannol (10 μmol/L; Calbiochem), anti-FcγRI antibodies (10 μg/mL; anti-CD64, clones 10.1 and H-250; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-FcγRII antibodies (10 μg/mL; anti-CD16, clone 3G8; Santa Cruz Biotechnology).

**Immunoblot Analyses**

Immunoblots were performed to assess Src kinase phosphorylation, FcγRIIB phosphorylation, and SHIP-1 phosphorylation. Antibodies used were anti-phospho-Src (Tyr416) (Cell Signaling Technology), anti-Src (Santa Cruz Biotechnology), antiphospho-FcγRIIB (Tyr292; Abcam), anti-FcγRIIB (Fitzgerald), antiphospho-SHIP-1 (Tyr1020; Cell Signaling Technology), and anti-SHIP-1 (Santa Cruz Biotechnology). Results shown were confirmed in three or more independent experiments.

**Statistical Analysis**

Analysis of variance with Neuman–Keuls post hoc testing was used to assess differences between multiple groups, and significance was set at \(P<0.05\). For an expanded Methods section, see the Online Supplement available at http://circres.ahajournals.org.

**Results**

**CRP Antagonism of eNOS and FcγRI**

To evaluate the potential role of FcγRI in endothelium in the actions of CRP, studies of eNOS activation by insulin were performed in BAEC (Figure 1). Under control conditions, CRP (25 μg/mL) caused an 82.7% decrease in eNOS activation by insulin, mirroring our previous findings.\(^4\) In contrast, in cells treated with either of two FcγRI-blocking antibodies, 10.1 or H-250, CRP had no effect on eNOS activation. However, blocking antibody to FcγRII did not alter CRP inhibition of eNOS activity. Thus, CRP antagonism of eNOS activation is FcγRI-dependent.

**CRP Actions and Src Kinase**

Knowing from previous work that CRP antagonism of eNOS is mediated by FcγRIIB,\(^4\) and now having also implicated
FcgRI, we determined how FcgRI are coupled to FcgRIIB and the ensuing signaling events prompted by CRP in endothelial cells. Because Src kinase or spleen tyrosine kinase (Syk) frequently links activating FcgR function to inhibiting FcgR function in immune response cells, we tested the requirement for these kinases in CRP inhibition of eNOS activity. In preliminary experiments we determined that eNOS activation by Ach in BAEC is Src-independent and Syk-independent (data not shown), making it feasible to use Ach as an agonist in studies of Src or Syk in CRP antagonism of eNOS. Ach increased eNOS activity, and this was fully inhibited by CRP as previously observed (Figure 2A). However, CRP did not inhibit Ach-stimulated eNOS activity in the presence of the Src inhibitor PP2. In parallel experiments, the antagonism of Ach-induced eNOS activation by CRP was unaffected by the Syk inhibitor piceatannol (Figure 2B), which effectively prevented Syk activation by heat-aggregated IgG in Raji cells (data not shown).

To further interrogate the potential role of Src in CRP action in endothelial cells, we evaluated whether CRP activates Src by immunoblot analysis of Src phosphorylation at Tyr416. Using high-density lipoprotein as a positive control for Src stimulation in endothelial cells, we observed that within 10 minutes of CRP treatment (25 μg/mL), Src phosphorylation was increased (Figure 3A). Heat-inactivated CRP (25 μg/mL) did not stimulate Src phosphorylation (data not shown), indicating that the response requires the intact protein. Dose–response studies further revealed that Src activation occurs at CRP concentrations as low as 2 μg/mL (Figure 3B), which is well within the range for modest chronic elevations in CRP that are associated with increased cardiovascular disease risk in humans, and which mirrors the dose–response for eNOS antagonism by CRP.

The role of FcgRI in CRP activation of Src was then interrogated in studies using primary mouse endothelial cells isolated from aortas of FcgRIIB−/− and FcgRIIB−/+ mice.

Whereas high-density lipoprotein caused comparable activation of Src in FcgRIIB−/− and FcgRIIB−/+ endothelial cells, CRP activation of Src was observed in FcgRIIB−/− endothelial cells but was absent in FcgRIIB−/+ endothelial cells (Figure 3C).

To test the coupling of CRP-induced FcgRI-mediated Src activation to FcgRIIB, BAECs were transfected with cDNA encoding human FcgRIIB, and 48 hours later the phosphorylation of FcgRIIB Tyr292 in the ITIM domain in response to CRP was evaluated by immunoblot analysis. This approach was used because the abundance of endogenous FcgRIIB in BAECs was insufficient to detect changes in receptor phosphorylation (data not shown). CRP caused a robust increase in transfected FcgRIIB phosphorylation, and this was fully prevented by Src inhibition with PP2 (Figure 4A). The requirement for Src in the activation of SHIP-1 was tested in nontransfected BAECs. CRP activated SHIP-1 phosphorylation, and this was prevented by Src inhibition with PP2 (Figure 4B). These cumulative findings indicate that through FcgRI, CRP activates Src, leading to FcgRIIB
Basis of Immune Complex Action in Endothelium

We previously demonstrated that similar to the pentraxins CRP and SAP, heat-aggregated IgG used to mimic immune complex causes eNOS antagonism. Although it has been shown that common Fc receptor-mediated processes underlie phagocytosis and cytokine secretion by leukocytes in response to pentraxins and immune complex, it is unknown whether CRP and immune complex induce similar FcγR-mediated events in endothelial cells. Similar to CRP (Figure 2A), heat-aggregated IgG used to mimic immune complex completely inhibited Ach-stimulated eNOS activity, and the suppression was fully reversed by PP2 antagonism of Src (Figure 5A). We further tested the effect of aggregated IgG on the phosphorylation of the ITIM of FcγRIIB in endothelial cells and found that similar to CRP (Figure 4A), aggregated IgG stimulated FcγRIIB phosphorylation and the phosphorylation was prevented by PP2 (Figure 5B). Aggregated IgG also stimulated SHIP-1 phosphorylation in endothelial cells.

Figure 3. C-reactive protein (CRP) stimulates Src through Fcγ receptor I (FcγRI). A, Bovine aortic endothelial cells (BAECs) were treated with CRP (25 μg/mL) for 0 to 30 minutes and Src activation was analyzed by immunoblot analysis for phospho-Src (Tyr416) and total Src. High-density lipoprotein (50 μg/mL) treatment for 10 minutes provided a positive control. Summary data for the ratio of pSrc to Src normalized to time 0 is provided. B, BAEC were treated with 0 to 25 μg/mL CRP for 10 minutes, and Src activation was analyzed. Summary data for the ratio of pSrc to Src normalized to time 0 is provided. C, Mouse primary aortic endothelial cells from FcγRII/" or FcγRII<sup>-/-</sup> mice were treated with high-density lipoprotein (50 μg/mL) for 10 minutes or CRP (25 μg/mL) for 15 minutes, and Src activation was analyzed. Summary data for the ratio of pSrc to Src normalized to basal is provided. Values are mean±SEM, n=4 to 6. *P<0.05 vs no CRP in A and B, *P<0.05 vs basal in C.
and the activation of SHIP-1 was completely inhibited in the presence of PP2 (Figure 5C). Thus, mirroring the events prompted by CRP, in endothelial cells aggregated IgG activates Src, leading to FcγRIIB activation and SHIP-1 activation.

**CRP, Re-Endothelialization and FcγR**

To determine the role of FcγRI in CRP actions on endothelium in vivo, mice null for the FcRγ-chain (Fcγγ−/−), which contains the immunoreceptor tyrosine-based activation motif domain of FcγRI, were crossed with TG-CRP mice and the resulting progeny were crossed. Carotid artery re-endothelialization was then evaluated in the four following groups of littermates: Fcγγ+/+, Fcγγ+/−, TG-CRP, Fcγγ−/−, and Fcγγ−/−; TG-CRP. Compared with Fcγγ+/− controls, re-endothelialization was impaired in Fcγγ+/−; TG-CRP mice (Figure 6A), as indicated by the larger area of remaining denudation that incorporated Evans blue dye in the latter group. Cumulative studies revealed that the area of remaining denudation was 4.0-fold greater in Fcγγ+/+; TG-CRP versus Fcγγ+/− mice (Figure 6B). In contrast, despite having elevated CRP, Fcγγ−/−; TG-CRP mice had normal re-endothelialization (Figure 6A, B).

To determine if partnership between FcγRI and FcγRIIB is required for CRP actions on endothelium in vivo, additional re-endothelialization experiments were performed in the following four groups of littermates: FcγγRIIB+/+, FcγγRIIB+/−; TG-CRP, FcγγRIIB−/−, and FcγγRIIB−/−; TG-CRP. Compared with FcγγRIIB+/− controls, re-endothelialization was impaired in FcγγRIIB+/−; TG-CRP mice (Figure 7A, B), with the area of remaining denudation increased 3.4-fold in the latter group. In contrast, although CRP was similarly elevated in FcγγRIIB−/−; TG-CRP mice, they had normal re-endothelialization (Figure 7A, B). These collective observations, which mirror the findings for Fc receptor coupling in the actions of CRP on cultured endothelial cells, indicate that both the γ-chain of FcγRI and FcγRIIB are required for CRP antagonism of re-endothelialization in vivo.

**Discussion**

CRP levels are strongly correlated with increased risk for cardiovascular disease and with endothelial dysfunction related to decreased NO bioavailability.1,3,20–22 We previously demonstrated that CRP potently antagonizes eNOS activation by diverse agonists at levels of CRP that have been associated with increased risk of cardiovascular disease,23 and that the inhibitory Fc receptor FcγRIIB is critically involved in this process.4 This represents a potentially major mechanism by which vascular NO bioavailability may be diminished under numerous disease conditions. Here, we show that the activating Fc Receptor, FcγRI, and functional coupling of FcγRI to FcγRIIB are required for CRP inhibition of eNOS in endothelial cells, thereby identifying another key cell surface receptor that participates in the negative regulation of NO production by the endothelium. We further show that, consistent with these observations in cell culture, both FcγRI and FcγRIIB are required for CRP to blunt endothelial repair in...
vivo, which is related to a diminution in bioavailable NO. Elucidation of these processes mediated by the FcγRI–FcγRIIB tandem provides further evidence that CRP is likely a causal factor in certain forms of cardiovascular disease, and is not merely a marker of chronic low-grade inflammation. In addition, because CRP impairs insulin signaling to eNOS in endothelial cells, which normally promotes blood flow that augments glucose disposal in skeletal muscle, these newly identified mechanisms are also potentially relevant to the insulin resistance that accompanies chronic inflammatory conditions, including obesity.

We further demonstrate that Src kinase is critically involved in the coupling of FcγRI to FcγRIIB in endothelial cells. In immune response cells in which Fc receptors serve their classical functions, Syk is the kinase that primarily links the activating phosphorylation of the immunoreceptor tyrosine-based activation motif within the γ subunit of FcγRI to the activating phosphorylation of the ITIM in FcγRIIB. Interestingly, whereas we now show that in response to CRP Src activation leads to eNOS antagonism, the stimulation of eNOS by numerous agonists including high-density lipoprotein, estrogen, and...
shear stress also entails Src activation, which precedes PI3 kinase and Akt activation that promotes eNOS Ser1177 phosphorylation. These disparate roles of Src may be explained by the participation of different Src family members. Alternatively, because Src activation entails poorly understood mechanisms that involve plasma membrane receptor clustering and Src interacts with a number of receptors, these disparate functions for Src may be explained by different responses by Src to Fc receptor clustering versus eNOS-activating receptor clustering, or differences in the mode of physical linkage between Src and Fc receptors versus eNOS-activating receptors. Having demonstrated an inhibitory role for Src in the regulation of eNOS for the first time, additional studies are now warranted to distinguish between these possibilities.

Recognizing that the classical ligand for Fc receptors is IgG, we previously demonstrated that heat-aggregated IgG mimicking immune complex causes eNOS antagonism to a degree that is comparable to that attained by CRP. We now show that similar to CRP, the actions of aggregated IgG on eNOS are mediated by Src activation causing Fc receptor IIITM phosphorylation and resulting activation of SHIP-1. Recognizing how eNOS contributes to insulin-mediated glucose disposal, these mechanisms may participate in the pathogenesis of not only the cardiovascular disease but also the insulin resistance that frequently complicates immune complex-mediated diseases such as rheumatoid arthritis or systemic lupus erythematosus.

From a therapeutic perspective, the identification of FcγRI as the initiator of CRP action in endothelial cells provides a new cell surface target for potential interventions. Studies in humans and in mice indicate that attenuated function of FcγRIIB likely contributes to the pathogenesis of systemic lupus erythematosus. Therefore, seeking to normalize CRP-related endothelial dysfunction by decreasing FcγRIIB activity, which can be accomplished by selective antibody-mediated blockade, has the risk of promoting autoimmune disease. Alternatively, strategies that selectively prevent FcγRI activation now can be considered to blunt CRP-induced endothelial dysfunction without shifting the balance of activating and inhibitory Fc receptor activity toward excessive immune...
function. Now having greater evidence of an important role for SHIP-1 in eNOS antagonism by both CRP and immune complex, the phosphatase also may warrant consideration as a therapeutic target in endothelial cells. Although there is clear evidence of a causal role for CRP in hypertension and endothelial dysfunction in mice, CRP has not yet been implicated as a contributing factor in the pathogenesis of such disorders in humans. However, now knowing that FcγRI and FcγRIIB are both important participants in CRP signaling in endothelium, it becomes apparent that there are multiple modifiers of CRP actions of relevance to vascular health. There are numerous polymorphisms in Fc receptors, some have known impact on receptor function, and there is also great variability in Fc receptor gene copy number. Thus, as we continue to identify the participating signaling molecules, genetic and molecular modifiers of CRP action should be considered in our assessment of the role of CRP in cardiovascular disease pathogenesis and our use of CRP as a risk factor in patient populations.

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Disclosures
None.

References
What is Known?

- Elevated levels of C-reactive protein (CRP) are associated with an increase in cardiovascular disease risk and endothelial dysfunction in humans.
- CRP plays a causal role in hypertension and endothelial dysfunction in mice.
- CRP antagonizes endothelial nitric oxide synthase (eNOS) through processes mediated by the IgG receptor FcγRIIB (FcγRIIB) and activation of the phosphatase SH2 domain-containing inositol 5'-phosphatase 1 (SHIP-1).

What New Information Does This Article Contribute?

- Inhibition of eNOS by CRP is mediated by the coupling of the activating Fcγ receptor I (FcγRI) to FcγRIIB by Src kinase that results in the activation of SHIP-1.
- Both FcγRI and FcγRIIB are required for CRP to blunt endothelial repair in vivo.
- Similar processes mediate eNOS antagonism by aggregated IgG that mimics immune complexes.

CRP levels are strongly correlated with increased risk for cardiovascular disease and with endothelial dysfunction. Previous studies both in cell culture and in mouse models have shown that CRP inhibits eNOS activation, and that the inhibitory Fc receptor FcγRIIB mediates this process. Nevertheless, how CRP activates FcγRIIB in endothelium is not known. This study shows that the activating Fc receptor FcγRI mediates CRP actions by coupling to FcγRIIB through the activation of Src kinase, and that both receptors are required for CRP to blunt endothelial repair in vivo. We demonstrate that similar mechanisms underlie eNOS antagonism by immune complexes. Three aspects of our work are novel and significant. First, we identify FcγRI as a new cell surface receptor that participates in the negative regulation of endothelial function. Second, we demonstrate for the first time an inhibitory role for Src in eNOS regulation. Last, our results raise the novel possibility that the FcγRI–FcγRIIB tandem in the endothelium plays an important role in the cardiovascular complications of immune complex-mediated diseases such as rheumatoid arthritis and systemic lupus erythematosus.
Coupling of Fcγ Receptor I to Fcγ Receptor IIB by Src Kinase Mediates C-Reactive Protein Impairment of Endothelial Function
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SUPPLEMENTAL MATERIAL

Expanded Methods:

Cell Culture and Transfection: Primary bovine aortic endothelial cells (BAEC) were harvested, propagated and used within nine passages. BAEC were maintained in Endothelial Cell Growth Medium-2 (EGM-2, Clonetics) with added growth supplements and 5% Fetal Bovine Serum (FBS), and they were placed in serum-free DMEM overnight prior to all experiments. In selected studies, BAEC were transfected with cDNA encoding hemagglutinin (HA)-tagged human FcγRIIB as described previously. Briefly, cells at 80% confluence in 6-well plates were incubated in Opti-MEM media (Invitrogen) containing 1µg cDNA/well and Lipofectamine 2000 (Invitrogen) for 4h, the media was replaced with EGM-2, and experiments performed 48h later. In other experiments, primary aortic endothelial cells were harvested from FcγRI+/- and FcγRI-/ mice and maintained as described in the Methods section.

eNOS Activation Assays: eNOS activation was assessed in whole cells at near-confluence in 6-well plates by measuring 14C-L-arginine conversion to 14C-L-citrulline over 15 min at 37°C. The incubations were initiated by adding 600ul/well of buffer containing 120mM NaCl, 4.2mM KCl, 2.5mM CaCl2, 1.3mM MgSO4, 7.5mM glucose, 10mM Hepes, 1.2mM Na2HPO4, 0.37mM KH2PO4, and 0.3nM 14C-L-arginine at pH 7.4. The reaction was stopped by adding 750ul of ice-cold buffer containing 15mM EGTA and 200mM Hepes at pH 5.5. The cells were freeze-fractured in liquid nitrogen for 2min and thawed at 37°C for 10min; this procedure was performed twice and cells were then scraped with a rubber spatula. 600ul of each well were then applied to 1.5ml Dowex AG50WX-8 (tris(hydroxymethyl)aminomethane (Tris) form) columns and eluted with 1ml of 40mM Hepes buffer, pH 5.5, containing 2mM EDTA and 2mM EGTA. The 14C-L-citrulline generated was collected in glass scintillation vials and quantified by liquid scintillation spectroscopy.

Immunoblot Analyses: Following treatments of BAEC at near-confluence in 6-well plates, whole cell lysates were collected in 0.5% SDS sample buffer containing 10% glycerol, 0.025% bromophenol blue, and 0.012% volume 2-mercaptoethanol (100µL/well). Equal volumes of whole cell lysates were loaded on SDS-PAGE gels and transferred to PVDF membranes (Immobilon-P, Millipore). After blocking with 5% bovine serum albumin in Tris-buffered saline TBS, 20 mM Tris, 137 mM NaCl, pH 7.6) with 0.1% Tween 20 (TBST) for 2 or 4h at room temperature, primary antibodies were added at final concentrations of either 1:50,000 for anti-phospho-FcγRIIB (Tyr292), or 1:1000 for all other antibodies and membranes were incubated overnight at 4°C. Following washing with TBST, horseradish peroxidase-labeled secondary antibodies were added at 1:10,000, and membranes were incubated for 2h at room temperature. Membranes were washed, ECL reactions were performed with either West Pico Chemiluminescent Substrate or West Dura Extended Duration Substrate (SuperSignal, Thermo Scientific), and the membranes were exposed to X-ray film. Images were captured digitally and densitometry was performed using...
image analysis software (FluorChem SP, AlphaEase FC software, Alpha Innotech).

**Carotid Artery Reendothelialization:** The care and use of all animals was approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Carotid artery reendothelialization following perivascular electric injury was studied in male mice at 11-13 weeks of age as previously described. Mice were anesthetized by intraperitoneal administration of avertin (0.02-0.04ml/g body weight of a combination of 40ml 2.5% tribromoethanol and 310μl tertiary amyl alcohol in 39.5ml water). The left common carotid artery was exposed by anterior incision of the neck and careful dissection. Electric current of 4 watts was applied through 2mm forceps (2watts/mm) for 3sec in micropolar mode (Force 2 Electrosurgical Generator, Valleylab). Mice were recovered and analgesia was provided by intraperitoneal administration of 0.45µg bupronex immediately after surgery and 24h later. Ninety-six h post-injury the animals were anesthetized with avertin, a thoracotomy was performed, and 100μL of 2.5% Evans blue dye was injected into the left ventricle and allowed to circulate for 4min. Mice then received 3mL of 4% formalin by intracardiac injection to flush and fix the circulatory system. Both right (sham-operated) and left (injured) common carotid arteries were removed, opened along their longitudinal axis to reveal the endothelial surface, and mounted on a microscope slide. Images were obtained and the area of denudation which incorporated the dye was quantified by image analysis (FluorChem SP, AlphaEase FC Software, Alpha Innotech) by an investigator blinded to group assignment.

**References**


