Bcr Kinase Activation by Angiotensin II Inhibits Peroxisome Proliferator-Activated Receptor γ Transcriptional Activity in Vascular Smooth Muscle Cells

Jeffrey D. Alexis, Nadan Wang, Wenyi Che, Nicole Lerner-Marmarosh, Abha Sahni, Vyacheslav A. Korshunov, Yiping Zou, Bo Ding, Chen Yan, Bradford C. Berk, Jun-ichi Abe

Abstract—Bcr is a serine/threonine kinase activated by platelet-derived growth factor that is highly expressed in the neointima after vascular injury. Here, we demonstrate that Bcr is an important mediator of angiotensin (Ang) II and platelet-derived growth factor–mediated inflammatory responses in vascular smooth muscle cells (VSMCs). Among transcription factors that might regulate Ang II–mediated inflammatory responses we found that ligand-mediated peroxisome proliferator-activated receptor (PPAR)γ transcriptional activity was significantly decreased by Ang II. Ang II increased Bcr expression and kinase activity. Overexpression of Bcr significantly inhibited PPARγ activity. In contrast, knockdown of Bcr using Bcr small interfering RNA and a dominant-negative form of Bcr (DN-Bcr) reversed Ang II–mediated inhibition of PPARγ activity significantly, suggesting the critical role of Bcr in Ang II–mediated inhibition of PPARγ activity. Point-mutation and in vitro kinase analyses showed that PPARγ was phosphorylated by Bcr at serine 82. Overexpression of wild-type Bcr kinase did not inhibit ligand-mediated PPARγ S82A mutant transcriptional activity, indicating that Bcr regulates PPARγ activity via S82 phosphorylation. DN-Bcr and Bcr small interfering RNA inhibited Ang II–mediated nuclear factor κB activation in VSMCs. DN-PPARγ reversed DN-Bcr–mediated inhibition of nuclear factor κB activation, suggesting that PPARγ is downstream from Bcr. Intimal proliferation in low-flow carotid arteries was decreased in Bcr knockout mice compared with wild-type mice, suggesting the critical role of Bcr kinase in VSMC proliferation in vivo, at least in part, via regulating PPARγ/nuclear factor κB transcriptional activity. (Circ Res. 2009;104:000-000.)

Key Words: signal transduction • smooth muscle cell • inflammation

It is well known that the renin–angiotensin system plays an important role in regulating pathophysiological processes of cardiovascular disease. Many clinical studies have shown that inhibition of the renin–angiotensin system reduces inflammation and oxidative stress. For example, treatment with the angiotensin (Ang) II type 1 receptor blocker valsartan reduced lipopolysaccharide (LPS)-stimulated interleukin (IL)-1β production by peripheral blood monocytes, and candesartan, another Ang II type 1 receptor blocker, reduced inflammation and insulin resistance in hypertensive patients.1,2 In the Valsartan Heart Failure Trial (Val-HeFT), valsartan treatment lowered plasma CRP concentrations.3 These clinical studies suggest that Ang II acts as an inflammatory mediator. In animal studies, it has been reported that Ang II–induced hypertension specifically increased the development of atherosclerosis in apolipoprotein (apo)E knockout mice.4 Interestingly, infusion of Ang II in apoE knockout mice results in abdominal aortic aneurysms formation, and the abdominal aortic aneurysms exhibit inflammatory infiltration, matrix metalloproteinase activation, thrombus formation, and oxidative stress, suggesting the profound impact of Ang II on atherosclerosis and inflammation.5,6 Ang II activates nuclear factor (NF)-κB, a key component of inflammation, in vascular smooth muscle cells (VSMCs). However, the exact mechanism of Ang II–mediated inflammation and NF-κB activation in VSMCs remains unclear.

The PPAR family consists of 3 different genes, PPARα, PPARβ/δ, and PPARγ. These receptors exert antiinflammatory activities in vascular and immune cells including endothelial cells, VSMCs, and monocytes. There are 2 isoforms of PPARγ: PPARγ1 and PPARγ2. PPARγ agonists include naturally occurring ligands such as 15-deoxy-D12,14-prostaglandin (15d-PGJ)2 and synthetic ligands such as the thiazolidinedione class of insulin-sensitizing drugs.7–9 PPARγ agonists inhibit the production of monocyte inflammatory cytokines (tumor necrosis factor [TNF]-α, IL-6, and IL-1β).10
and inhibit IFNγ, TNF-α, and IL-2 production by human CD4+ T cells. PPARγ agonists have also been shown to inhibit VSMC growth, migration, and DNA synthesis and to inhibit neointimal proliferation following arterial injury. PPARγ contains a mitogen-activated protein kinase consensus recognition site at serine 82. Phosphorylation of PPARγ by mitogen-activated protein kinase has been shown to reduce growth factor–mediated PPARγ transcriptional activity.

Bcr is a serine/threonine kinase originally defined as the breakpoint of the Philadelphia chromosome translocation associated with chronic myelogenous leukemia. Bcr is expressed in many cell types and its cDNA sequence predicts several functional domains including serine/threonine kinase activity, a region that binds Src-homology 2 (SH2) domains, and a GTPase-activating function for the small GTP-binding protein Rac.

We previously reported that Bcr mediates platelet-derived growth factor (PDGF) activation of Elk-1 in VSMCs. We also demonstrated that Bcr expression is increased in proliferating VSMCs of the neointima. Because inflammation is an important component of intimal formation, we studied the contribution of Bcr to vascular inflammation and intimal proliferation. In the present study, we found that increased Bcr expression and activation mediated by Ang II induces inflammatory responses and enhances VSMC proliferation in part via a Bcr-mediated inhibitory effect against PPARγ transcriptional activity.

Materials and Methods

Cell Culture

Rat and mouse VSMCs were isolated as described previously or were purchased from Cell Applications Inc. VSMCs were maintained in DMEM. Cells were treated with ciglitazone (Biomol), pioglitazone (Takeda Pharmaceuticals, North America Inc, Lincolnshire, Ill), PDGF (R&D Systems), and Ang II (MP Biomedicals) as described in individual experiments.

Plasmids and Transfection

Bcr wild-type (WT) and dominant-negative Bcr (DN-Bcr) (Y328F) plasmids were prepared as described previously. The single or double mutations of PPARγ were created with the QuikChange site-directed mutagenesis kit (Stratagene) as described previously. For transient expression experiments, cells were transfected with the Lipofectamine Plus method (Invitrogen) as described previously.

For small interfering (si)RNA experiments, VSMCs were transfected with Bcr siRNA oligonucleotides (Invitrogen) using RNAiFect reagent (Qiagen).

Immunoprecipitation and Western Blot

After treatment with reagents, the cells were washed with PBS and harvested in 0.5 mL of lysis buffer as described previously. For immunoprecipitation, cell lysates were incubated with mouse anti-Bcr antibody (10 μL) as described previously. For Western analysis, the blots were incubated for 4 hours at room temperature with Bcr antibody (Santa Cruz Biotechnology) or α-tubulin antibody (Sigma), followed by incubation with horseradish peroxidase–conjugated secondary antibody (Amersham Life Science).

Bcr In Vitro Kinase Assay

Immunoprecipitation was performed using Bcr antibody, and in vitro kinase activity was measured at 30°C for 20 minutes in a reaction mixture including 0.1 mg/mL indicated substrates.

Immunofluorescence

VSMCs were fixed with 4% paraformaldehyde, permeabilized with 0.1% PBS-Triton, and stained with relevant primary antibodies, followed by secondary antibodies as indicated. Nuclei were stained with DAPI (Sigma). Cells were visualized with an Olympus (BX-51) fluorescent microscope.

[3H]Thymidine Incorporation Assay

Measurement of [3H] thymidine incorporation into DNA was performed as described.

Carotid Ligation and Immunohistochemistry

Mice were used in accordance with the guidelines of the NIH and the American Heart Association for the care and use of laboratory animals. All procedures were approved by the University of Rochester Animal Care Committee. Mice were anesthetized with an intraperitoneal injection of ketamine (130 mg/kg) and xylazine (8.8 mg/kg) in saline (10 mL/kg). The left external and internal carotid branches were ligated so that left carotid blood flow was reduced to flow via the occipital artery. Carotid arteries were harvested 2 weeks after ligation. Cross-sections were stained with hematoxylin/coxin and were analyzed using MCID image software (MCID Elite 6.0, Imaging Research). Representative samples were evaluated with Ki-67 antibody (DAKO, 1:500 dilution).

Statistics

Numeric data are expressed as means ± SEM or SD as indicated in the figure legends. Statistical analysis was performed with the StatView 5.0 package (ABACUS Concepts, Berkeley, Calif). Differences were analyzed with a 1-way or a 2-way repeated-measures

![Figure 1. Ang II–inhibited PPARγ transcriptional activity in VSMCs. VSMCs were cotransfected with PPARγ and reporter plasmids. After pretreatment with Ang II or vehicle incubation, VSMCs were stimulated by either ciglitazone or vehicle at indicated dose (A) or pioglitazone (100 μmol/L) or vehicle (B) for 16 hours, the cells were harvested, and dual-luciferase reporter assay was performed. For all figures, the data are representative of triplicates using 2 or more different preparations of SMCs. Results are means ± SD. *P<0.05, **P<0.01.](http://circres.ahajournals.org/lookup/suppl/doi:10.1161/01.RES.0000321076.00933.9a/-/media/derivative/11006B.png)
Ang II Inhibits PPARγ Transcriptional Activity in VSMCs

It has been reported that PPARγ agonists can inhibit the development of hypertension in Ang II–infused rats, but it remains unclear whether Ang II can inhibit PPARγ transcriptional activity. Therefore, we examined the effect of Ang II on PPARγ activity and PPARγ expression. Using 2 different PPARγ agonists ciglitazone and pioglitazone, we demonstrated that Ang II inhibits PPARγ transcriptional activity in VSMCs (Figure 1A and 1B). Ang II did not alter Ang II expression (data not shown). Because PPARγ activation has a critical role in regulating inflammatory responses,10,30 these data suggest a mechanism of Ang II–mediated inflammation is via inhibiting PPARγ transcriptional activity.

Ang II Increased Bcr Expression and Bcr Kinase Activity in VSMCs

Previously, we reported that Bcr kinase activation can regulate Elk-1, which may have a significant impact on inflammation.20 To determine the role of Bcr kinase on Ang II–mediated inhibition of PPARγ transcriptional activity, we investigated whether Ang II could regulate Bcr expression and Bcr kinase activity in VSMCs. Western blotting demonstrated that 200 nmol/L Ang II increased Bcr expression within 3 hours (Figure 2A). Ang II also rapidly stimulated Bcr kinase activity, detected by Bcr autophosphorylation as reported previously,20 with peak (2.0±0.12-fold increase versus no treatment) at 2 minutes (Figure 2B), suggesting the possible involvement of Bcr kinase in Ang II–mediated signaling.

Critical Role of Bcr Kinase on Ang II–Mediated Inhibition of PPARγ Transcriptional Activity

To investigate whether Bcr kinase is involved in Ang II–mediated inhibition of PPARγ transcriptional activity, VSMCs were cotransfected with a PPARγ reporter plasmid and either Bcr WT or a dominant-negative form of Bcr (Y328F). After stimulation with the PPARγ ligand ciglitazone (5 µmol/L or 10 µmol/L) or vehicle for 16 hours the cells were harvested and dual-luciferase reporter assay performed. Overexpression of Bcr WT inhibited PPARγ transcriptional activity (Figure 3A). In contrast, DN-Bcr did not result in a change of PPARγ transcriptional activity in VSMCs (Figure 3A). This effect of WT-Bcr on PPARγ activity did not appear to be just an effect limited to exogenous PPARγ agonists because a similar result was obtained when we overexpressed PPARγ in the absence of exogenous PPARγ ligands (Figure 3B). Previously, we reported that PDGF-induced Bcr kinase activity is involved in extracellular signal-regulated kinase (ERK)1/2 activation. It may be possible that overexpression of Bcr inhibits PPARγ activity via ERK1/2 activation, but we did not find significant ERK1/2 activation by Bcr overexpression alone.20 In addition, overexpression of WT Bcr does not increase c-Jun transcriptional activity, which represents c-Jun N-terminal kinase (JNK) activity (Figure I in the online data supplement). Therefore, a mechanism other than ERK1/2 or JNK activation is most likely involved in this PPARγ regulation.

To further confirm the role of Bcr kinase in Ang II–mediated inhibition of PPARγ transcriptional activity, we examined whether knockdown of Bcr would inhibit Ang II–mediated inhibition of PPARγ activation. Following cotransfection of VSMCs with DN-Bcr or Bcr siRNA and reporter plasmids, Ang II inhibition of PPARγ activity was significantly reversed by both DN-Bcr and deletion of Bcr expression with Bcr siRNA, suggesting that the effect of Ang II on PPARγ is mediated largely by Bcr (Figure 3C through 3E).
Bcr Phosphorylates PPARγ via Serine 82 and Inhibits PPARγ Transcriptional Activity

Because phosphorylation of PPARγ (S82) inhibits PPARγ transcriptional activity, we hypothesized that Bcr kinase directly phosphorylates PPARγ. To examine this hypothesis, we used VSMCs to perform an in vitro kinase assay with glutathione S-transferase (GST)-PPARγ WT as substrate. VSMCs were treated with Ang II, and Bcr in vitro kinase assay was performed with GST-PPARγ WT. After 2 minutes of Ang II stimulation, Bcr kinase significantly phosphorylated GST-PPARγ WT (Figure 4A) with a time course similar to the Bcr autophosphorylation assay in Figure 2B. IgG was also nonspecifically phosphorylated, but it did not relate to Bcr kinase activity. We observed a phosphorylated protein around 60 kDa (asterisk), which correlated well with Bcr kinase activation induced by Ang II. We believe that this band represents another Bcr kinase substrate that coimmunoprecipitated with Bcr in VSMCs. Several candidate proteins have been identified using mass spectrometry analysis, but characterizing these proteins is beyond the scope of the present study.

To determine the possible role of S82 phosphorylation by Bcr kinase, we generated a point mutation replacing serine with alanine and created a GST-tagged fusion protein with
the PPARγ S82A mutant. Following immunoprecipitation of VSMCs with Bcr antibody, Bcr in vitro kinase assay was performed with GST-PPARγ WT, S82A mutant, and GST control as substrate (Figure 4B). In vitro kinase assay revealed that Bcr phosphorylates PPARγ WT, but phosphorylation of GST-PPARγ S82A was significantly reduced compared with GST-PPARγ WT, suggesting that S82 is 1 of the phosphorylation sites of Bcr kinase. No phosphorylation of GST alone was observed.

To examine whether the inhibition of PPARγ activity by Bcr kinase is via phosphorylation of PPARγ S82, we determined the effect of Bcr kinase on PPARγ transcriptional activity with mutation of S82. We overexpressed WT Bcr kinase with PPARγ WT or S82A mutant and PPRE-luc reporter gene. The cells were incubated with the PPARγ agonist ciglitazone (5 μmol/L) 24 hours after transfection. After 16 hours of ciglitazone stimulation, luciferase PPARγ transcriptional activity was assayed. As shown in Figure 4C, ciglitazone stimulated transcriptional activity of both PPARγ WT and PPARγ S82 by ≈3-fold. Bcr WT significantly inhibited PPARγ WT transcriptional activity, whereas PPARγ S82A transcriptional activity was not decreased by Bcr WT. Surprisingly, we found that Bcr WT could increase PPARγ S82A transcriptional activity, which may reflect a positive effect of Bcr WT on PPARγ transcriptional activity via a S82 phosphorylation-independent mechanism. Combined with our in vitro kinase assay result, these data suggest that Bcr inhibits PPARγ activation by PPARγ S82 phosphorylation.

Bcr Localizes in the Nucleus in VSMCs

Because PPARγ is a nuclear receptor, we next examined whether we could detect Bcr in the nucleus. As shown in supplemental Figure II, by using an anti-Bcr antibody, we observed significant immunostaining for Bcr in the nucleus in VSMCs. To confirm the specificity of Bcr antibody for endogenous Bcr, we used Bcr siRNA and determined whether Bcr siRNA, specifically designed to inhibit Bcr expression,
could reduce immunostaining detected by Bcr antibody used in this study. Bcr siRNA, but not control siRNA, significantly decreased Bcr immunostaining in the nucleus as shown in supplemental Figure II, supporting the specificity of anti-Bcr antibody. We intentionally transfected Bcr siRNA at moderate transfection efficiency (70 to 80%) to select the Bcr downregulated cells from nontransfected cells, as performed previously. The beauty of this method is that we can compare transfected and nontransfected cells in the same optical field, meaning that both cells are under the same condition, and we can observe the immunofluorescence signals of the cells under the same conditions. Therefore, the residual immunostaining in the cytosol should be nonspecific (supplemental Figure II). We did not observe significant changes in Bcr localization in cells stimulated by Ang II and PDGF-B (data not shown).

**Ang II Induced NF-κB Activation via Bcr Kinase Activation**

Because it is well known that PPARγ ligands have comprehensive antiinflammatory effects, we next examined the effect of Bcr and PPARγ on Ang II mediated NF-κB activation. The proinflammatory effect of Ang II is mediated in part by NF-κB. To test the effect of PPARγ on Ang II-mediated NF-κB activation, VSMCs were transfected for 28 hours with a NF-κB reporter plasmid. VSMCs were treated with ciglitazone for 30 minutes and then stimulated with Ang II for 16 hours. Ciglitazone inhibited Ang II mediated NF-κB activation in a dose-dependent manner (Figure 5A). We then examined whether knockdown of Bcr would inhibit Ang II–mediated NF-κB activation. Following cotransfection of VSMCs with DN-Bcr or Bcr siRNA and reporter plasmids, we found that DN-Bcr, as well as deletion of Bcr expression with Bcr siRNA, inhibited Ang II–mediated NF-κB activation in a dose-dependent manner. These data suggest that phosphor-ylation of S82 by Bcr kinase inhibits PPARγ activation. The proinflammatory effect of Ang II is downstream of Bcr (Figure 5C). We next examined the effect of PPARγ S82A mutant on Bcr induced NF-κB activation. Bcr overexpression dose-dependently increased NF-κB activation in VSMCs (Figure 6A). Overexpression of PPARγ S82A mutant, but not PPARγ WT, blocked Bcr WT–mediated NF-κB activation (Figure 6B and 6C). These data also suggest that phosphorylation of S82 by Bcr kinase inhibits PPARγ transcriptional activity, and mutation of S82 enables PPARγ to inhibit Bcr-induced NF-κB activation.

**Bcr siRNA Inhibits Ang II/PDGF-Induced DNA Synthesis**

Ang II is an important regulator of VSMC growth and induces both protein synthesis and DNA synthesis in VSMCs and enhances PDGF induced DNA synthesis. As shown in supplemental Figure III, we found that knockdown of Bcr by Bcr siRNA significantly blocked Ang II/PDGF-induced DNA synthesis assessed by [3H]thymidine incorporation. We used the combination of Ang II and PDGF to stimulate the cells, because we found that the combination of Ang II (200 nmol/L) and PDGF (10 ng/mL) maximized DNA synthesis.

**Intimal Proliferation After Decreased Blood Flow in Bcr Knockout Mice**

Our group has developed a reproducible mouse model of flow-dependent vascular remodeling that resembles human
intima–media thickening. In response to decreased blood flow intimal thickening occurs, which involves inflammation and VSMC proliferation. Based on the significant role of Bcr in VSMC inflammation and proliferation in vitro, we hypothesized that Bcr plays an important role in intimal thickening associated with decreased flow.

Immunohistochemical analysis demonstrated no difference between sham-operated WT and Bcr knockout animals (Figure 7a and 7d). In ligated arteries, vascular remodeling was seen in WT animals (Figure 7a versus 7b) but less so in Bcr knockout animals (Figure 7d versus 7e). This difference in vascular remodeling was secondary to greater neointimal proliferation in WT animals compared with knockout animals (Figure 7c and 7f). These histological findings were confirmed by morphometry (Figure 7g through 7i). In addition, with Ki-67 staining, we showed a reduction in cell prolifera-

Figure 6. Bcr-induced NF-κB activation was inhibited by PPARγ S82 mutant but not by PPARγ WT in VSMCs. A, Rat VSMCs were transfected with pNF-κB-Luc plasmids with control reporter vector. Bcr WT was transfected with indicated amounts of transfected DNA, and NF-κB transcriptional activity was performed as described in Figure 5. B and C, VSMCs were cotransfected with Bcr WT with or without PPARγ WT or PPARγ S82A. After 24 hours of transfection, the VSMCs were harvested and NF-κB activation was detected by luciferase assay. **P<0.01.

Figure 7. Knockdown or absence of Bcr inhibits cellular proliferation in vitro and in vivo. a through f, Hematoxylin/eosin staining of carotids from Bcr knockout and WT animals 2 weeks after ligation. a, Left carotid artery from sham-operated WT animal (light microscope magnification, ×10). b and c, Ligated carotid from WT animal (b, ×10; c, ×40). d, Left carotid artery from sham-operated Bcr knockout animal (×10). e, Ligated carotid artery from Bcr knockout animal (e, ×10; f, ×40). Arrows delineate the intima (c and f). Morphometric measurements of intimal area, medial area, and intima-to-media ratio 2 weeks after carotid ligation (g through i) in WT animals (n=7) and knockout animals (n=6). Shown is mean±SEM. *P<0.05.
The major findings of this study are that Bcr kinase activation by Ang II inhibits PPARγ activation and that Ang II–induced NF-κB activation occurs in part via Bcr kinase activation and subsequent inhibition of PPARγ activation. These data suggest that Bcr inhibits PPARγ activation via phosphorylation of S82. Furthermore, to our knowledge this is the first report to show that activation of Bcr kinase plays an important role in arterial proliferative disease in vivo. Ang II is an inflammatory mediator that activates NF-κB, a key component of inflammation. Previously reported data show that crosstalk between NF-κB and PPARγ is important in the proinflammatory effects of NF-κB.36 Specifically, NF-κB has been shown to block PPARγ ligand-induced transactivation in adipocytes. Our data using VSMCs and previous reports30,37 show the converse, that PPARγ inhibits NF-κB activity. Therefore, we propose that crosstalk between NF-κB and PPARγ, which are regulated by Bcr kinase, is important in regulating VSMC inflammatory gene expression. Given our novel findings that Bcr kinase inhibits PPARγ transcriptional activation and enhances NF-κB, coupled with our finding that PPARγ inhibits NF-κB activity, we believe that Bcr acts as a set point mechanism that regulates the sensitivity of VSMCs to inflammatory stimuli.

In this study, we demonstrate that Bcr is a major regulator of SMC that sits at the cross roads of inflammation and proliferation (Figure 8D). Our findings that Bcr inhibits PPARγ transcriptional activation (Figure 3) and that knock-down of Bcr with Bcr siRNA or DN-Bcr reverses Ang II inhibition of PPARγ (Figure 3) demonstrate that Bcr is a positive regulator of Ang II–mediated inflammation. We also found that both Bcr siRNA and DN-Bcr block Ang II–mediated NF-κB activation (Figure 5), demonstrating that Bcr regulation of NF-κB is a key component of regulation of inflammation by Bcr. Furthermore, both our in vitro and in vivo studies showing that knock down or absence of Bcr reduces Ang II/PDGF-induced [3H]thymidine incorporation and reduces cell growth and intimal thickening (Figures 7 and 8 and supplemental Figure III) demonstrate that Bcr regulates proliferation.

Our data suggest that the proinflammatory and proliferative effects of Bcr are mediated, at least in part, by inhibition of PPARγ and suggest that Ang II–mediated Bcr kinase activation inhibits PPARγ by phosphorylation of S82. Our demonstration of nuclear localization of Bcr in VSMCs is consistent with this concept. It remains unclear whether there are distinct differences between nuclear and cytoplasmic Bcr. Interestingly, Bcr contains a putative nuclear localization signal at amino acid 802 to 819 (http://myhits.isb-sib.ch/cgi-bin/motif_scan), but the functional consequence of this domain needs further investigation.

Overexpression of Bcr can inhibit PPARγ activation without showing any ERK1/2 activation, suggesting that Bcr inhibited PPARγ activation in an ERK1/2-independent manner. PPARγ plays an important role in regulating inflammation. PPARγ is a negative regulator of macrophage activation,30 and PPARγ agonists have been demonstrated to inhibit the production of monocyte inflammatory cytokines.10 The PPARγ agonist 15d-PGJ2 has been shown to inhibit transcription factors including NF-κB.30 The antiproliferative effect of PPARγ has several possible
mechanisms. One is a direct result of its antiinflammatory effect because cytokines and chemokines may promote lesion progression in a paracrine fashion. In addition, the PPARY agonist troglitazone has been shown to inhibit basic fibroblast growth factor–induced DNA synthesis in VSMCs and to inhibit intimal proliferation in a rat aortic balloon injury model. Troglitazone was shown to inhibit c-fos induction and to inhibit transactivation of the serum response element that regulates c-fos expression, but the exact inhibitory target of PPARY agonists against inflammation and proliferation remains unclear.

Our results do not exclude the possibility of an effect of Bcr on inflammation and proliferation that is independent of PPARY (Figure 8D). Indeed, as noted, we did find a phosphorylated protein around 60 kDa that correlated well with Bcr kinase activation induced by Ang II. Future studies will focus on PPARY-independent effects of Bcr signaling.

In conclusion, our data suggest that Bcr is an important regulator of inflammation and proliferation in VSMCs and that Bcr plays a key role in arterial proliferative disease. This effect of Bcr is mediated, in part, by inhibition of PPARY transcriptional activation via phosphorylation of PPARY by Bcr.

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Disclosures

None.

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“Bcr kinase activation by Angiotensin II inhibits PPARγ transcriptional activity in vascular smooth muscle cells”

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**Online Figure I.** *Bcr WT does not activate JNK.* After co-transfection with c-Jun plasmid and reporter plasmid as well as MEKK plasmid or Bcr WT (500 ng) or pSG5 control plasmid (500 ng), rat VSMC were serum starved for 24 hours. Cells were then harvested and dual luciferase assay performed.
Online Figure I. Bcr WT does not activate JNK. After co-transfection with c-Jun plasmid and reporter plasmid as well as MEKK plasmid or Bcr WT (500 ng) or pSG5 control plasmid (500 ng), rat VSMC were serum starved for 24 hours. Cells were then harvested and dual luciferase assay performed.

Online Figure II. Bcr nuclear localization. Mouse VSMC were transfected with Bcr siRNA or control siRNA and immunostained for Bcr (green stain) or DAPI (blue stain). Merged images show that green Bcr and dark blue DAPI on merging become light blue. VSMC were transfected with siRNA at moderate transfection efficiency (70-80%) to select Bcr down-regulated cells from non-transfected cells to allow comparison of transfected and non-transfected cells in the same optical field. Arrows show cells in which Bcr expression is not down regulated by siRNA suggesting that these cells were not transfected by siRNA. Bar is 50 µm.

Online Figure III. Knockdown of Bcr inhibits cellular proliferation in vitro. Following transfection with Bcr siRNA or control siRNA, VSMC were treated with AngII 200 nM and PDGF 10 ng/ml or vehicle for 24 hours and pulse-labeled with [3H] thymidine during the last 1 hour of incubation. **p <0.01.
Online Figure III. Knockdown of Bcr inhibits cellular proliferation in vitro. Following transfection with Bcr siRNA or control siRNA, VSMC were treated with AngII 200 nM and PDGF 10 ng/ml or vehicle for 24 hours and pulse-labeled with [³H] thymidine during the last 1 hour of incubation. **p <0.01.
Expanded Materials and Methods

Plasmids and transfection

Bcr wild-type and dominant negative Bcr (Y328F) plasmids were prepared as previously described. The single or double mutations of PPARγ were created with the QuikChange site-directed mutagenesis kit (Stratagene) as previously described. All constructs were verified by DNA sequencing. For transient expression experiments, cells were transfected with the lipofectamine plus method (Invitrogen) as previously described. For NF-κB luciferase studies, VSMC were co-transfected with pNF-κB Luc plasmid, pRL-TK plasmid and DN-Bcr or other cDNAs as indicated in each experiment. Similarly, for PPARγ relative luciferase activity, VSMC were co-transfected with either reporter plasmid PPRE-Luc, PPARγ and pRL-TK plasmid, or pBind PPARγ and PG5 Luc with other plasmids as indicated in each experiment. After treatment, cells were harvested and dual-luciferase reporter assay performed. For siRNA experiments, mouse VSMC were transfected with Bcr siRNA oligonucleotides (Invitrogen) using RNAiFect reagent (Qiagen). The sequences of the oligonucleotides used were as follows: sense: 5’-CCCAGACCCUGCAGAUAUGAUUA-3’ and anti-sense: 5’-UAACAUAGUAUCCGCAGGGUCUGGG-3. Glutathione S-transferase (GST)- PPARγ wild type and PPARγS82A mutant were created by cloning PCR-amplified DNA fragments into the EcoRI and XhoI sites of the pGEX-KG vector (Amersham).

PathDetect in vivo Signal Transduction Pathway Reporting System

c-Jun activity was measured by PathDetect Signal Transduction Pathway trans-Reporting Systems (Stratagene). Cells were cotransfected with pFR-Luc reporter plasmid and pFA2-cJun
plasmid and with other plasmids as indicated in the figures. After 24 hours of serum starvation, the cells were harvested and were assessed for luciferase activity.

**Immunoprecipitation, Bcr in vitro kinase assay, and Western blot**

After treatment with reagents, the cells were washed with PBS and harvested in 0.5 mL of lysis buffer (50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 µM Na$_3$VO$_4$, 10 mM HEPES, pH 7.4, 0.1% Triton X-100, 500 µM phenylmethanesulfonfyl fluoride, and 10 µg/ml leupeptin) and flash-frozen on liquid nitrogen. After allowing the cells to thaw, cells were scraped off the dish, sonicated and centrifuged at 10,000 rpm (4°C for 10 min), and protein concentration was determined using the Bradford protein assay (Bio-Rad). For in vitro kinase assay, immunoprecipitation was done by incubating cell lysates with mouse anti-Bcr antibody (10 µl) at 4°C overnight. The cell lysates were then incubated with 40 µl of a 1:1 ratio of protein A/protein G sepharose (Invitrogen) for 1 hour on a roller system at 4°C. The beads were washed two times with 1 ml of lysis buffer, 2 times with 1 ml of LiCl wash buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, 0.1% Triton X-100, and 1 mM dithiothreitol), and two times in 1 ml of washing buffer (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM MgCl$_2$, 1 mM dithiothreitol, and 0.1% Triton X-100). The reaction mixture including 0.1 mg/ml of indicated substrates was then placed in a thermomixer and in vitro kinase activity was measured at 30°C at 1100 rpm for 30 minutes. For Western analysis the blots were incubated for 2 hours at room temperature with Bcr antibody (Santa Cruz) or α-tubulin antibody (Sigma) followed by incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Life Science).
Carotid ligation and immunohistochemistry

Male and female mice were used in accordance with the guidelines of the National Institutes of Health and the American Heart Association for the care and use of laboratory animals. All procedures were approved by the University of Rochester Animal Care Committee. C57BL/6 /129S2 /Black Swiss Bcr knockout mice (Jackson Labs) were crossed with Black Swiss mice (Taconic) to generate Bcr +/- heterozygote animals. Non-transgenic littermate mice bred from Bcr +/- pairs were used in the experiments. Mice were anesthetized with an intraperitoneal injection of ketamine (130 mg/kg) and xylazine (8.8 mg/kg) in saline (10 mL/kg) and maintained at 37 °C on a heating pad. The left external and internal carotid branches were ligated so that left carotid blood flow was reduced to flow via the occipital artery. Carotid arteries were harvested two weeks after ligation. Cross sections were stained with hematoxylin and eosin and were analyzed using MCID image software (MCID Elite 6.0, Imaging Research). Representative samples were evaluated with Ki-67 antibody (DAKO, 1:500 dilution).

References


2. Akaike M, Che W, Marmarosh NL, Ohta S, Osawa M, Ding B, Berk BC, Yan C, Abe J. The hinge-helix 1 region of peroxisome proliferator-activated receptor gamma1 (PPARgamma1) mediates interaction with extracellular signal-regulated kinase 5 and

