Endothelial Shc Regulates Arteriogenesis Through Dual Control of Arterial Specification and Inflammation via the Notch and Nuclear Factor-κ–Light-Chain-Enhancer of Activated B-Cell Pathways

Daniel T. Sweet,* Zhongming Chen,* Christopher S. Givens, A. Phillip Owens III, Mauricio Rojas, Ellie Tzima

Rationale: Arteriogenesis, the outward remodeling of preexisting collateral arteries, is critical for recovery and restoration of blood supply to ischemic tissue after occlusion of a vessel. Arteriole–arteriole anastomoses, termed collaterals, act as a natural bypass mechanism to maintain blood supply to downstream tissue even when major arteries, such as the femoral artery, become blocked.1 Blood flow through collateral arteries in a healthy animal is negligible; however, after an occlusion, the steep pressure gradient between the preocclusive and postocclusive regions of the vessel causes blood to rush into preexisting collaterals and induces outward remodeling of the vessel. Outward remodeling of preexisting collaterals is driven primarily by the sudden spike in hemodynamic forces, especially shear stress, resulting from the increase in flow through collaterals.2 Changes in hemodynamic forces are sensed by endothelial cells (ECs),3–5 which initiate signal transduction pathways that induce cell proliferation and inflammation, including the upregulation of cell adhesion molecules on ECs and leukocyte accumulation in the vessel wall.6

ECs are equipped with numerous mechanoreceptors capable of responding to shear stress, including caveolae, ion channels, integrins, receptor tyrosine kinases, the apical glycocalyx, primary cilia, heterotrimeric G proteins, and intercellular adhesion molecules on ECs and leukocyte accumulation in the vessel wall.6

Methods and Results: Conditional knockout mice in which Shc is deleted from endothelial cells were subjected to femoral artery ligation. Hindlimb perfusion recovery was attenuated in Shc conditional knockout mice compared with littermate controls. Reduced perfusion was associated with blunted collateral remodeling and reduced capillary density. Bone marrow transplantation experiments revealed that endothelial Shc is required for perfusion recovery because loss of Shc in bone marrow–derived hematopoietic cells had no effect on recovery. Mechanistically, Shc deficiency resulted in impaired activation of the nuclear factor κ–light-chain-enhancer of activated B-cell–dependent inflammatory pathway and reduced CD45+ cell infiltration. Unexpectedly, Shc was required for arteriogenesis through the remodeling arteriole by mediating upregulation of the arterial endothelial cell marker ephrinB2 and activation of the Notch pathway. In vitro experiments confirmed that Shc was required for shear stress–induced activation of the Notch pathway and downstream arterial specification through a mechanism that involves upregulation of Notch ligands delta-like 1 and delta-like 4.

Conclusions: Shc mediates activation of 2 key signaling pathways that are critical for inflammation and arterial specification; collectively, these pathways contribute to arteriogenesis and the recovery of blood perfusion. (Circ Res. 2013;113:32-39.)

Key Words: arteriogenesis ■ NF-κB ■ Notch ■ Shc ■ shear stress

Original received March 20, 2013; revision received May 5, 2013; accepted May 9, 2013. In April 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.5 days.

From the Department of Genetics, Curriculum in Genetics and Molecular Biology (D.T.S., E.T.), Department of Cell and Molecular Physiology (Z.C., C.S.G., E.T.), Department of Medicine, Division of Hematology and Oncology (A.P.O.), and McAllister Heart Institute (M.R., E.T.), University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.301407/-/DC1.

*D.T.S. and Z.C. contributed equally to this work.
Correspondence to Ellie Tzima, PhD, 6341C Medical Biomolecular Research Building, Campus Box 7545, 111 Mason Farm Road, Chapel Hill, NC 27599. E-mail etzima@med.unc.edu

© 2013 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.113.301407
junctons.4 We previously identified a mechanosensory complex located at cell–cell junctions comprising platelet endothelial cell adhesion molecule-1, vascular endothelial (VE)-cadherin, and VE growth factor receptor-2 that is necessary and sufficient for EC response to shear stress in vitro,7 vascular remodeling,9 and arteriogenesis in vivo.9

The adaptor protein Shc mediates signaling cascades downstream of a multitude of receptor tyrosine kinases for various growth factors, cytokines, hormones, and, recently, mechanical force.10,11 Global knockout of the Shc1 gene in mice causes embryonic lethality at embryonic day 11.5 as a result of defects in embryonic heart development.12,13 Conditional knockout mice in which Shc was deleted from ECs displayed a defect in postnatal angiogenesis.14 Using an in vitro shear stress system that models the sudden increase in hemodynamic forces in collaterals immediately after femoral artery ligation, we previously demonstrated that the onset of shear stress activates Shc and induces its association with components of the mechanosensory complex (VE-cadherin and VE growth factor receptor-2), as well as integrins.15 Here, we hypothesize that endothelial Shc is required for shear stress–driven arteriogenesis and blood flow recovery in response to femoral artery ligation. Our data show impaired plantar perfusion recovery in Shc flox/flox; Tie2-Cre mice, which was dependent on Shc expression in ECs but not hematopoietic cells. Furthermore, we demonstrate that Shc is required for activation of the nuclear factor–κ-light-chain-enhancer of activated B-cell (NF-κB) pathway and downstream inflammation in collateral arteries. Surprisingly, Shc is also required for shear stress–induced activation of the Notch pathway and downstream expression of the arterial EC marker ephrinB2 through a mechanism that involves upregulation of Notch ligands delta-like 1 (Dll-1) and Dll-4.

Methods

The detailed materials and methods are described in the online-only Data Supplement.

Results

She Is Required for Plantar Perfusion Recovery After Hindlimb Ischemia

To determine the role of endothelial Shc in perfusion recovery after ischemia, Shc flox/flox; Tie2-Cre and Shc flox/flox littersmates were subjected to hindlimb ischemia by ligation of the femoral artery, which triggers shear stress–mediated adaptive remodeling of preexisting collaterals from the deep femoral artery. Blood perfusion of hind paws (plantar) was noninvasively measured using laser Doppler imaging before surgery (pre), immediately after surgery (acute), and at various time points throughout the 3-week recovery period after surgery (Figure 1A). Plantar perfusion was quantified from the Doppler images and normalized to the sham control side of the same animal to determine the percentage of perfusion of the ligated hindlimb compared with the unligated sham hindlimb. Strikingly, Shc flox/flox; Tie2-Cre mice displayed attenuated perfusion recovery as early as 5 days postsurgery (Figure 1B). The defect in perfusion recovery was exacerbated at each time point until day 21, when Shc flox/flox; Tie2-Cre mice showed a 40% reduction in plantar perfusion when compared with Shc flox/flox controls. Importantly, in wild-type mice, femoral artery ligation induced rapid Shc phosphorylation in ECs that line preexisting collateral arteries (Figure 1C; Online Figure I), indicating that endothelial Shc is activated in this model. These data demonstrate that Shc is activated in ECs during collateral remodeling and that Shc is required for perfusion recovery after hindlimb ischemia.

She Is Required for Collateral Remodeling

Plantar perfusion recovery after femoral artery ligation requires 2 EC-dependent vascular processes: arteriogenesis and angiogenesis.2,16 Ligation of the femoral artery causes a sudden increase in blood flow and hemodynamic force through preexisting collateral arteries in the gracilis muscles, causing flow-induced outward vascular remodeling, therefore allowing more blood to be carried by the collateral artery. Simultaneously, ischemia in tissues distal to the ligation, such as the gastrocnemius muscle, induces angiogenesis to increase vascular density and blood perfusion. To test whether Shc is required for arteriogenesis and angiogenesis, we examined the gracilis and gastrocnemius muscles before and 3 weeks after ligation. At day 21 after ligation, the collaterals in Shc flox/flox; Tie2-Cre mice were ≈30% smaller than those of control Shc flox/flox littermates, indicating defective arteriogenesis (Figure 1D). Importantly, no difference existed between the 2 genotypes in basal gracilis collateral size or vascular architecture of the gracilis muscle in unligated animals (Online Figure II). Similarly, induction of angiogenesis in the ischemic gastrocnemius muscle was defective in Shc flox/flox; Tie2-Cre mice at day 21. Capillary density increased by ≈50% in control Shc flox/flox mice, whereas Shc flox/flox; Tie2-Cre mice were refractory to induction of angiogenesis (Online Figure III). Because arteriogenesis and collateral remodeling are the largest contributors to perfusion recovery,17 we focused our attention on the role of Shc in collateral growth.

She Is Required for shear stress–Induced NF-κB–Dependent Inflammation in Collaterals

The attenuated plantar perfusion recovery and collateral remodeling in Shc flox/flox; Tie2-Cre mice suggest a role for Shc in arteriogenesis. The sharp increase in hemodynamic forces in the collateral induces EC proliferation and inflammation, 2 EC-dependent processes that underlie arteriogenesis. We assayed EC proliferation in collaterals of Shc flox/flox; Tie2-Cre and Shc flox/flox mice by nuclear proliferating cell nuclear antigen (PCNA) staining. Proliferation in

---

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>Dll-1</td>
<td>delta-like 1</td>
</tr>
<tr>
<td>Dll-4</td>
<td>delta-like 4</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor–κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>vascular endothelial cadherin</td>
</tr>
</tbody>
</table>
collaterals 3 days after surgery was decreased in Shc flox/flox; Tie2-Cre mice compared with Shc flox/flox controls (Figure 2A). Similarly, we assayed the role of Shc in activation of inflammation in response to femoral artery ligation. Collaterals were stained for infiltration of CD45-positive leukocytes, an important mediator of collateral remodeling. Shc flox/flox; Tie2-Cre mice exhibited a significant decrease in CD45-positive leukocyte infiltration compared with Shc flox/flox controls (Figure 2B), indicating a role for Shc in inflammation in response to hindlimb ischemia. CD45-positive cell recruitment after femoral artery ligation requires activation of NF-κB in ECs, so we tested the role of Shc in NF-κB activation in this model. Although Shc flox/flox mice showed significant activation of NF-κB as early as 24 hours after ligation, Shc flox/flox; Tie2-Cre mice displayed defects in NF-κB activation in ECs (Figure 2C; Online Figure IV). Interestingly, we also observed upregulation of p65 expression in ECs in Shc flox/flox mice that was absent in Shc flox/flox; Tie2-Cre mice. This defect in p65 nuclear localization coincided with a decrease in upregulation of the NF-κB–dependent adhesion molecule, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1 (Figure 2D; Online Figure V).

Together, these data indicate that Shc is required for EC proliferation and inflammation during collateral remodeling, both of which are critical for recovery from hindlimb ischemia.

Shc Expression in ECs, but Not Hematopoietic Cells, Is Required for Plantar Perfusion Recovery, Arteriogenesis, and Leukocyte Adhesion

The Tie2 promoter used to drive Cre expression in these experiments is expressed primarily in ECs and in a significant portion of bone marrow (BM)–derived hematopoietic cells. To determine whether Shc in ECs or hematopoietic cells determines perfusion recovery, we performed BM transplants. Four groups of mice (Shc flox/flox recipients [flox] reconstituted with Shc flox/flox [flox] or Shc flox/flox; Tie2-Cre [knockout] BM and Shc flox/flox; Tie2-Cre [knockout] recipients reconstituted with Shc flox/flox or Shc flox/flox; Tie2-Cre BM) were subjected to femoral artery ligation surgery, and plantar perfusion recovery was assessed by laser Doppler imaging (Figure 3A and 3B). The recovery of plantar perfusion was significantly decreased in Shc flox/flox; Tie2-Cre mice reconstituted with either Shc flox/flox or Shc flox/flox; Tie2-Cre BM, whereas the recovery of the Shc flox/flox
mice reconstituted with either \textit{Shc flox/flox} or \textit{Shc flox/flox; Tie2-Cre} BM was normal. Similarly, remodeling of collaterals after femoral artery ligation was defective in both the \textit{Shc flox/flox; Tie2-Cre} mice reconstituted with \textit{Shc flox/flox} BM and the \textit{Shc flox/flox; Tie2-Cre} mice reconstituted with \textit{Shc flox/flox; Tie2-Cre} BM (Figure 3C). These results suggest that Shc expressed in ECs, but not hematopoietic cells, determines arteriogenesis and plantar perfusion recovery.

To specifically test whether Shc expression in ECs and leukocytes is required for shear stress–induced leukocyte adhesion to the endothelium, we performed in vitro leukocyte adhesion assays. ECs isolated from \textit{Shc flox/flox} or \textit{Shc flox/flox; Tie2-Cre} mice were subjected to inflammatory shear stress to induce cell adhesion molecule expression and adhesion of monocytic cells (THP-1 cells) that express shRNA against Shc or a nonspecific target was assayed (Figure 3D). As expected, shear stress induced a 3-fold increase in shNS monocytic cell adhesion to \textit{Shc flox/flox} ECs. Interestingly, \textit{Shc flox/flox} ECs incubated with shShc monocytic cells exhibited a similar 3-fold induction, whereas no induction of adhesion was observed in \textit{Shc flox/flox; Tie2-Cre} ECs incubated with either shNS or shShc THP-1 cells (Figure 3D). Together, these in vitro data show that Shc expressed in ECs, but not hematopoietic cells, is required to support shear stress–induced monocytic cell adhesion. Importantly, activation of the NF-κB pathway is required for this response because adhesion to the endothelium was blocked when performed in the presence of compound A, a specific inhibitor of IκB kinase-β\textsuperscript{21} (Online Figure VI).

\textbf{Notch-Dependent Collateral EC Arterial Specification Requires Shc}

The EC phenotype is plastic and heterogeneous throughout the vascular tree, and the expression of arterial-specific and venous-specific genes is the consequence of local hemodynamic cues that may regulate vessel remodeling\textsuperscript{22,23}. Because ECs in collaterals quickly change from a low-flow environment to a high-shear-stress environment after femoral artery ligation\textsuperscript{24,25}, we hypothesized that collateral ECs adopt an arterial identity to suit the new arterial-like blood flow environment. To test this, we stained collateral tissue sections for a marker of arterial ECs, ephrinB2\textsuperscript{26}. EphrinB2 expression was upregulated in \textit{Shc flox/flox} collateral ECs within 1 to 3 days after ligation; however, ephrinB2 upregulation was attenuated in \textit{Shc flox/flox; Tie2-Cre} mice, suggesting that Shc is required for arterial specification of ECs in response to changes in the hemodynamic environment in collaterals (Figure 4A; Online Figure IV). Because ephrinB2 is transcriptionally regulated by the Notch transcription factor\textsuperscript{27}, we next examined activation of Notch in collaterals. In control

---

\textbf{Figure 2. Shc is required for collateral endothelial cell (EC) proliferation and inflammation.} Proliferation and inflammation in the vessel wall were assessed by analyzing cross-sections of collateral arteries 3 days after femoral artery ligation (or sham) surgery. A, Sections were stained with proliferating cell nuclear antigen (PCNA) to mark proliferating cells (red), smooth muscle α-actin (green), and 4′,6-diamidino-2-phenylindole (DAPI; blue). Quantitation (right) is shown as % PCNA-positive ECs. B, Quantitation of CD45-positive cell accumulation in the vessel wall 3 days after surgery. C, Staining of collaterals for NF-κB subunit p65 (red), smooth muscle α-actin (green), and DAPI (blue). Quantitation (right) displayed as % ECs with nuclear (active) p65 localization. D, Staining of collaterals for vascular cell adhesion molecule-1 (VCAM-1; red), smooth muscle α-actin (green), and DAPI (blue). Scale bars=20 μm. For all quantitation, values are provided as mean±SEM. *P<0.05. n=8 \textit{Shc flox/flox} and n=8 \textit{Shc flox/flox; Tie2-Cre}. 

---
**Shc** flox/flox mice, femoral artery ligation induced Notch activation (Notch intracellular domain nuclear localization) in collateral ECs, whereas Notch activation was impaired in Shc flox/flox, Tie2-Cre mice (Figure 4B and 4C; Online Figure IV). These data indicate that Shc is important for activation of the Notch pathway and arterial specification of the remodeling collateral arterioles.

ECs in collateral arteries experience several mechanical and chemical stimuli simultaneously during arteriogenesis, making it difficult to delineate the exact role of Shc during collateral remodeling. Therefore, we tested the role of Shc in shear stress–induced Notch activation and ephrinB2 upregulation using our in vitro system. The onset of shear stress induced expression of the Notch target genes ephrinB2, HES-1, and Deltex-1 in ECs isolated from Shc flox/flox mice. In contrast, shear stress–induced upregulation of Notch target genes was impaired in Shc knockout cells (Figure 4D). Importantly, shear stress–induced expression of Notch target genes was impaired in wild-type ECs treated with the γ-secretase inhibitor DAPT (Online Figure VII), indicating that shear stress–induced expression of these genes is Notch-dependent. To gain mechanistic insights into the role of Shc in shear stress–induced Notch activation, we assessed the expression of Notch ligands. Notch ligands Dll-1 and Dll-4 were upregulated by shear stress in vitro in Shc flox/flox but not in Shc flox/flox, Tie2-Cre ECs (Figure 4E).

Shc-dependent upregulation of Dll-4 was confirmed in collateral ECs 3 days after femoral ligation (Figure 4F), indicating that Shc controls activation of the Notch pathway by mediating shear stress–induced upregulation of canonical Notch ligands Dll-4 and possibly Dll-1.

**Discussion**

Restoration of blood flow to ischemic tissue downstream of an occlusion is a highly complex process requiring activation of several signaling pathways to induce simultaneous angiogenesis and arteriogenesis. Here, we show that the adaptor protein Shc is instrumental in orchestrating EC signaling during postischemic neovascularization after ligation of the femoral artery. Shc flox/flox, Tie2-Cre mice in which Shc was removed from ECs displayed a marked reduction in restoration of blood flow after femoral artery ligation. Histological analyses revealed defects in arteriogenesis because of impaired collateral remodeling. Mechanistically, Shc mediates vessel inflammation and activation of the transcription factor NF-κB as well as proliferation, both of which are critical for arteriogenesis. Unexpectedly, Shc is also required for arterial specification of the remodeling collateral arterioles by mediating shear stress–induced upregulation of Notch ligands and downstream activation of the Notch pathway.
including upregulation of the arterial EC marker ephrinB2 (Figure 4G).

The Notch pathway is critical for embryogenesis\textsuperscript{28} and development of the cardiovascular system,\textsuperscript{29,30} but its role in adult physiology is less well-defined. Mice heterozygous for Notch-1 or the Notch ligand Dll-1 exhibit reduced plantar perfusion recovery after femoral artery ligation because of attenuated collateral remodeling, similar to the phenotype observed in Shc flox/flox; Tie2-Cre mice.\textsuperscript{31,32} Similarly, the inhibition of Dll-4 delayed plantar perfusion recovery after femoral artery ligation because of disorganized, low-perfused angiogenic sprouting in the ischemic capillary network.\textsuperscript{34} Although the deletion of Shc does not affect basal collateral vessel organization, it is required for Dll-4 expression during collateral remodeling and in response to the onset of shear stress in vitro. Previous studies suggested that the Notch pathway is activated downstream of vascular endothelial growth factor, which is produced by ischemic tissue and drives angiogenesis. Here, we introduce an alternative model in which shear stress directly activates the Notch pathway in preexisting collateral arterioles and results in upregulation of Notch target genes during arteriogenic remodeling and ECs in culture. We show that activation of the Notch pathway by shear stress requires Shc, which mediates signal transduction downstream of the mechanosensory complex (vascular endothelial cadherin [VE-Cad] and VE growth factor receptor-2 [VEGFR2]) and integrins. Shc is required for shear stress–induced nuclear factor-κB activation, downstream cell adhesion molecule upregulation (vascular cell adhesion molecule-1 [VCAM-1]), and inflammation (blue arrows). In addition, Shc is required for shear stress–induced notch ligand upregulation (Dll-1 and Dll-4), downstream canonical Notch activation and Notch target gene expression (ephrinB2, HES-1, and Deltex-1), and arterial specification (red arrows). MCP-1 indicates monocyte chemoattractant protein-1; NICD, Notch intracellular domain; PECAM-1, platelet endothelial cell adhesion molecule-1.
and work of others suggest that shear stress induces the expression of Notch ligands, DII-1 and DII-4, which in turn activate Notch in trans it through the canonical pathway. Similarly, it remains unclear whether arterial specification of ECs in remodeling arterioles is required for, or is merely a consequence of, the remodeling process. EC-specific knockout of the arterial EC marker ephrinB2 is embryonic-lethal,13 precluding any experiments to address this question in the adult mouse.

Hemodynamic forces, such as fluid shear stress, are the primary morphogenetic cue that induces collateral remodeling after femoral artery ligation. ECs are responsible for sensing shear stress and, thus, are equipped with numerous mechanoreceptors capable of sensing and responding to changes in shear stress. Two such mechanoreceptors, integrins and a mechanosensory complex composed of EC-specific proteins platelet endothelial cell adhesion molecule-1, VE-cadherin, and vascular endothelial growth factor receptor-2, are required for activation of inflammation in response to flow and, thus, regulation of perfusion recovery in response to ischemic insult. We previously have shown that Shc binds to both integrins and the mechanosensory complex after the onset of flow15 and mediates signaling from these mechanoreceptors. Here, we show that Shc is required for the EC response to changes in shear stress in vivo. Endothelial Shc relays signals from these mechanoreceptors into whole-vessel responses, such as inflammation and arterial specification, both of which are critical for arteriogenesis and the recovery of blood flow to ischemic tissues after an occlusion (Figure 4G).

Acknowledgments

The authors thank K.S. Ravichandran for generous donation of Shc flox mice, A.S. Baldwin for generous donation of compound A, K. McNaughton and the University of North Carolina Histology Research Core Facility for histology support, and Tzima Laboratory colleagues for critical reading of the manuscript.

Sources of Funding

This work was supported by grants T32 HL069768 (D.T. Sweet) and HL088632 (E. Tzima) from the National Institutes of Health, and grants 3490004 and 0635228 N (Dr Tzima) and predoctoral fellowship 2230268 (D.T. Sweet) from the American Heart Association. E. Tzima thanks K.S. Ravichandran for generous donation of Shc flox mice and E. Tzima Laboratory colleagues for critical reading of the manuscript.

Disclosures

None.

References

Novelty and Significance

What Is Known?
- Collateral arteries reroute blood flow around an occluded artery to maintain perfusion of downstream tissue.
- Collateral arteries form during embryonic development but are unused in healthy animals unless there is an occlusion. After an occlusive event, preexisting collaterals remodel to larger, mature vessels.
- Collateral remodeling or arteriogenesis is driven, in part, by shear stress imposed on endothelial cells by blood flow.

What New Information Does This Article Contribute?
- Endothelial, but not hematopoietic, Shc is required for collateral remodeling and perfusion recovery after occlusion of the femoral artery.
- Shc regulates inflammatory signaling in collateral endothelial cells by mediating nuclear factor-κ-light-chain-enhancer of activated B-cell activation.
- Shc regulates activation of the Notch pathway and downstream arterial specification of remodeling collaterals.

Arteriogenesis, the outward remodeling of collateral arteries in response to an increase in blood flow, is essential for maintaining tissue perfusion after occlusion of a large artery. Arteriogenesis is driven by increased shear stress, but the molecular mechanism by which endothelial cells sense and respond to shear stress is incompletely understood. We conditionally deleted Shc from endothelial cells and hematopoietic cells and examined the response to femoral artery occlusion. We found that mice lacking Shc exhibited impaired perfusion recovery and collateral remodeling after femoral occlusion. In bone marrow transplantation experiments, we found that endothelial, but not hematopoietic, Shc is required for perfusion recovery. We found that Shc is the required nuclear factor-κ-light-chain-enhancer of activated B-cell activation in remodeling collaterals and for mediating shear stress–induced Notch activation via upregulation of Notch ligands δ-like 1 and δ-like 4. This leads to expression of the arterial marker ephrinB2 and arterial specification of the remodeling arteriole. These findings contribute to our understanding of arteriogenesis, and how it could be enhanced to prevent tissue death after vascular occlusions.
Activated B-Cell Pathways

Light-Chain-Enhancer of \( \kappa \)- and Inflammation via the Notch and Nuclear Factor-κ Light-Chain-Enhancer of Activated B-Cell Pathways

Daniel T. Sweet, Zhongming Chen, Christopher S. Givens, A. Phillip Owens III, Mauricio Rojas and Ellie Tzima

Circ Res. 2013;113:32-39; originally published online May 9, 2013; doi: 10.1161/CIRCRESAHA.113.301407

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/1/32

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/05/09/CIRCRESAHA.113.301407.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS:

Mice
Shc-flox mice were a kind gift from Dr. Kodi Ravichandran at University of Virginia. Tie2-Cre (B6.Cg-Tg(Tek-cre)12Flv/J) mice were purchased from Jackson Labs. All housing, breeding and experimental procedures using mice were in accordance with national guidelines and regulations and were approved by the Institutional Animal Care and Use Committee at the University of North Carolina-Chapel Hill. Male Shc flox/flox; Tie2-Cre and age-matched littermates (Shc flox/flox, 10-14 weeks) were used for all experiments. To genotype animals, DNA was isolated from ears at weaning and PCR performed. All analyses were conducted by observers blinded to animal genotype.

Femoral artery ligation surgery
The surgery procedure was performed on the right side as described previously. Briefly, animals were anesthetized with 1.125% isoflurane supplemented with oxygen, and body temperature was maintained at 37°C. Hair was removed from the hindquarters with a depilating cream. The femoral artery was exposed aseptically through a 2mm incision and isolated from vein and nerve. The femoral artery was ligated with 7-0 ligatures proximal to the bifurcation of the popliteal artery and distal to the lateral caudal femoral artery (LCFA) for the less severe ischemia mode. The incision was closed after the wound was irrigated with sterile saline.

Laser-Doppler Imaging
The animals were placed for 5min at a 37°C chamber before the measurements to avoid vasoconstriction by anesthetic heat loss. A Laser Doppler imager was used to estimate relative blood flow. Ratios of occluded over non-occluded values were compared. Noninvasive measurements of superficial hindlimb perfusion were obtained before ligation, immediately after ligation (acute), 7 and 21 days after ligation. Plantar perfusion was quantified within anatomically defined regions of interest (ROIs). All ROIs were drawn by an investigator blind to animal genotype. Data are reported as perfusion ratios of ligated vs. sham control side.

Morphometry
Collateral arteries were harvested from mice as described previously. Briefly, animals were transcardially perfused at 100 mmHg with PBS containing 10 nmol/l sodium nitroprusside and 10U/ml heparin 3 weeks after hindlimb ischemia. PBS was followed by 2% PFA for 20 min. We harvested the anterior and posterior gracilis muscles which contain two preexisting collaterals. The midzone of the muscles (i.e., the 5-mm-wide centermost section) was trimmed. A section of the calf (gastrocnemius/soleus) muscle was also harvested for the examination of capillary density (described below). Samples were embedded in paraffin and 5µm thick cross sections were H. & E. stained. Lumen diameter of collateral arteries was measured as previously described. H&E stained cross-sections within 0.5mm from the midzone of the collateral arteries in anterior and posterior gracilis muscles were digitized at 60x magnification. Lumen circumference (C) was measured interactively using NIH Image J package. For each mouse, four arteries were studied, two from the surgery-operated side and two from the sham control side. At least 4 cross-sections from each mouse were measured and the average lumen diameter of collateral vessel was used.
**Immunohistochemistry**
We used antibodies to NF–κB (1:200, BD Pharmingen, San Diego, CA), VCAM-1 (1:200, Santa Cruz), CD45 (1:100, BD Pharmingen), PCNA (1:1000, Abcam, Cambridge, MA), ephrinB2 (1:200, R & D systems, Minneapolis, MN), Cleaved Notch-1 (1:100, Abcam), Phospho-Shc (1:100, BD Transduction) and Smooth Muscle α-Actin(SMC α-actin, 1:1000, Sigma-Aldrich). Antigen retrieval was performed for cross sections with Retrogen (BD Pharmingen), except for NFκB and PCNA antibodies. Thyramide signal amplification (TSA, Perkins Elmar Inc, Waltham, MA) was performed for NFκB, VCAM-1, CD45, ephrinB2 and Cleaved Notch-1 staining, per manufacturer's instruction. Primary antibody was incubated at 4°C overnight, followed by 60 minutes for biotinylated secondary antibody (1:500), and 30 minutes for ABC complex (Elite ABC kit, Vector Laboratories, Burlingame, CA). Cy3-thyramide was used to visualize the peroxidase-binding sites. To visualize collateral media, the slides were further incubated with mouse anti-SMC α-actin for 2 hours, followed by the incubation of Cy5-goat anti-mouse Ig G (1:100) for one hour. The slides were counter stained with DAPI to visualize cellular nuclei.

**Leukocyte density**
Leukocytes in the adventitia and periadventitia of preexisting collaterals were detected with CD45 antibody as described above. CD45-positive cells having a blue nucleus surrounded by Cy3 fluorescence (from Cy3-thyramide) on their surface were counted by an observer blinded to the identity of the randomly arranged slides. Average leukocyte density was determined from 4 sections from each animal.

**Capillary density**
Capillary density was counted as described previously. Briefly, muscle fibers were harvested from the right and left side, from the m. adductor and m. gastrocnemius. The plasma membrane of capillary ECs in tissue sections was labeled with Alexa 568-IsolectinB4 (Invitrogen, CA). Micrographs were obtained with the Nikon fluorescence microscope using a 20x objective lens. Digitized images were analyzed with an image analysis package, Image J. The total number of capillaries was counted on 5 random optical fields for each mouse. Results are expressed in capillaries per muscle fiber.

**Bone marrow transplants**
Bone marrow transplants were performed as previously described. Recipient Shc flox/flox and Shc flox/flox; Tie2-Cre mice (6-8 weeks old) were irradiated with a total of 11 Gy (2 doses of 550 rads 4 hours apart) using a Cs\textsuperscript{137} irradiator. Irradiated mice were repopulated with bone marrow (approx. 2 × 10\textsuperscript{5} cells per animal) harvested from Shc flox/flox and Shc flox/flox; Tie2-Cre donor mice via retroorbital injection. Mice were allowed to recover for 4 weeks and then subjected to femoral artery ligation surgery as described above.

**Monocyte adhesion experiments in vitro**
THP-1 cells were transduced with a lentivirus as previously described. Successfully transduced cells express GFP and were collected by flow cytometry and resuspended in serum-free RPMI 1640 medium. Confluent dishes of serum starved MLECs were either kept static or exposed to 8hrs oscillatory shear stress (+/- 5 dynes/cm\textsuperscript{2}) using a cone and plate viscometer. Then 1 million THP-1 cells per dish were added onto the monolayers of ECs and incubated at 37°C for 25 min. The unbound cells were rinsed off
with PBS and the bound cells were fixed with 2% formaldehyde. To quantify the assays, five random fields under the 5x objective on an inverted microscope (DMIRB; Leica) were counted for each assay, and representative images were acquired using a RETIGA 1300 camera.

For assays using Compound A to inhibit the NF-κB pathway, the above protocol was used with the following modifications: Uninfected THP-1 cells were labeled with 1 µM CellTracker green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) and for 20 min at 37°C, then washed and resuspended in serum-free RPMI 1640. Confluent dishes of MLEC were pre-treated with 5 µM Compound A or equal volume DMSO (vehicle) for 1 hr in serum-free RPMI and then exposed to shear stress in the presence of the inhibitor.

**Cell culture and shear stress assays**

*Shc flox/flox* and *Shc flox/flox; Tie2-Cre* MLEC (isolation protocol in 5) were subjected to shear stress in vitro using a previously described cone and plate viscometer 6. 100-mm tissue culture dishes were coated in 10µg/ml Fibronectin, then MLEC were seeded and grown to confluence in EGM-2 (Lonza) with 10% FBS. Then, MLEC were serum starved overnight in M-199 (Gibco) with 0.5% FBS and exposed to an arterial level of unidirectional laminar shear stress (15 dyn/cm²) in the starvation medium for 4 hours or kept static as controls. For inhibitor experiments, MLEC were pre-treated for 1 hr with 20µM DAPT (Selleck Chem) or DMSO vehicle control in starvation media, then exposed to shear for 4 hrs in the presence of inhibitor. Immediately after treatment, dishes were washed 1x in cold PBS and frozen. MLEC were lysed in TriZol for subsequent RNA isolation.

**Real-time reverse transcription PCR**

Total RNA was extracted from MLEC using Trizol reagent (Invitrogen, Inc, Carlsbad, CA) following manufacturer’s protocol. Total RNA (5µg) was reverse-transcribed into cDNA with random primers and SuperScript II (Invitrogen) per manufacturer’s instruction. The primer pairs were as following (“-F”: forward, “-R”: reverse): GAPDH-F: 5’-GGC ATT GCT CTC AAT GAC AA -3’; GAPDH-R: 5’-TGT TGC TGT AGC GTT ATT CA -3’; ephrinB2-F: 5’-GCG GGA TCC AGG AGA TCC CCA CTG GGA CT -3’; ephrinB2-R: 5’- GTG CGC AAC TTT TCT TGA TGG TTT GAA TTA TG -3’; HES-F: 5’- ACT GAG CAG TGG TTT GAG TAT TAT GTC T -3’; Deltex-F: 5’- CAC TGG CCC TGT CCA CCC AGC CTT GGC AGG -3’; Deltex-R: 5’- GGG AAG GCG GGC AAC TCA GGC CTC AGG -3’; Delta-like 1-F: 5’- GAC AC CAA GTA CCA GTG GTA TG -3’; Delta-like 1-R: 5’- AAC CTG GTT CTC AGC AGC AGT C -3’; Delta-like 4-F: 5’- CGG GTC ATC TGC AGT GAC AAC -3’; Delta-like 4-R: 5’- ATG TGA ATT GGT GCT ATT GGC TTC GTC -3’; Jagged-1-F: 5’- CCA GCG GTC ATC TGC AGT GAC AAC -3’; Jagged-1-R: 5’- GCT GTG GTT CTG AGC TGC AAA G -3’; Jagged-2-F: 5’- GAT GCC CAC CGG GAT TGT AG -3’; Jagged-2-R: 5’- TCT CAC AGG GTG GCA AG -3’.

SYBR Green I based real-time PCR (Absolute SYBR Green ROX Mix, Thermo Fisher Scientific, Surrey, UK) was performed in a Rotor Gene thermal cycler (Qiagen, Foster City, CA) with the following thermal parameters: 95°C 15 min, followed by 40 cycles of 95°C for 30sec, 57°C for 30sec, 72°C for 30sec. Data were analyzed using relative real-time PCR quantification based on the ΔΔCt method.

GAPDH was the endogenous reference gene for ephrinB2, HES, Deltex, Delta-like 1, Delta-like 4, Jagged-1 and Jagged-2 and the control was *Shc flox/flox* static MLEC.

**Statistical analysis**

Values are presented as means ± SE. Differences was
determined by Student t-test (between two groups) and one-way ANOVA (among multiple groups). A value of $P<0.05$ was considered to indicate statistical significance.
SUPPLEMENTAL ONLINE FIGURES:

A. 

Online Figure I: Shc is transiently activated 1-3 days after femoral artery ligation (A) Staining of gracilis collaterals 3 and 21 days after femoral artery ligation surgery, or in unligated sham mice. Sections were stained phospho-Shc Y239,240 (red) and Smooth Muscle α-actin (green) and DAPI (blue).
Online Figure II: Loss of endothelial Shc does not alter baseline collateral lumen area or gracilis muscle vascular architecture

(A) Baseline collateral lumen area of gracilis collaterals was measured in H&E stained sections from unligated mice. Values are provided as mean +/- SEM. * = p<0.05 from 2 mice per genotype. (B) Vascular architecture in the gracilis muscle was assessed by staining sections with Smooth Muscle α-Actin to mark blood vessels and counting the number of large arteries, large veins, small arterioles and small venules per gracilis muscle in unligated mice. Values are provided as mean +/- SEM. from 2 mice per genotype
Online Figure III: Shc is required for ischemia induced angiogenesis following femoral artery ligation

(A) Angiogenesis response was measured in sham and ligated hindlimbs 3 weeks after femoral artery ligation surgery in Shc flox/flox and Shc flox/flox; Tie2-Cre mice. Gastrocnemius muscle was cross-sectioned and stained with TRITC-Wheat Germ Agglutinin. Capillary density was quantified as number of capillaries per muscle fiber in 5 random fields per mouse. Quantitation is shown in (B). Values are provided as mean +/- SEM. * = p<0.05. n= 8 Shc flox/flox and 8 Shc flox/flox; Tie2-Cre.
Staining of gracilis collaterals 1,3 and 21 days after femoral artery ligation surgery, or in unligated sham mice. Sections were stained for (A) NF-κB subunit p65 (red), (B) VCAM-1 (red), (C) ephrinB2 (red) or (D) cleaved Notch-1 (red) and Smooth Muscle α-actin (green) and DAPI (blue).
Online Figure V: Activation of Akt and MCP-1 by femoral artery ligation require Shc
Staining of gracilis collaterals 3 and 21 days after femoral artery ligation surgery, or in unligated sham mice. Sections were stained for (A) Akt (red) or (B) Monocyte Chemoattractant Protein-1 (red) and Smooth Muscle α-actin (green) and DAPI (blue).
Online Figure VI: The NF-κB pathway is required for shear stress induced monocyte adhesion to the endothelium.

Control Shc flox/flox MLECs were pre-treated with DMSO (Vehicle) or Compound A and exposed to shear stress or left as a static controls in the presence of lentivirus-infected THP-1 cells.
Online Figure VII: Shear stress-induced Notch target gene expression is Notch-dependent

Relative mRNA expression of Notch target genes Ephrin-B2, HES-1 and Deltex-1 in wild-type ECs. ECs were treated with 20µM DAPT (γ-secretase inhibitor) or DMSO (vehicle) and exposed to shear stress or kept static as a control. Values are mean +/- SEM (n=3 independent experiments). *= p<0.05.
SUPPLEMENTAL REFERENCES


