Notch-Dependent Regulation of the Ischemic Vasodilatory Response—Brief Report

Alex C.Y. Chang, Alexandre Patenaude, Katherine Lu, Megan Fuller, Michelle Ly, Alastair Kyle, Saeid Golbidi, Yingjin Wang, Keith Walley, Andrew Minchinton, Ismail Laher, Aly Karsan

Objective—We have recently described that Notch activates nitric oxide (NO) signaling in the embryonic endocardium. Both Notch signaling and NO signaling have been shown to be important during adult arteriogenesis. Notch has been shown to be required for remodeling of the collateral vessels, whereas NO is required for the initial vasodilatory response to ischemia. Whether Notch also has an impact on the vasodilatory phase of arteriogenesis after ischemia is not known. We tested the hypothesis that endothelial cell-Notch function is required for NO induction and vasodilation, in response to ischemia in the adult vasculature.

Methods and Results—We observed a significant decrease in NO levels in the dorsal aorta using a mouse model where Notch was inhibited in endothelial cell in a Tet-inducible fashion. In a femoral artery ligation model, inhibition of endothelial cell-Notch reduced reperfusion and NO generation, as quantified by laser Doppler perfusion imaging and by phosphoendothelial NO synthase, nitrotyrosine, and phosphovasodilator-stimulated phosphoprotein staining, respectively.

Conclusion—Endothelial Notch activation is required for NO production and reactive vasodilation in a femoral artery ligation model. (Arterioscler Thromb Vasc Biol. 2013;33:510-512.)

Key Words: arteriogenesis • ischemia • nitric oxide • notch • vasodilation

Postnatal arteriogenesis is the expansion of existing collateral vessels secondary to mechanical stress or chemical stimuli and is recognized to be important for vascular reperfusion after ischemic injury.1 During the initial vasodilatory phase, obstruction of the main artery causes increased blood flow through collateral vessels, via nitric oxide (NO)-dependent vasodilation.2 Notch signaling has been shown to play a critical role in postnatal arteriogenesis, but the mechanism of its action is not completely defined.3 Recently, we have shown that Notch activation induces NO synthesis through the release of a paracrine factor that results in phosphorylation and activation of endothelial NO synthase (eNOS).4

In this study, we examined the effect of endothelial cell (EC)-Notch inhibition on postischemic vasodilatation using a conditional mouse model, where the pan-Notch inhibitor, dominant negative Mastermind-like 1 (dnMAML), is expressed under the control of a vascular endothelial (VE)-cadherin promoter. We demonstrate that postnatal EC-Notch inhibition results in a significant decrease in basal NO in large vessels. EC-Notch inhibition blocks reperfusion of ischemic tissue because of attenuation of NO release after ischemia. These findings suggest that Notch activation is required for the initial vasodilatory phase in response to ischemia.

Materials and Methods

Animals and Hind Limb Ischemia Model

All protocols were approved by the Committee on Animal Care of the University of British Columbia (Vancouver, British Columbia). The tetracycline operator site (TetOS)-dnMAML1-green fluorescent protein (GFP) (dnMAML) transgenic mouse was generated as previously described.5 VE-cadherin-tTA (VEtTA) mice were a kind gift of L. Benjamin, Harvard Medical School (Boston, MA). Endothelial Notch inhibition was achieved as previously described.6 All of the animals were provided with doxycycline (50 μg/mL) in the drinking water to suppress dnMAML-GFP expression until 6 weeks of age. Animals were subjected to 2 weeks of Notch inhibition, followed by measurement of mean arterial pressure,6 myograph recording,6 or femoral ligation6 procedures, as previously described. For femoral ligation, mice were anesthetized under isoflurane, and from the inguinal ligament the artery was ligated distal to the third arterial branch and proximal to the fourth, the latter being just proximal to a major vessel bifurcation. This model produces less severe ischemia than standard models, as previously shown.7 Littermates were used as controls. Blood flow was monitored by laser Doppler perfusion imaging (PeriScan PIM II, Perimed, Las Vegas, NV) preligation and postligation. The extent of reperfusion was calculated by taking the perfusion ratio of the ischemic leg over the nonischemic leg using LDPI 2.6 (Perimed; Las Vegas, NV). See the online-only Data Supplemental Methods for reagents and tissue isolation.

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510
results and discussion

ec-notch inhibition does not affect basal vascular function

we have previously identified a novel link between notch and the no pathway. as targeted deletion of notch1 results in embryonic death, to determine the role of notch in the maintenance of blood pressure in the adult, we conditionally inhibited notch signaling in the endothelium starting at 6 weeks of age using vete-tet<sup>os</sup>-dnMAML mice. We did not detect a difference in mean arterial pressure between vete-tet<sup>os</sup>-dnMAML and littermate controls (data not shown) suggesting that notch activation is not critical for basal vascular homeostasis.

endothelial notch activation is required for vasodilation postischemia

previous work has shown that shear stress, through vascular endothelial growth factor induction, activates notch in EC. To evaluate the effect of endothelial notch inhibition in response to ischemia-related vasodilation, we subjected vete-tet<sup>os</sup>-dnMAML and littermate control mice to hind limb ischemia (HLI) using a selective femoral artery ligation model. Relative blood flow measurements by laser Doppler imaging revealed comparable perfusion in both vete-tet<sup>os</sup>-dnMAML and littermate controls before surgery (Figure 1A and 1B). Serial readings in littermate controls revealed rapid reperfusion in the ischemic leg immediately after and at 1- and 2-days post-HLI (Figure 1B). However, reperfusion was blunted in vete-tet<sup>os</sup>-dnMAML animals post-HLI (Figure 1B). To confirm that EC-expression of dnMAML-GFP blocked downstream notch activation, we stained for GFP to detect dnMAML expression, notch intracellular domain, and the notch downstream target Hey2 in the ischemic collaterals after 1 day. We observed colocalization of GFP staining with CD31 expression in the vete-tet<sup>os</sup>-dnMAML animals, confirming the expression of the dnMAML-GFP transgene specifically in the endothelium (Figure 1C). notch cleavage was present in both vete-tet<sup>os</sup>-dnMAML and littermate controls as expected and evidenced by presence of the notch intracellular domain form (Figure 1C). However, notch activity—as determined by upregulation of the target Hey2—was absent in vete-tet<sup>os</sup>-dnMAML animals, indicating that notch signaling was blocked (Figure 1C). These findings suggest that notch inhibition perturbs the initial vasodilatory phase that occurs consequent to ischemia.

endothelial notch activation is required for no generation and signaling postischemia

To understand the effect of EC-notch inhibition on reperfusion in the HLI model, we examined the level of NO and vascular function of thoracic dorsal aortas of the animals. There were significantly lower levels of NO in vete-tet<sup>os</sup>-dnMAML aortas compared with littermate controls (Figure 2A). However, we did not observe defects in NO-dependent vasodilation secondary to acetylcholine stimulation (Figure 1A in the online-only Data Supplement). Further, vascular contraction in response to phenylephrine was not altered indicating that notch blockade permits normal levels of endothelial-derived NO production through an independent mechanism (Figure 1B in the online-only Data Supplement). These results suggest that although EC-Notch inhibition decreases basal NO levels, compensatory mechanisms exist to maintain vascular homeostasis in the unperturbed stated.

We next examined the collateral arteries to determine whether there was a difference in vascular smooth muscle cell coverage of the vessels at 1- and 2-days after HLI (Figure II in the online-only Data Supplement). Quantification of the surface area of vascular smooth muscle cell showed no significant differences between vete-tet<sup>os</sup>-dnMAML and littermate controls (Figure II in the online-only Data Supplement) suggesting that vascular smooth muscle cell remodeling has not yet occurred at this stage, as previously reported. Quantification of eNOS activation (phospho-eNOS staining) revealed a significant difference between vete-tet<sup>os</sup>-dnMAML and littermate control animals in the ischemic collateral vessels at 1 day post-HLI (Figure 2B and Figure IIIA in the online-only Data Supplement). The lack of eNOS activation in vete-tet<sup>os</sup>-dnMAML ischemic leg was confirmed by a significant reduction in nitrotyrosine staining (surrogate for NO production).
revealed in VE-TEA-Tet<sup>OS</sup>-dnMAML animals compared with the littermate controls in the ischemic collateral vessels at 2 days post-HLI (Figure 2C and Figure IIB in the online-only Data Supplement). However, this difference in NO production between the VE-TEA-Tet<sup>OS</sup>-dnMAML and littermate controls was not observed in the nonischemic limbs (Figure 2C), suggesting that NO production occurs secondary to ischemia, and that Notch signaling is required for NO induction in this stressed state. To demonstrate downstream activation of the NO pathway, phosphorylation of vasodilator-stimulated phosphoprotein was examined. Phosphovasodilator-stimulated phosphoprotein-Ser157 staining in ischemic collaterals was significantly higher in littermate controls than VEtTA-TetOS-dnMAML animals and was localized to the EC (Figure 2D). Immunofluorescence quantification for NO (nitrotyrosine) and downstream target phosphovasodilator-stimulated phosphoprotein-Ser157 (D) was achieved by normalizing the number of positively stained pixels to cell count (DAPi) and are plotted as box-and-whisker plots.

Figure 2. Notch is required for nitric oxide (NO) signaling after hind limb ischemia (HLI). A, NO levels in VE-cadherin-tTA (VEtTA)-tetracycline operator site (Tet<sup>OS</sup>)-dominant negative Mastermind-like (dnMAML) and littermate control aortas were measured using the Griess reaction. Results are normalized to either total protein (μg) or total protein relative to body weight (g) and expressed as the mean±SE Immunofluorescence quantification for endothelial NO synthase (eNOS) activation (B, phospho-eNOS) was achieved by normalizing the number of positively stained pixels to endothelium (CD31). Immunofluorescence quantification for NO (nitrotyrosine) and downstream target phosphovasodilator-stimulated phosphoprotein-Ser157 (D) was achieved by normalizing the number of positively stained pixels to cell count (DAPi) and are plotted as box-and-whisker plots.

Heterozygous loss of Notch1 or the Notch ligand Dll1 results in defective arteriogenesis after femoral ligation. In both models, revascularization of the ischemic leg was blunted in animals with attenuated Notch activation. Our current studies suggest that immediately after ischemia, activated Notch induces EC NO production, which initiates reperfusion through vasodilation. Over the longer term, remodeling of the vessel results in a permanent expansion of the collateral. We have previously shown that Notch is also important in the remodeling phase, but secondary to activation in a smooth muscle cell precursor rather than in EC. Thus, Notch activation appears to play a role in reperfusion after ischemia, both in the immediate vasodilatory response as well as in longer term remodeling. Our findings suggest that inhibition of EC-Notch decreases eNOS activation and NO production in these animals, and when challenged with ischemia, EC-Notch inhibited animals fail to generate a vasodilatory response.

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**Disclosures**

None.

**References**

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Supplemental Material

Reagents

Rabbit anti-Hey2 and rabbit anti-phospho-Vasp-Ser157 were acquired from Abcam (Cambridge, MA). Rabbit anti-cleaved Notch1 and rabbit phospho-eNOS-S1177 were acquired from Cell Signaling Technologies (Beverly, MA). Rat anti-mouse CD31 antibody was acquired from BD Biosciences (Franklin Lakes, NJ). Rabbit anti-GFP and Alexa Fluor secondary antibodies were acquired from Invitrogen (Carlsbad, CA). Rabbit anti-nitrotyrosine was acquired from Chemicon/Millipore (Billerica, MA). Rabbit anti-smooth muscle actin (Acta2) was acquired from NeoMarkers/Termo Scientific (Fremont, CA). DAPI (4',6-diamidino-2-phenylindole) was acquired from Sigma-Aldrich (St. Louis, MO). The Griess reagent kit was obtained from Promega (Madison, WI).

Supplemental Methods

Tissue Sampling and Immunofluorescence Staining

Thoracic dorsal aortas were cleaned of surrounding tissue, cut into 2 mm rings, four aortic rings per animal were mounted into individual 5 mL wire myograph chambers (Multi Myograph Model 610 M, Danish Myo-tech, Aarhus, Denmark) and vascular contractility were measured as previously described\(^1\). Remaining thoracic dorsal aortas were collected and NO levels were measured using the Griess reaction kit according to manufacturer’s instructions.

Following HLI, mice were euthanized and perfused with 2% Heparin in PBS, in situ fixed with 2% PFA, and both the ischemic and non-ischemic thighs were excised. The tissues were then fixed with 4% PFA overnight, decalcified with 14% EDTA solution overnight, and dehydrated in 30% sucrose PBS overnight. The semi-membranous muscle containing collateral arteries was excised and separated from the femoral artery and embedded in OCT (Sakura, Japan). 10 μm transverse cryosections of ligated and contralateral non-ligated femoral arteries, and ischemic and contralateral collateral arteries were collected for each animal. Immunofluorescence staining and image capture were performed as previously described\(^2\).
Statistics

All immunofluorescence quantifications are shown as box-and-whisker plots with boxes depicting 10-90 percentiles of values obtained and the whiskers representing the range of values. Statistical estimates of significance were calculated using the Mann-Whitney t-test (n = 6 mice per group, total of 32-64 sections per group) using GraphPad Prism software. Remaining data are shown as the mean ± S.E. of multiple experiments and $P$ values were calculated using the two-tailed Student's t test. All statistical analyses were considered to be significant at $P < 0.05$. 
Supplemental Figures

Supplemental Figure I. Long-term endothelial Notch inhibition does not affect the vasodilatory response in explanted aortas. (A) Concentration-dependent relaxation to acetylcholine (Ach) with and without L-NAME (n = 5) as determined in wire myograph study of explanted aortas. (B) Concentration-dependent contraction to phenylephrine (PE) normalized to 80 mmol/L potassium chloride with and without L-NAME (n = 5).

Supplemental Figure II. EC-Notch inhibition does not affect vessel remodeling at early times post-ischemia. (A) Representative micrographs of SMA and CD31 staining of collaterals 2 days post-HLI. (B) Surface area of VSMC vessel coverage was determined by quantifying SMA immunostaining as previously described3.

Supplemental Figure III. EC-Notch inhibition results in attenuated NO pathway activation. Representative micrographs of (A) phospho-eNOS (1 day post-HLI), (B) nitro-tyrosine (2 days post-HLI), and (C) phospho-Vasp-Ser157 (2 days post-HLI), co-stained with CD31 of ischemic collaterals.
Supplemental Figure II

A

CD31/SMA/DAPI

Littermate controls

VEATA-TetOS-dnMAML-GFP

B

Box plots showing surface area for non-ischemic and ischemic limbs at 1 and 2 days post-ischemia.

Littermate controls

VEATA-TetOS-dnMAML-GFP
Supplemental Figure III

**A**
Littermate controls
VEiTA-TetOS-dnMAML-GFP

**B**
CD31/PeNOS/DAPI

**C**
phospho-VASP-Ser157/DAPI

Supplemental Figure III
