

# VEGF Receptor-2–Linked PI3K/Calpain/SIRT1 Activation Mediates Retinal Arteriolar Dilations to VEGF and Shear Stress

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**PURPOSE.** Vasomotor responses of retinal arterioles to luminal flow/shear stress and VEGF have a critical role in governing retinal blood flow possibly via nitric oxide synthase (NOS) activation. However, the cellular mechanism for flow-sensitive vasomotor activity in relation to VEGF signaling in retinal arterioles has not been characterized. We used an isolated vessel approach to specifically address this issue.

**METHODS.** Porcine retinal arterioles were isolated, cannulated, and pressurized to 55 cm H<sub>2</sub>O luminal pressure by two independent reservoir systems. Luminal flow was increased stepwise by creating hydrostatic pressure gradients across two reservoirs. Diameter changes and associated signaling mechanisms corresponding to increased flow and VEGF receptor 2 (VEGFR2) activation were assessed using videomicroscopic, pharmacological, and molecular tools.

**RESULTS.** Retinal arterioles developed basal tone under zero-flow condition and dilated concentration-dependently to VEGF<sub>165</sub>. Stepwise increases in flow produced graded vasodilation. Vasodilations to VEGF<sub>165</sub> and increased flow were abolished by endothelial removal, and inhibited by pharmacological blockade of VEGFR2, NOS, phosphoinositide 3-kinase (PI3K), calpains, or sirtuin-1 (SIRT1) deacetylase. A VEGF<sub>165</sub> antibody blocked vasodilation to VEGF<sub>165</sub> but not flow. Immunostaining indicated that VEGFR2 was expressed in the endothelial and smooth muscle layers of retinal arterioles.

**CONCLUSIONS.** Ligand-dependent and ligand-independent activation of VEGFR2 in the endothelium mediates NO-dependent dilations of porcine retinal arterioles in response to VEGF<sub>165</sub> and luminal flow/shear stress, respectively. It appears that NOS stimulation via PI3K, calpain proteases, and SIRT1-dependent deacetylation downstream from VEGFR2 activation contributes to these vasodilator responses.

**Keywords:** retinal arterioles, endothelium, vasodilation, vascular endothelial growth factor, shear stress

The retinal microcirculation lacks autonomic innervation so the regulation of retinal blood flow is largely dependent on alteration of vascular tone of the retinal arterioles in response to local mechanical<sup>1,2</sup> and chemical<sup>3</sup> stimuli. The shear stress generated by the flowing blood in a vessel exerts a tangential force on the endothelial surface, leading to activation of intracellular signal cascades and modulation of vascular function.<sup>4</sup> Dilation of resistance arterioles to increased luminal flow or shear stress, termed flow-induced vasodilation, has been indicated to have a key role in the regulation of local blood flow in many organ systems,<sup>5</sup> including the retina.<sup>2,6</sup> The increase in luminal flow has been shown to directly elicit endothelium-dependent nitric oxide (NO)-mediated dilation of isolated coronary,<sup>7</sup> mesenteric,<sup>8</sup> and skeletal muscle arterioles.<sup>9</sup> We have demonstrated recently that elevated flow also causes

dilation of porcine and human retinal arterioles via activation of NO synthase (NOS).<sup>10</sup>

An important chemical factor produced and released by the endothelium<sup>11,12</sup> and neural-glial cells<sup>13,14</sup> in the retina under normal and ischemic/hypoxic conditions is VEGF. Although several different sizes of VEGF exon splice variants have been identified, the 165-amino-acid VEGF (VEGF<sub>165</sub>) is the predominant human isoform.<sup>15</sup> It is a multifunctional protein that has been shown to promote hyperpermeability,<sup>16</sup> angiogenesis,<sup>17</sup> and hypotension<sup>18,19</sup> in in vivo studies via activation of its VEGF receptor 2 (VEGFR2; also known as Flk-1 or KDR). The observed hypotensive effect suggests that VEGF<sub>165</sub> promotes dilation of arterioles to reduce vascular resistance. In accord, in vitro studies have shown that VEGF<sub>165</sub> dilates isolated coronary<sup>20–22</sup> and retinal arterioles.<sup>23</sup> Interestingly, VEGFR2 has been implicated as one of several mechanosensors triggered

by flow/shear stress leading to transduction of mechanical stimuli into intracellular molecular signals in cultured endothelial cells.<sup>24–27</sup> However, underlying cellular and molecular signaling events contributing to dilation of retinal arterioles in response to flow and VEGF<sub>165</sub> are largely unknown. A better mechanistic understanding of these vasomotor responses is critical, considering the apparent prominent roles of blood flow and VEGF<sub>165</sub> dysregulation in the development of retinal diseases, such as diabetic retinopathy<sup>3,28</sup> and age-related macular degeneration.<sup>29,30</sup> Therefore, in the present study we used an isolated vessel approach *in vitro*, which excludes neural-glial, metabolic, and humoral influences, to specifically address roles of the endothelium and VEGFR2 signaling in dilations of porcine retinal arterioles to luminal flow/shear stress and VEGF<sub>165</sub>.

## METHODS

### Animal Preparation

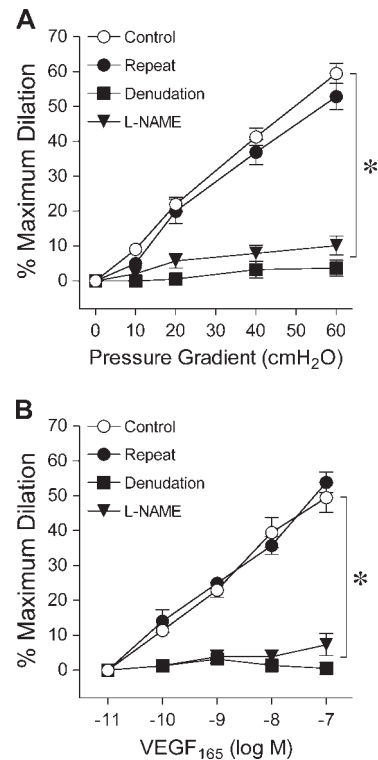
All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Scott & White Institutional Animal Care and Use Committee. Pigs of either sex (age range, 8–12 weeks; weight, 8–12 kg) purchased from Real Farms (San Antonio, TX, USA) were sedated with Telazol (4.4 mg/kg, intramuscularly), anesthetized with 2% to 4% isoflurane, and intubated. The procedure used for harvesting eyes has been described previously.<sup>31</sup>

### Isolation and Cannulation of Microvessels

The techniques used for identification, isolation, cannulation, pressurization, and visualization of the retinal vasculature have been described previously.<sup>31</sup> In brief, the isolated retinal arterioles (~40–60 μm *in situ*) were cannulated with a pair of glass micropipettes and pressurized to 55 cm H<sub>2</sub>O intraluminal pressure without flow by two independent pressure reservoir systems.<sup>10</sup> Vasomotor activity of isolated vessels was recorded continuously using videomicroscopic techniques throughout the experiments.<sup>10,31</sup>

### Study of Vasomotor Function

Cannulated, pressurized arterioles were bathed in physiological saline solution (PSS)-albumin (0.1%) at 36°C to 37°C to allow the development of basal tone (stable within 90 minutes). The vascular response to increased flow was studied under constant intraluminal pressure using dual-reservoir techniques as described previously.<sup>10</sup> In brief, the luminal flow was produced by simultaneously moving the pressure reservoirs in opposite directions of the same magnitude, which generates a pressure gradient (ΔP; range, 10–60 cm H<sub>2</sub>O) across the length of the vessel without changing intraluminal pressure.<sup>7</sup> We have demonstrated previously that the luminal flow is increased linearly with increasing ΔP and the range of mean volumetric flows for ΔP between 0 and 60 cm H<sub>2</sub>O is 0 to 34.8 nL/s (0–2.1 μL/min),<sup>7</sup> corresponding to the range reported in human retinal arterioles < 60 μm in diameter *in vivo*.<sup>32</sup> In another cohort of vessels, concentration-dependent vasomotor responses to human recombinant VEGF<sub>165</sub> (10 pM to 0.1 μM; Cell Signaling, Beverly, MA, USA) were evaluated in the absence of flow. Preliminary studies showed that the vasodilations to flow and VEGF<sub>165</sub> were reproducible and did not deteriorate at least 30 and 90 minutes after the first exposure to these stimuli, respectively (Fig. 1), so these minimal time frames were followed for all mechanistic studies



**FIGURE 1.** Vasodilator responses of isolated and pressurized porcine retinal arterioles to increased flow and VEGF<sub>165</sub>. **(A)** Retinal arterioles were exposed to stepwise increases in pressure gradient; that is, flow, in the absence (Denudation,  $n = 6$ ) or presence ( $n = 6$ ) of endothelium, or before and after a 30-minute washout period (Repeat,  $n = 6$ ) or administration of L-NAME ( $n = 5$ ). **(B)** Concentration-dependent vasodilation to VEGF<sub>165</sub> was examined in the absence (Denudation,  $n = 5$ ) or presence ( $n = 5$ ) of endothelium, or before and after a 90-minute washout period (Repeat,  $n = 5$ ) or administration of L-NAME ( $n = 5$ ). \* $P < 0.05$  versus Control.

delineated below. Vessels were exposed to each concentration of agonist or level of flow for 4 to 5 minutes until a stable diameter was maintained.

The following studies were performed to elucidate the possible cellular mechanisms involved in retinal arteriolar dilations to flow and VEGF<sub>165</sub>. First, the role of endothelium was evaluated in vessels following air bolus injection to remove endothelial cells.<sup>33</sup> Vasodilations to agonists were evaluated and compared in intact and denuded vessels from the same animal. The denuded vessels that exhibited normal basal tone, showed no vasodilation to endothelium-dependent vasodilator bradykinin (1 nM),<sup>10,33</sup> and showed unaltered response to endothelium-independent vasodilator sodium nitroprusside (10 μM) were accepted for data analysis. Second, the contributions of NOS, VEGFR2, phosphatidylinositol 3-kinase (PI3K), and sirtuin-1 (SIRT1) deacetylase were examined following at least a 30- to 60-minute incubation with their specific inhibitors L-NAME<sup>10</sup> (10 μM), SU1498<sup>27,34</sup> (1 μM; EMD Millipore, Billerica, MA, USA), wortmannin<sup>27</sup> (0.1 μM), and EX527<sup>35</sup> (5 μM; Bio-Techne/Tocris, Minneapolis, MN, USA), respectively. Third, the contribution of calpains was assessed following a 30-minute incubation with their cognate inhibitors MG132<sup>36</sup> (2 μM; Bio-Techne/Tocris) and PD150606<sup>37,38</sup> (2 μM; Bio-Techne/Tocris). Finally, vasodilator responses were obtained in another cohort before and after intraluminal administration of a VEGF<sub>165</sub> antibody<sup>39</sup> (1 μg/mL, sc-57496; Santa Cruz Biotechnology, Dallas, TX, USA).

The vasodilations to bradykinin and sodium nitroprusside also were assessed to test the function of endothelium and vascular smooth muscle, respectively, in the presence of inhibitors delineated above. We have shown previously that L-NAME does not alter dilation of porcine retinal arterioles to sodium nitroprusside.<sup>31</sup> In addition, endothelium-dependent NO-mediated vasodilation to SIRT1 activator resveratrol (30  $\mu$ M)<sup>40,41</sup> was examined in the presence of EX527, wortmannin, and MG132.

### Western Blot Analysis

Retinal arterioles of similar size to those used for functional studies were isolated and homogenized in lysis buffer. The protein content of each sample was quantified and separated by electrophoresis as described previously.<sup>42</sup> For electrophoresis, 5  $\mu$ g protein were loaded in each lane. Blotting and detection of proteins were done as described previously using a mouse anti-VEGFR2 primary antibody (1:250, sc-393163; Santa Cruz Biotechnology). After incubation with an anti-mouse secondary antibody (1:1000, sc-2005; Santa Cruz Biotechnology), the proteins were visualized via enhanced chemiluminescence (Pierce, Rockford, IL, USA).

### Immunohistochemical Analysis

Frozen sections (10- $\mu$ m thick) of retinal arterioles were fixed in cold acetone for 10 minutes and then immunolabeled with a mouse anti-VEGFR2 antibody (1:100, sc-393163; Santa Cruz Biotechnology) and a goat anti-endothelial NOS (eNOS) antibody (1:100, AF950; R&D Systems, Minneapolis, MN, USA). Images were observed using fluorescence microscopy as reported previously.<sup>42</sup>

### Chemicals

All drugs were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) except as specifically stated. Sodium nitroprusside, VEGF<sub>165</sub>, VEGF<sub>165</sub> antibody, and L-NAME were dissolved in PSS, whereas SU1498, wortmannin, MG132, PD150606, resveratrol, and EX527 were dissolved in dimethyl sulfoxide (DMSO). Subsequent concentrations of drugs in DMSO were diluted in PSS. The final concentrations of DMSO in the vessel lumen did not exceed 0.05% by volume. The 0.05% DMSO had no significant effect on vessel viability, vasodilator responses, or maintenance of tone (data not shown).

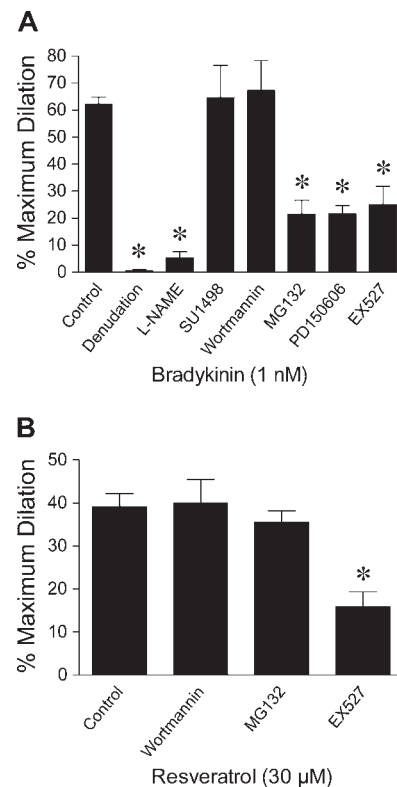
### Data Analysis

At the end of each functional experiment, the vessel was relaxed with 0.1 mM sodium nitroprusside in ethylenediaminetetraacetic acid (EDTA, 1 mM)-Ca<sup>2+</sup>-free PSS to obtain its maximum diameter at 55 cm H<sub>2</sub>O intraluminal pressure.<sup>31</sup> Diameter changes in response to vasodilator agonists and luminal flow were normalized to this maximum vasodilation and expressed as percent maximum dilation. Data are reported as mean  $\pm$  SEM and *n* value represents the number of vessels (1 per pig per treatment group) studied. Student's *t*-test or ANOVA followed by Bonferroni multiple-range test was used to determine the significance of experimental interventions, as appropriate. A value of *P* < 0.05 was considered significant.

## RESULTS

### Roles of Endothelium and NOS in Vasodilations to Flow and Agonists

In this study, all vessels (*n* = 170) developed a similar level of basal tone (constricted to 50  $\pm$  7% of maximum diameter) in

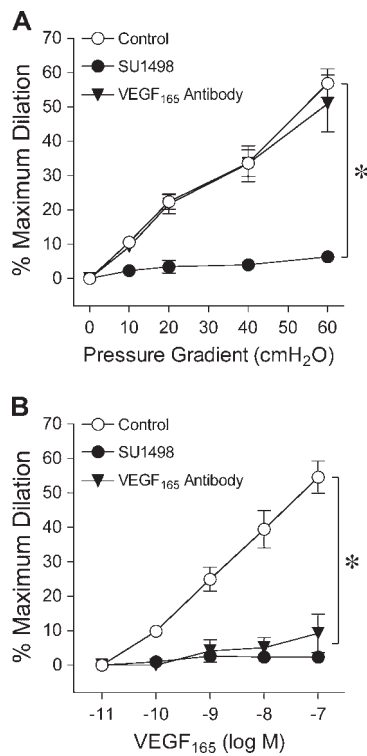


**FIGURE 2.** Vasodilator responses of isolated and pressurized porcine retinal arterioles to bradykinin and resveratrol. (A) Vasodilation to bradykinin (1 nM) was examined in the absence (Denudation, *n* = 5) or presence (*n* = 5) of endothelium, or before and after administration of L-NAME (*n* = 5), SU1498 (*n* = 4), wortmannin (*n* = 5), MG132 (*n* = 5), PD150606 (*n* = 5), or EX527 (*n* = 8). (B) Vasodilation to resveratrol (30  $\mu$ M) was examined before and after administration of wortmannin (*n* = 5), MG132 (*n* = 5), or EX527 (*n* = 5). \**P* < 0.05 versus Control.

the absence of luminal flow. The average resting and maximum diameters of the vessel were 47  $\pm$  9 and 95  $\pm$  13  $\mu$ m, respectively. The retinal arterioles exhibited graded dilation when the pressure gradient, and thus luminal flow, was increased in a stepwise manner (Fig. 1A). Under control conditions, the highest flow elicited nearly 60% of maximum dilation. In another cohort, vessels exhibited concentration-dependent dilation to VEGF<sub>165</sub> with approximately 50% of maximum dilation to the highest concentration of 0.1  $\mu$ M (Fig. 1B). Disruption of the endothelium did not affect resting basal tone (Control, 47  $\pm$  2% versus Denudation, 51  $\pm$  3%, *n* = 11, *P* = 0.24) but abolished vasodilations to flow (Fig. 1A), VEGF<sub>165</sub> (Fig. 1B), and bradykinin (Fig. 2A). The vasodilation to sodium nitroprusside (10  $\mu$ M) was not altered by endothelial removal (Control, 81  $\pm$  6% versus Denudation, 73  $\pm$  4%, *n* = 6, *P* = 0.28). In a similar manner as endothelial denudation, exposure to NOS inhibitor L-NAME nearly abolished vasodilations to flow (Fig. 1A), VEGF<sub>165</sub> (Fig. 1B), and bradykinin (Fig. 2A) without significantly affecting basal tone (Control, 52  $\pm$  3% versus L-NAME, 50  $\pm$  2%, *n* = 15, *P* = 0.18) and vasodilation to sodium nitroprusside (10  $\mu$ M; Control, 75  $\pm$  3% versus L-NAME, 79  $\pm$  6%, *n* = 4, *P* = 0.51).

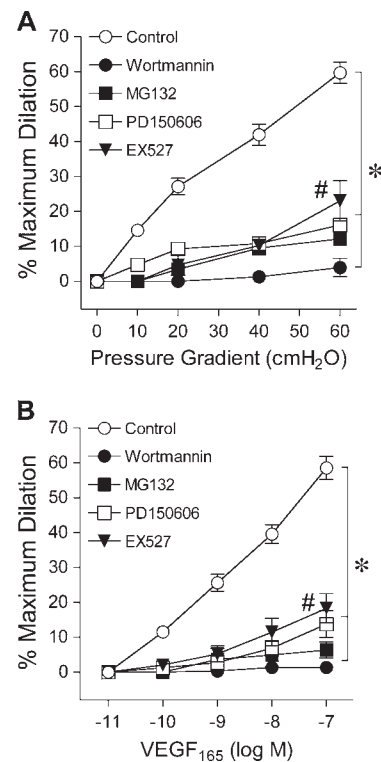
### Roles of VEGFR2, PI3K, Calpain, and SIRT1 in Vasodilations to Flow and Agonists

Intraluminal administration of VEGFR2 inhibitor SU1498 abolished vasodilations to flow (Fig. 3A) and VEGF<sub>165</sub> (Fig. 3B) but did not affect vasodilation to bradykinin (Fig. 2A) or



**FIGURE 3.** (A) Retinal arterioles were exposed to stepwise increases in pressure gradient; that is, flow, before and after intraluminal administration of SU1498 ( $n = 6$ ) or VEGF<sub>165</sub> antibody ( $n = 5$ ). (B) Concentration-dependent vasodilation to VEGF<sub>165</sub> was examined before and after intraluminal administration of SU1498 ( $n = 6$ ) or VEGF<sub>165</sub> antibody ( $n = 5$ ). \* $P < 0.05$  versus Control.

basal tone (Control,  $46 \pm 1\%$  versus SU1498,  $47 \pm 1\%$ ,  $n = 15$ ,  $P = 0.26$ ). By contrast, luminal exposure to a VEGF<sub>165</sub> antibody did not alter flow-induced vasodilation (Fig. 3A) or basal tone (Control,  $52 \pm 1\%$  versus VEGF<sub>165</sub> antibody,  $53 \pm 1\%$ ,  $n = 10$ ,  $P = 0.10$ ), whereas vasodilation to VEGF<sub>165</sub> was inhibited (Fig. 3B). In the presence of PI3K inhibitor wortmannin, vasodilations to flow (Fig. 4A) and VEGF<sub>165</sub> (Fig. 4B) were abolished, while basal tone (Control,  $49 \pm 1\%$  versus Wortmannin,  $50 \pm 2\%$ ,  $n = 20$ ,  $P = 0.32$ ), and vasodilations to bradykinin (Fig. 2A) and resveratrol (Fig. 2B) were maintained. Significant attenuation of vasodilations to bradykinin (Fig. 2A), flow (Fig. 4A), and VEGF<sub>165</sub> (Fig. 4B) was observed in the presence of calpain inhibitor MG132, whereas basal tone (Control,  $50 \pm 2\%$  versus MG132,  $51 \pm 2\%$ ,  $n = 20$ ,  $P = 0.73$ ) and vasodilation to resveratrol (Fig. 2B) were unaltered. An additional calpain inhibitor, PD150606, also diminished vasodilator responses to bradykinin (Fig. 2A), flow (Fig. 4A), and VEGF<sub>165</sub> (Fig. 4B), without altering basal tone (Control,  $50 \pm 1\%$  versus PD150606,  $50 \pm 1\%$ ,  $n = 19$ ,  $P = 0.91$ ). In another cohort of vessels, the SIRT1 inhibitor EX527 did not influence basal tone (Control,  $48 \pm 1\%$  versus EX527,  $49 \pm 1\%$ ,  $n = 24$ ,  $P = 0.31$ ) but significantly reduced vasodilations to bradykinin (Fig. 2A), resveratrol (Fig. 2B), flow (Fig. 4A), and VEGF<sub>165</sub> (Fig. 4B). The vasodilations to the highest flow (i.e., pressure gradient of 60 cm H<sub>2</sub>O) and concentration of VEGF<sub>165</sub> ( $0.1 \mu\text{M}$ ) in the presence of EX527 were significantly greater than those following wortmannin treatment (ANOVA/Bonferroni multiple-range test). Pharmacological inhibitors wortmannin, EX527, and MG132 had no effect on endothelium-independent vasodilation to NO donor sodium nitroprusside ( $10 \mu\text{M}$ ; Control,  $74 \pm 3\%$ ; Wortmannin,  $70 \pm 3\%$ ; EX527,  $74 \pm 7\%$ ; MG132,  $67 \pm 4\%$ ), suggesting that the ability of the smooth



**FIGURE 4.** (A) Retinal arterioles were exposed to stepwise increases in pressure gradient; that is, flow, before and after intraluminal administration of wortmannin ( $n = 5$ ), MG132 ( $n = 6$ ), PD150606 ( $n = 5$ ), or EX527 ( $n = 5$ ). (B) Concentration-dependent vasodilation to VEGF<sub>165</sub> was examined before and after intraluminal administration of wortmannin ( $n = 5$ ), MG132 ( $n = 4$ ), PD150606 ( $n = 8$ ), or EX527 ( $n = 7$ ). \* $P < 0.05$  versus Control; # $P < 0.05$  EX527 versus Wortmannin.

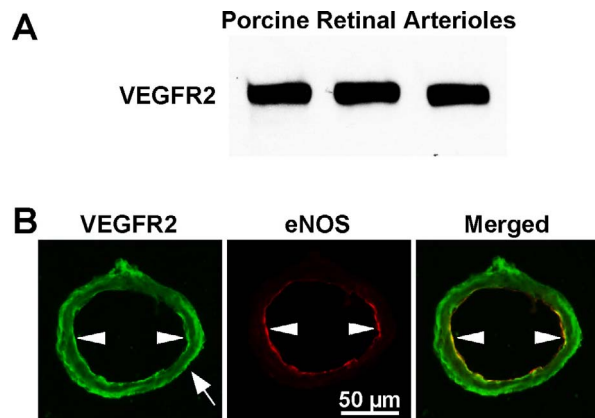
muscle to relax in response to NO remained intact following these treatments.

### Protein Expression and Localization of VEGFR2

Immunoblot staining revealed expression of VEGFR2 in retinal arterioles (Fig. 5A). For cellular localization of protein, tissue immunofluorescence analysis showed VEGFR2 staining in the endothelial and smooth muscle layers in intact retinal arterioles, with the former showing overlap with eNOS staining in the endothelium (yellow staining in merged image; Fig. 5B).

### DISCUSSION

The present study provides the first direct evidence of endothelium-dependent dilations of retinal arterioles to elevated luminal flow and VEGF<sub>165</sub>. Our findings also indicated that activation of VEGFR2 in the endothelium of retinal arterioles mediates NO-dependent dilations in response to stimuli via NOS activation. Molecular events downstream from VEGFR2 activation, such as PI3K signaling, calpain protease activity, and SIRT1-dependent deacetylation, appear to be responsible for the NOS activation. The sequential signaling pathway for these vasodilator responses is delineated in Figure 6 and discussed below. These results, which provide new insight into fundamental molecular mechanisms contributing to vasomotor regulation of retinal arterioles by VEGF<sub>165</sub> and shear stress, are critical because clinical reports indicate that NO produced

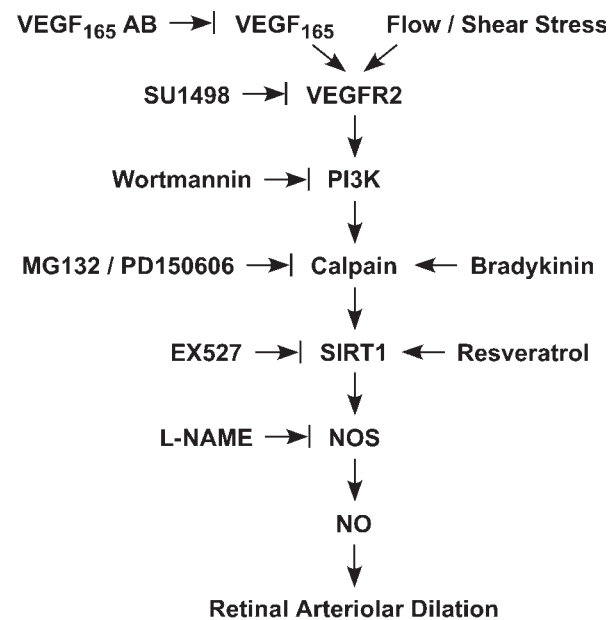


**FIGURE 5.** (A) Immunoblot detection of VEGFR2 protein expression in retinal arterioles from three pigs. (B) Immunofluorescence detection of VEGFR2 in an isolated porcine retinal arteriole. In the presence of anti-VEGFR2 (green) or anti-eNOS (red) antibodies, high levels of immunostaining were detected in the endothelium for VEGFR2 and eNOS, and in the smooth muscle layer for VEGFR2. The merged image shows overlap staining (orange-yellow) in the endothelial layer. White arrowheads denote endothelial cells and white arrow denotes smooth muscle cells. Data shown are representative of three separate experiments.

from NOS can influence retinal vascular tone and blood flow in humans.<sup>43-46</sup>

The flow-induced vasodilator response is thought to contribute to local flow regulation by recruiting blood flow to the tissue when metabolic demand is increased (e.g., functional hyperemia) or oxygen supply to the tissue is inadequate (e.g., reactive hyperemia and hypoxia).<sup>5</sup> In the clinic, flow-induced dilation of the brachial artery via ultrasound measurement following transient forearm ischemia has been used widely as an index to assess endothelial function<sup>47,48</sup> and a diminished response has been reported in type 1 diabetic patients.<sup>49-51</sup> Although physiological corroboration of this vascular phenomenon in the retina is lacking, evidence from intact animal studies suggests that hypoxia<sup>2</sup> and acute elevation of blood flow secondary to blood pressure elevation<sup>6</sup> are likely to elicit flow/shear stress-induced NO-mediated dilation of retinal arterioles. Additional studies have demonstrated that NO is released from cultured bovine retinal endothelial cells subjected to shear stress,<sup>52</sup> but the direct functional contribution of the endothelium in flow-induced dilation of retinal microvessels has not been established. To this end, we used an isolated vessel approach in the current study and found that dilation of porcine retinal arterioles to physiological levels of luminal flow<sup>32</sup> is abolished by either endothelial disruption or NOS blockade. These interventions also caused similar inhibition of vasodilation to bradykinin, which we have previously established as an endothelium-dependent NO-mediated vasodilator of human and porcine retinal arterioles.<sup>10,33</sup> In concert, our current findings support the notion that luminal flow causes endothelium-dependent dilation of retinal arterioles via activation of NOS and consequent NO release.

The retinal vasomotor tone also may be regulated by endogenous chemical substances such as VEGF<sub>165</sub>. Experimental studies have shown that intravitreal administration of VEGF<sub>165</sub> elicits retinal vasodilation in rats,<sup>53</sup> rabbits,<sup>54</sup> and monkeys.<sup>55</sup> In addition, a recent isolated vessel study showed that VEGF (molecular size was not reported) caused dilation of porcine retinal arterioles precontracted with endothelin-1, although the confounding effect of the precontractor on vasomotor function was not assessed.<sup>23</sup> At the clinical level,



**FIGURE 6.** The proposed signaling pathway involved in VEGF<sub>165</sub> and flow/shear stress-induced dilations of retinal arterioles. Evidence from our studies provides support for ligand-dependent and ligand-independent activation of VEGFR2 in the endothelium mediating NO-dependent dilations of retinal arterioles in response to VEGF<sub>165</sub> and increased flow/shear stress, respectively. The activation of endothelial VEGFR2 leads to stimulation of PI3K and downstream link to calpain proteases and subsequent SIRT1-dependent deacetylation of NOS for NO production and arteriolar dilation. Bradykinin also can elicit a calpain/SIRT1-dependent vasodilator response, whereas resveratrol activates SIRT1 downstream from calpain leading to NO-mediated vasodilation. Blockade of the proposed signaling pathways by their respective inhibitors is indicated with vertical lines in reference to the direction of the arrows. AB, antibody.

evidence has shown that intravitreal injection of anti-VEGF antibodies (bevacizumab/Avastin or ranibizumab/Lucentis: nonselectively bind and inhibit VEGF isoforms) can significantly reduce retinal arteriolar diameter<sup>29,56-59</sup> and retinal blood flow<sup>29</sup> in humans with retinal disorders, such as branch retinal vein occlusion<sup>56</sup> and neovascular age-related macular degeneration.<sup>29,57-59</sup> In colon cancer patients, retinal vasodilation to flickering light, an indication of NOS function in response to retinal metabolic activation,<sup>46,60</sup> was significantly reduced by intravenous administration of bevacizumab.<sup>61</sup> Conversely, some clinical studies observed that a significant change in retinal arteriolar diameter was not apparent under resting<sup>62,63</sup> or flicker-stimulated<sup>63</sup> conditions in patients with diabetic macular edema subjected to intravitreal anti-VEGF therapy. Although the reason for these inconsistent clinical findings on anti-VEGF therapy remains unclear, we recently demonstrated a compromised endothelium-dependent, NO-mediated dilation of retinal arterioles in diabetic pigs.<sup>64</sup> Because VEGF<sub>165</sub> and shear stress exert NO-mediated vasodilation as shown in the present study, the observed absence of a significant vasomotor effect of anti-VEGF therapy in those diabetic patients<sup>62,63</sup> is consistent with our findings in diabetic pigs.

In the present study, we found that the retinal arterioles, with basal myogenic tone, were sensitive to VEGF<sub>165</sub> with threshold dilation in the high picomolar range. These vasoactive levels are slightly above the mean vitreous VEGF<sub>165</sub> concentrations in the range of approximately  $5 \times 10^{-13}$  to  $1 \times 10^{-11}$  M (determined from reported mean values in pg/mL using conversion factor of 40,000 g/mol for VEGF<sub>165</sub>) obtained from control subjects<sup>65-69</sup> in clinical studies of human retinal

disease. Although the VEGF<sub>165</sub> concentration is expected to be significantly higher at the local retinal vascular wall, its actual level remains to be determined. In fact, the mean vitreous VEGF<sub>165</sub> concentrations reported in human retinal pathology (diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion)<sup>65-70</sup> vary from approximately  $2 \times 10^{-11}$  to  $2 \times 10^{-9}$  M and are within the range that elicited retinal arteriolar dilation in the current study. Notably, the  $1 \times 10^{-10}$  to  $1 \times 10^{-9}$  M VEGF<sub>165</sub> caused approximately 10% to 20% dilation of retinal arterioles, which would result in a nearly 30% to 110% reduction in resistance to blood flow through an individual vessel in the retinal vascular bed based on Poiseuille's equation (resistance inversely proportional to fourth power of vessel radius).<sup>71</sup> This alteration of tone at the level of arterioles < 60  $\mu$ m in diameter in the retinal microcirculation is likely to contribute significantly to blood flow change, because a marked pressure drop (i.e., 50%) across these vessels was observed in the feline retina.<sup>72</sup> In regards to cell signaling, the vasodilator response of retinal arterioles was abolished by endothelial denudation and NOS blockade, supporting the essential role of endothelial NOS in mediating VEGF<sub>165</sub>-induced vasodilation.

At the level of the resistance vasculature, previous pharmacological evidence suggests that VEGF<sub>165</sub> causes dilation of rat coronary arterioles via activation of VEGFR2.<sup>22</sup> Similarly, selective blockade of tyrosine phosphorylation and activity of VEGFR2 with SU1498<sup>34</sup> inhibited dilation of retinal arterioles to VEGF<sub>165</sub> in the present study. Interestingly, administration of a VEGFR2 antibody in normal mice caused a significant increase in systemic blood pressure,<sup>73</sup> suggesting a tonic regulation of microvascular resistance by endogenous VEGF and VEGFR2 under resting conditions. It is worth noting that the influence of VEGFR2 signaling on vascular tone may extend beyond ligand activation, because VEGFR2 blockade reduces flow-induced dilation of hamster cheek pouch arterioles *in vivo*<sup>27</sup> and of rat coronary arterioles *in vitro*.<sup>22</sup> A comparable mechanism appears to be prevalent in the retinal microcirculation, because VEGFR2 blockade with SU1498 abolished flow-induced dilation of retinal arterioles in the present study. The ability of SU1498 to inhibit dilation of retinal arterioles to VEGF<sub>165</sub> without altering dilation to bradykinin receptor activation supports the specific action of this antagonist on VEGFR2. Additional data showing that treatment of retinal arterioles with a VEGF<sub>165</sub> antibody inhibited vasodilation to VEGF<sub>165</sub> but did not alter flow-induced vasodilation provide inferential evidence that the flow-stimulated VEGFR2 activation is ligand-independent. Evidence for prominent endothelial VEGFR2 protein expression in retinal arterioles was provided by immunohistochemical analysis. Although VEGFR2 expression also was detected in the smooth muscle layer, the lack of vascular reactivity to VEGF<sub>165</sub> and shear stress in the absence of endothelium implies its nonvasomotor function, possibly involvement in migration,<sup>74</sup> for this cellular receptor. Collectively, our functional and molecular data demonstrated that endothelial VEGFR2 activation mediates ligand-dependent dilation to VEGF<sub>165</sub> and ligand-independent dilation to luminal flow in retinal arterioles.

The underlying mechanisms for flow-sensitive vasomotor activity in relation to VEGFR2 signaling in retinal arterioles are largely unknown. A reasonable target to consider was calpains, which are a family of cytoplasmic calcium-dependent proteases that can translocate to the cell membrane upon activation by shear stress<sup>75</sup> or VEGF<sub>165</sub>.<sup>76</sup> Our present data showed that blockade of calpains attenuates flow- and VEGF<sub>165</sub>-induced vasodilations, which provides the first functional evidence for a role of these proteases in vasomotor regulation. Moreover, it appears that the calpain-dependent pathway is triggered in response to other endothelium-dependent agonists, because

vasodilation to bradykinin also was diminished by calpain blockade. The two major calpain isoforms are calpain 1 ( $\mu$ -calpain) and calpain 2 (M-calpain),<sup>77</sup> which are blocked by the nonselective calpain inhibitors, MG132 and PD150606, used in the present study. Previous studies in cultured human retinal microvascular endothelial cells reported that VEGF<sub>165</sub> treatment caused a greater increase in calpain 2 than calpain 1 activity.<sup>78</sup> The contribution of a specific calpain isoform linked to endothelial NO-mediated dilation in retinal arterioles merits future research.

Another signaling molecule that was found to be involved in the vasomotor regulation in the present study was PI3K, which has been suggested to be phosphorylated and activated by VEGFR2 following shear stress<sup>26</sup> and VEGF<sup>79</sup> stimulation in cultured endothelial cells. We found that PI3K inhibitor wortmannin abolishes dilations of retinal arterioles to VEGF<sub>165</sub> and flow, suggesting that these responses are dependent on PI3K signaling. These findings are consistent with previous studies showing that wortmannin blocks NO production in endothelial cells stimulated with VEGF<sub>165</sub><sup>76</sup> or flow/shear stress.<sup>27</sup> However, its relationship with calpain remains unclear. In the current study, the inability of PI3K blockade to inhibit calpain-dependent dilation of retinal arterioles to bradykinin (Fig. 2) suggests that calpain activation is downstream from PI3K in response to VEGF<sub>165</sub> and flow (Fig. 6). This idea is supported by earlier evidence showing that shear stress elicited PI3K-dependent activation of calpain 2 in human umbilical vein endothelial cells,<sup>80</sup> although its signaling to NOS activation was not studied.

The activity of eNOS can be regulated in part at the post-translational level by acetylation of the enzyme. Accumulating evidence indicates that SIRT1-dependent deacetylation of lysine residues in the calmodulin-binding domain of eNOS leads to increased enzyme activity.<sup>41,81</sup> The SIRT1 enzyme is a member of the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases, which are involved in regulation of metabolism, stress responses, and senescence.<sup>82</sup> *In vitro* studies have shown that blockade of SIRT1 diminishes endothelium-dependent relaxation of vasculatures.<sup>41,83</sup> In addition, exposure of cultured human umbilical vein endothelial cells to laminar shear stress has been shown to promote SIRT1-dependent eNOS deacetylation.<sup>81</sup> The present findings underpin the notion that SIRT1 contributes to the transduction of a hemodynamic signal to NO-mediated vasodilation, because selective SIRT1 inhibitor EX527<sup>35,84</sup> reduced retinal arteriolar dilation to increased flow. Support for the inhibitory action of EX527 on SIRT1 linked to eNOS activation was provided by the ability of the drug to attenuate vasodilation to resveratrol, a polyphenolic activator of SIRT1<sup>41,85</sup> that we have previously shown causes endothelium-dependent NO-mediated dilation of porcine retinal arterioles.<sup>40</sup> It appears that SIRT1 deacetylation of eNOS may be a general mechanism contributing to regulation of NO production in retinal arterioles, because vasodilations to NO-mediated agonists bradykinin and VEGF<sub>165</sub> also were diminished. In addition, the arteriolar dilation to SIRT1 agonist resveratrol remained during calpain blockade, suggesting that SIRT1 activation occurs downstream from calpain in response to VEGFR2-dependent stimuli (Fig. 6). The inability of EX527 to completely prevent vasodilation to the NO-mediated stimuli may be due to a lower than maximally effective concentration of the drug.<sup>35</sup> It is worth noting that we were limited to 5  $\mu$ M EX527 because higher concentrations of this drug contained high levels of solvent DMSO that caused nonspecific inhibition on vasodilator function of retinal arterioles. Nonetheless, our data supported a significant role for SIRT1 in NO-mediated dilation of retinal arterioles in response to chemical and mechanical stimuli, and contributed

to the emerging evidence on the protective roles of SIRT1 in ocular disease development.<sup>86</sup>

In summary, we found that VEGFR2 activation in the endothelium of porcine retinal arterioles leads to NO-mediated dilations in response to VEGF<sub>165</sub> and shear stress stimulations. The underlying molecular events downstream from VEGFR2 activation linking to NOS stimulation appear to include a PI3K/calpain/SIRT1 cascade. Prudent evaluation of this signaling pathway in retinal arterioles under pathological conditions is warranted because aberrant endothelial<sup>44,64</sup> and arteriolar<sup>3,28</sup> functions may contribute to pathogenesis of retinal ischemic diseases.

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