

# Role of Arginase in Selective Impairment of Endothelium-Dependent Nitric Oxide Synthase-Mediated Dilatation of Retinal Arterioles during Early Diabetes

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**PURPOSE.** Retinal vasomotor activity can be regulated by two major endothelial enzymes, nitric oxide synthase (NOS) and cyclooxygenase (COX). The vascular arginase also consumes a NOS substrate and thus impedes NOS-mediated vasodilation. Diabetes mellitus exhibits vascular complications in the retina with elevated oxidative stress and compromised NOS-mediated vasodilation. However, the underlying molecular mechanisms remain unclear, and the effect of diabetes on COX-mediated vasodilation is unknown. Herein, we examined the relative impact of diabetes on retinal arteriolar dilations to COX and NOS activation and the roles of arginase and superoxide in diabetes-induced vasomotor dysfunction.

**METHODS.** Retinal arterioles were isolated from streptozocin-induced diabetic pigs (2 weeks of hyperglycemia,  $433 \pm 27$  mg/dL) or age-matched control pigs ( $97 \pm 4$  mg/dL). The vasodilations to bradykinin (NOS activator) and histamine (NOS/COX activator) were examined in vitro.

**RESULTS.** Retinal arteriolar dilations to histamine and bradykinin were significantly reduced after 2 weeks of diabetes. The NOS inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) attenuated the dilations of control vessels, but not diabetic vessels, to histamine. In the presence of L-NAME and COX inhibitor indomethacin, histamine-induced dilations of control and diabetic vessels were reduced similarly. Treatment of diabetic vessels with arginase inhibitor nor-NOHA, but not superoxide dismutase mimetic TEMPOL, preserved both histamine- and bradykinin-induced dilations in an L-NAME-sensitive manner.

**CONCLUSIONS.** Arginase, rather than superoxide, impairs endothelium-dependent NOS-mediated dilatation of retinal arterioles during diabetes, whereas vasodilation mediated by COX remains intact. Blockade of vascular arginase may improve endothelial function of retinal arterioles during early onset of diabetes.

**Keywords:** arginase, diabetes, nitric oxide, retinal arterioles, vasodilation

Microvascular complications are major contributors to the pathologic development of retinopathy in patients with diabetes mellitus and consequently cause vision loss.<sup>1</sup> Several studies have demonstrated reduced retinal blood flow in the early stages of experimental<sup>2-5</sup> and human type 1 diabetes,<sup>6-8</sup> suggesting that dysfunction of resistance arterioles, the major microvascular site for blood flow regulation, may contribute to the development of retinopathy. It is well documented that the vascular endothelium plays a key role in regulating vasomotor function of retinal arterioles. Two important enzymes in the vascular endothelium that influence vasomotor function are nitric oxide synthase (NOS) and cyclooxygenase (COX). Activation of NOS produces NO,<sup>9,10</sup> whereas COX activation leads to the generation of prostanoids such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prostaglandin I<sub>2</sub> (PGI<sub>2</sub>).<sup>11</sup> These three vasoactive molecules can cause dilation of retinal arterioles.<sup>12-14</sup> Clinical evidence has shown that both NOS<sup>15-18</sup> and COX<sup>19</sup> play important roles in retinal blood flow regulation in healthy

humans, and blockade of these two enzymes reduces retinal perfusion. Endothelial dysfunction, manifested as impaired endothelium-dependent NOS-mediated dilatation, has been implicated as an early event in the development of diabetic complications in the retinal arterioles.<sup>20,21</sup> However, there is limited information about the molecular mechanisms contributing to the NOS deficiency in retinal arterioles,<sup>20,22</sup> and the effect of diabetes on COX-mediated dilatation of these microvessels remains unknown.

A potential factor affecting retinal microvascular function during diabetes is oxidative stress.<sup>23</sup> Elevation in oxidant molecules such as superoxide anions is associated with diabetic retinopathy.<sup>24</sup> Superoxide has the ability to scavenge and reduce the vasodilator function of NO,<sup>25</sup> and to diminish the production of COX-derived PGI<sub>2</sub>.<sup>26</sup> Oxidative stress in the endothelium may also lead to upregulation of arginase, an enzyme that can compete with NOS for their common substrate L-arginine.<sup>27,28</sup> Increased arginase activity limits the availability of the precursor L-arginine for

endothelial NOS to produce NO.<sup>29–31</sup> A previous *in vivo* study reported that pharmacologic blockade of arginase or deletion of one copy of the arginase I gene in diabetic mice preserved dilation of retinal arterioles in response to systemic administration of endothelium-dependent agonist acetylcholine.<sup>32</sup> However, whether superoxide contributes to the impact of arginase on retinal arteriolar dilation during diabetes has not been investigated. Therefore the objective of this study was to examine the impact of type 1 diabetes on endothelium-dependent dilations of retinal arterioles in response to NOS and COX activations, and to investigate the contributions of arginase and superoxide to these vasodilator responses. Disturbance of the retinal microcirculation by type 1 diabetes was studied in pigs, which resemble human retinal structure<sup>33</sup> and vasomotor function.<sup>20</sup> We used an isolated vessel approach, excluding the confounding influences from hemodynamic, neural glial, and humoral factors, to directly examine the effect of diabetes on endothelium-dependent vasodilation.

## METHODS

### Porcine Diabetes Model

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Baylor Scott & White Institutional Animal Care and Use Committee. Domestic (Yorkshire) male pigs (8–12 weeks old, 8–17 kg) were purchased from Real Farms (San Antonio, TX, USA). Type 1 diabetes was induced by selective ablation of pancreatic  $\beta$ -cells with intravenous injection of streptozocin (STZ; Zanosar, 200 mg/kg in saline solution) via an ear vein (20 diabetic pigs), as described in detail in our previous studies.<sup>20,22</sup> The control group was intravenously injected with saline solution (24 control pigs). The pigs were maintained for a period of 2 weeks. The health condition and body weight were closely monitored in all pigs. Fasting blood glucose levels were obtained every other day in the morning with a Bayer Contour glucometer. Pigs were treated with insulin (Humulin 70/30, 2–8 units; Lilly; Indianapolis, IN, USA) if the fasting blood glucose was sustained above 600 mg/dL to keep the level between 250 and 600 mg/dL, but insulin was not given 48 hours before terminal surgery. After the 2-week time period, pigs were sedated with Telazol (4–8 mg/kg, intramuscularly), anesthetized with 2% to 5% isoflurane, and intubated for eye harvesting as described previously.<sup>34,35</sup>

### Isolation and Cannulation of Retinal Arterioles

The techniques used for visualization, identification, isolation, cannulation, and pressurization of the retinal vasculature have been described elsewhere.<sup>34,35</sup> In brief, the isolated retinal arterioles (~20–60  $\mu$ m in situ diameter) were cannulated on each end with glass micropipettes containing a physiological saline solution (PSS)–albumin (1%)<sup>34</sup> and pressurized to 55 cmH<sub>2</sub>O intraluminal pressure without flow by two independent pressure reservoir systems. The inner diameter of retinal arterioles was recorded using videomicroscopic techniques throughout the experiments.<sup>36</sup>

### Study of Vasomotor Function

Cannulated and pressurized arterioles were bathed in PSS-albumin at 36°C to 37°C to allow the development

of basal tone (stable within 90 minutes). To evaluate the effect of diabetes on vasomotor function, endothelium-dependent vasodilation to histamine (0.1 to 30  $\mu$ mol/L)<sup>37</sup> and bradykinin (1 pmol/L to 10 nmol/L)<sup>25</sup> were established in vessels isolated from the diabetic and euglycemic control pigs. Vessels were exposed to each concentration of agonist for 2 to 3 minutes until a stable diameter was maintained. The roles of NOS and COX in vasodilations were examined using the specific inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 10  $\mu$ mol/L, 40-minute incubation)<sup>37,38</sup> and indomethacin (10  $\mu$ mol/L, 40-minute incubation),<sup>35,37</sup> respectively. For some vessels, the relationship between NOS and arginase in the vasodilations to histamine and bradykinin was evaluated in the presence of L-NAME with or without the arginase inhibitor nor-NOHA (0.1 mmol/L intraluminal treatment for 90 minutes; Cayman Chemical; Ann Arbor, MI, USA).<sup>28,39</sup> To assess the involvement of arginase and superoxide in the diabetes-induced effect, the vasodilator responses were examined after intraluminal treatment of vessels with nor-NOHA (0.1 mM)<sup>28,39</sup> or superoxide dismutase mimetic TEMPOL (1 mmol/L),<sup>38,39</sup> respectively, for 90 minutes.

### Chemicals

All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) except as specifically stated. Indomethacin was dissolved in ethanol, whereas all other drugs were dissolved in PSS. The final concentration of ethanol in the vessel bath did not exceed 0.1% by volume. The 0.1% ethanol had no significant effect on vessel viability, vasodilator responses, or maintenance of basal tone (data not shown).

### Data Analysis

At the end of each experiment, the maximum diameter of the vessels was obtained at 0.1 mmol/L sodium nitroprusside in the presence of calcium-free PSS with ethylenediamine tetra-acetic acid (1 mmol/L).<sup>34</sup> Diameter changes in response to vasodilator agonists 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 (one per pig per treatment group) studied. Student's *t*-test or analysis of variance followed by the Bonferroni multiple-range test was used to determine the significance of experimental interventions, as appropriate. A value of *P* < 0.05 was considered significant.

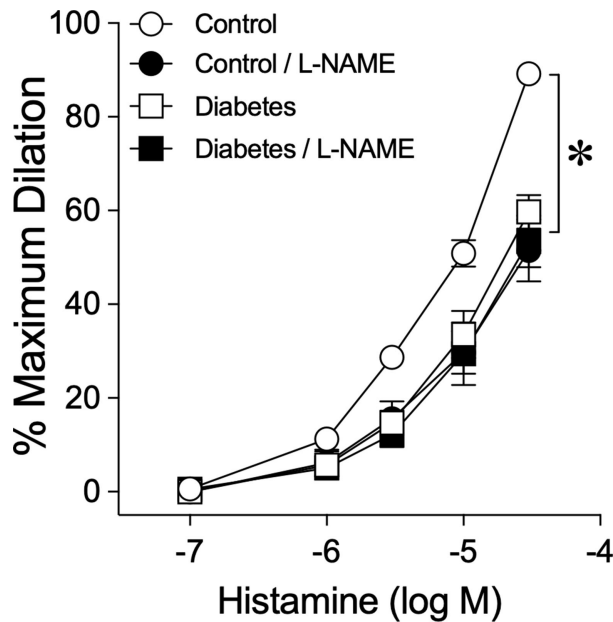
## RESULTS

### Animal Model of Diabetes Mellitus

Two weeks after STZ injection, blood glucose in pigs elevated from 89  $\pm$  12 mg/dL to 433  $\pm$  27 mg/dL. Pigs injected with saline solution (control) had comparable blood glucose levels after 2 weeks: 89  $\pm$  4 mg/dL versus 97  $\pm$  4 mg/dL after saline injection. The body weight gain was less for diabetic (before STZ injection: 12.5  $\pm$  0.5 kg; 2 weeks after STZ: 14.2  $\pm$  0.6 kg) than for control pigs (before saline injection: 13.3  $\pm$  0.5 kg; 2 weeks after saline injection: 24.2  $\pm$  0.7 kg).

### Effect of Diabetes on NOS- and COX-mediated Vasodilations

Retinal arterioles from control (*n* = 26 vessels) and diabetic (*n* = 26 vessels) pigs developed a comparable level of basal

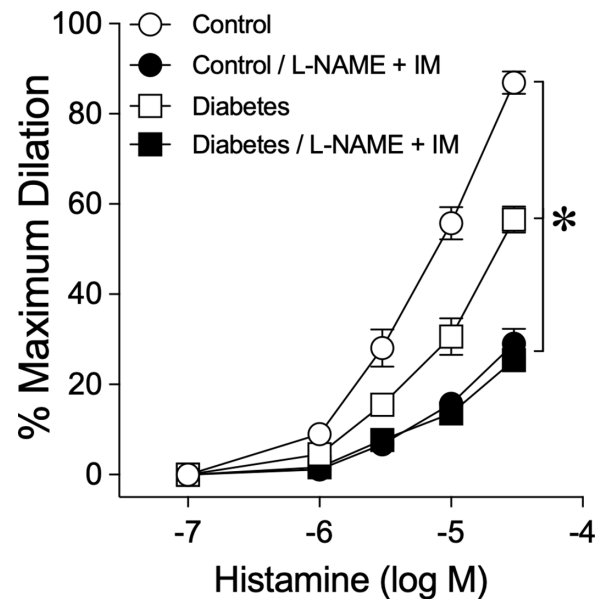


**FIGURE 1.** Diabetes impairs NOS-mediated dilation of retinal arterioles. Concentration-dependent dilation of isolated and pressurized retinal arterioles to histamine was examined in the absence or presence of NOS inhibitor L-NAME after 2 weeks of euglycemia (control;  $n = 6$ ) or hyperglycemia (diabetes;  $n = 6$ ) in pigs. \* $P < 0.05$  versus control.

tone (Control:  $56 \pm 2\%$  of maximum diameter,  $96 \pm 2 \mu\text{m}$  versus Diabetes:  $58 \pm 2\%$  of maximum diameter,  $97 \pm 2 \mu\text{m}$ ;  $P = 0.47$ ). In one cohort, retinal arterioles from control pigs dilated concentration-dependently to histamine with maximum dilation of about 90% at  $30 \mu\text{mol/L}$  (Fig. 1). In the presence of NOS inhibitor L-NAME, the resting basal tone was not altered (Control:  $63 \pm 3\%$  of maximum diameter versus Control/L-NAME:  $61 \pm 3\%$  of maximum diameter;  $P = 0.06$ ), but the histamine-induced vasodilation was significantly reduced (Fig. 1). After 2 weeks of diabetes, the dilation of retinal arterioles to histamine was compromised (60% maximum dilation at  $30 \mu\text{mol/L}$ ) in a manner comparable to that produced by L-NAME (Fig. 1). This residual vasodilation to histamine was insensitive to L-NAME (Fig. 1). In both control and diabetic vessels, combined treatment with L-NAME and indomethacin reduced the dilation to histamine in a similar manner with maximum dilation of about 25% at  $30 \mu\text{mol/L}$  (Fig. 2).

### Roles of Arginase and Superoxide in Diabetes-induced Vasodilator Dysfunction

In another cohort, the relative contributions of arginase and superoxide to the diabetes-induced impairment of vasodilation were assessed. After 2 weeks of diabetes, the dilations of retinal arterioles to histamine (Fig. 3A) and bradykinin (Fig. 3B) were significantly reduced. Intraluminal treatment with arginase inhibitor nor-NOHA restored the dilations of diabetic vessels to histamine (Fig. 3A) and bradykinin (Fig. 3B), in a manner sensitive to L-NAME (Figs. 3A and 3B). In contrast to nor-NOHA, superoxide dismutase mimetic TEMPOL did not improve the dilations of diabetic vessels to histamine (Fig. 4A) and bradykinin (Fig. 4B). Administration of nor-NOHA (Fig. 3) or TEMPOL (Fig. 4) had no effect



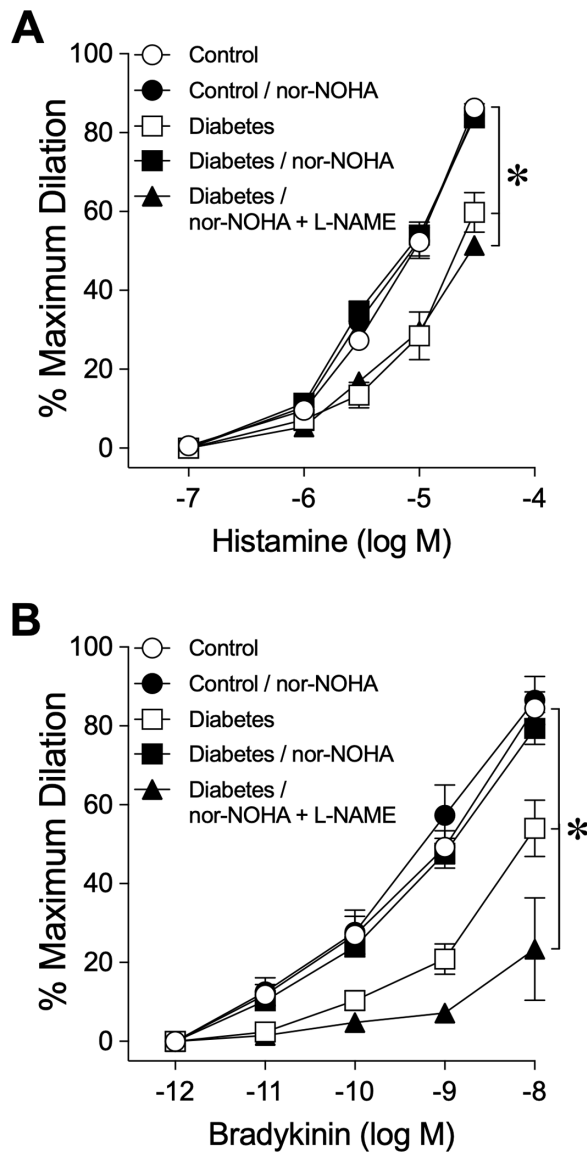
**FIGURE 2.** Diabetes does not alter COX-mediated dilation of retinal arterioles. Concentration-dependent dilation of isolated and pressurized retinal arterioles to histamine was examined in the absence or presence of both NOS inhibitor L-NAME and COX inhibitor indomethacin after 2 weeks of euglycemia (control;  $n = 5$ ) or hyperglycemia (diabetes;  $n = 5$ ) in pigs. \* $P < 0.05$  among control, diabetes, and vessels treated with L-NAME + IM.

on the responsiveness of control retinal arterioles to either histamine or bradykinin. Neither nor-NOHA nor TEMPOL altered the basal tone of control or diabetic vessels.

### DISCUSSION

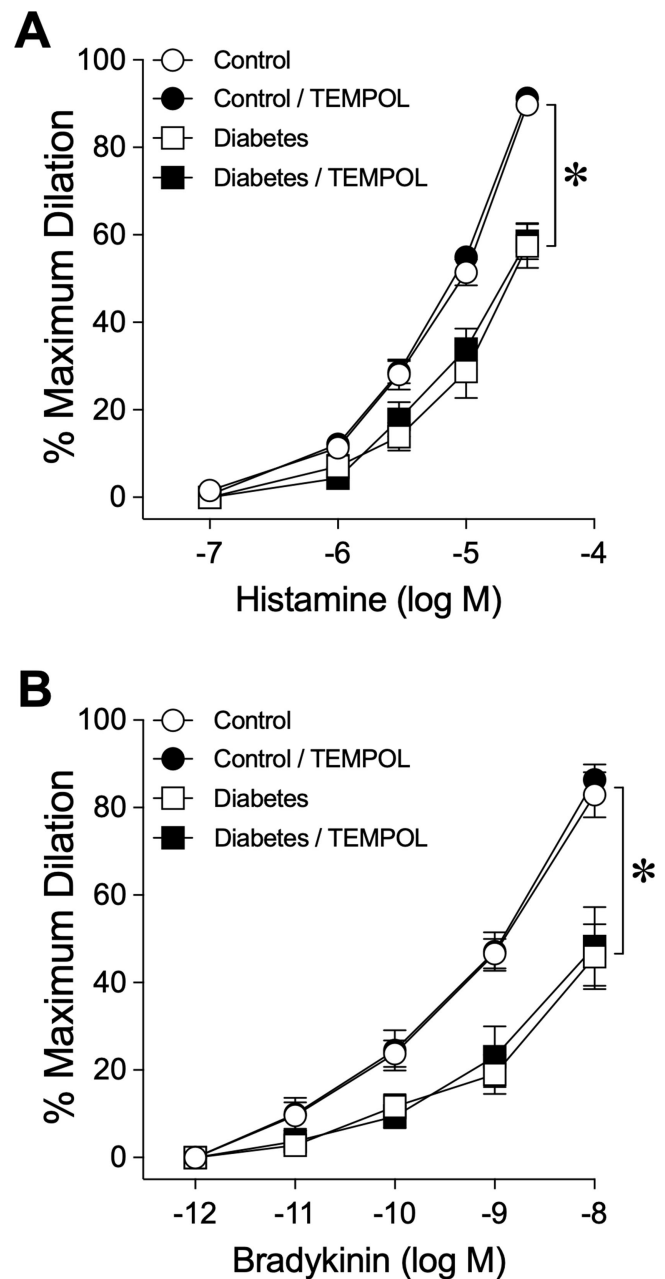
Diminished vasodilator capacity of retinal arterioles has been reported in animals and patients with type 1 diabetes,<sup>20,21,40</sup> but there is limited insight on the molecular mechanisms responsible for the dysfunction of these microvessels that regulate retinal blood flow. The salient findings of this study are that endothelium-dependent NOS-mediated dilation rather than COX-mediated dilation of retinal arterioles is impaired within 2 weeks of type 1 diabetes in pigs; and that pharmacologic blockade of arginase activity but not superoxide production improves the endothelial NOS signaling for vasodilation. Therefore the early onset of diabetes selectively compromises retinal endothelial NOS-mediated dilation by elevating arginase activity in retinal arterioles.

In the present study, we used an isolated and pressurized vessel preparation to evaluate the impact of type 1 diabetes in pigs on endothelium-dependent dilation of retinal arterioles. This *in vitro* approach allowed us to directly assess the endothelial NOS and COX pathways. We used two endogenous vasodilator agonists, histamine<sup>37,41–43</sup> and bradykinin,<sup>25,44</sup> as pharmacologic tools to assess endothelial function of retinal arterioles. Histamine and bradykinin may exert a local impact on regulation of retinal arteriolar tone based on the existence of histamine-generating enzymes and the kallikrein-kinin system in the retinal tissue of several vertebrates including human.<sup>43,45</sup> Previous studies from our and other laboratories have shown that bradykinin causes dilation of healthy porcine retinal arterioles by activating



**FIGURE 3.** Blockade of arginase activity improves dilation of retinal arterioles isolated from diabetic pigs. **(A)** The histamine-induced dilation was compared between euglycemic control ( $n = 6$ ) and diabetic ( $n = 6$ ) vessels. In another cohort of control ( $n = 6$ ) and diabetic ( $n = 6$ ) vessels, dilation to histamine was examined in the presence of arginase inhibitor nor-NOHA. The histamine-induced dilation of nor-NOHA-treated diabetic vessels was also assessed after incubation with L-NAME ( $n = 5$ ).  $*P < 0.05$  versus control. **(B)** The bradykinin-induced dilation was compared between euglycemic control ( $n = 6$ ) and diabetic ( $n = 6$ ) vessels. In another cohort of control ( $n = 9$ ) and diabetic ( $n = 9$ ) vessels, dilation to bradykinin was examined in the presence of nor-NOHA. The bradykinin-induced dilation of nor-NOHA-treated diabetic vessels was also assessed after incubation with L-NAME ( $n = 5$ ).  $*P < 0.05$  among control, diabetes, and diabetes / nor-NOHA + L-NAME.

NOS but not COX.<sup>25,44</sup> In contrast, we also have shown that histamine-induced dilation of porcine retinal arterioles is dependent on activation of both NOS and COX.<sup>37</sup> The present findings corroborate these earlier results by showing that the NOS inhibitor L-NAME attenuated the dilation of retinal arterioles from control nondiabetic pigs to histamine (Fig. 1), supporting the role of NO in this vasodilation. The remaining portion of histamine-induced vasodilation, i.e.,



**FIGURE 4.** Scavenging of superoxide does not improve dilation of retinal arterioles isolated from diabetic pigs. **(A)** The histamine-induced dilation was compared between euglycemic control ( $n = 6$ ) and diabetic ( $n = 6$ ) vessels. In another cohort of control ( $n = 6$ ) and diabetic ( $n = 6$ ) vessels, dilation to histamine was examined in the presence of superoxide dismutase mimetic TEMPOL.  $*P < 0.05$  versus control. **(B)** The bradykinin-induced dilation was compared between euglycemic control ( $n = 6$ ) and diabetic ( $n = 6$ ) vessels. In another cohort of control ( $n = 6$ ) and diabetic ( $n = 6$ ) vessels, dilation to bradykinin was examined in the presence of TEMPOL.  $*P < 0.05$  versus control.

insensitive to L-NAME, was reduced by adding indomethacin (Fig. 2), suggesting the contribution of COX to this vasodilation. Based on the extent of inhibitions, it appears that NOS and COX contribute equally to the dilation of retinal arterioles to histamine.

We have previously shown that endothelium-dependent NOS-mediated dilation of retinal arterioles in response to



bradykinin is reduced after 2 weeks of hyperglycemia in diabetic pigs.<sup>22</sup> It appears that diabetes attenuates the ability of the endothelium in these vessels to produce and/or release NO via NOS, because vasodilation to the NO donor sodium nitroprusside was found to be unaltered.<sup>22</sup> Clinical studies have shown that local stimulation of metabolic activity in the retina with flickering light causes NOS-mediated dilation of retinal arterioles<sup>18</sup> but is compromised by type 1 diabetes while the dilation of these vessels to systemic administration of NO donor glyceryl trinitrate remains intact.<sup>40</sup> Although the cumulative data point to NOS deficiency, it remains unclear whether other endothelium-dependent vasodilator pathways are affected by type 1 diabetes. Impaired vasodilation to bradykinin (i.e., NOS-dependent mechanism) was observed in the present study along with diminished vasodilation to histamine (Fig. 3), which exhibits a COX-mediated component. The relatively rapid onset of endothelial vasodilator dysfunction in response to histamine within 2 weeks of diabetes in our pig model (Fig. 1) is consistent with previous evidence showing diminished retinal blood flow augmentation following intravitreal administration of histamine in 1-week diabetic rats.<sup>2</sup> Using pharmacologic tools, we elucidated that NOS-mediated dilation was selectively obliterated in the diabetic arterioles because their dilation to histamine was suppressed to the level identical to that produced by L-NAME in control vessels, and addition of L-NAME failed to exert further inhibition of histamine-induced dilation in diabetic vessels (Fig. 1). However, addition of indomethacin, in the presence of L-NAME, further reduced the dilation of diabetic retinal arterioles to histamine to the same level of blockade observed in control vessels (Fig. 2). These data suggested that the COX-mediated vasodilator pathway was not altered by 2 weeks of diabetes. Although it is unclear whether prolonged diabetes (more than 2 weeks) will affect COX-mediated vasomotor activity, NOS-mediated vasodilation appears to be more susceptible to diabetic insult. We have not identified the specific prostanoid(s) involved in mediating the indomethacin-sensitive vasodilator response to histamine, but the preserved COX-mediated dilation of retinal arterioles during diabetes is seemingly in line with the lack of a direct effect of high glucose on PGI<sub>2</sub> release from cultured human retinal endothelial cells.<sup>46</sup> Collectively, our findings support the notion that short-term duration of diabetes promotes endothelial dysfunction by diminishing NOS, but not COX, signaling in retinal arterioles.

The ability of NOS in retinal arterioles to generate NO may be limited by increased activity of vascular arginase. Both NOS and arginase in the endothelium utilize L-arginine as a substrate, so the activity of the enzyme arginase can influence the availability of this amino acid for the production of NO.<sup>29–31</sup> Experimental evidence suggests that elevated arginase activity contributes to the reduced acetylcholine-stimulated endothelium-dependent NO-mediated dilation of retinal arterioles in diabetic mice *in vivo* and of central retinal arteries isolated from diabetic rats.<sup>32</sup> The current study extends these earlier findings to retinal arterioles in diabetic pigs and supports the impact of arginase on vasodilator responses to additional endothelial NO-mediated agonists, histamine and bradykinin. We found that pharmacologic blockade of arginase activity with its specific inhibitor nor-NOHA<sup>28,47</sup> did not alter the dilation of retinal arterioles from nondiabetic pigs to histamine and bradykinin, but restored these vasodilator responses in vessels from diabetic pigs. These improved vasodilations

were blocked by L-NAME (Fig. 3), indicating that arginase inhibition can provide adequate NO for the dilation of diabetic vessels during NOS stimulation. Because arginase inhibition did not alter vasomotor function of nondiabetic control vessels, the elevated arginase activity or its protein expression might have contributed to the observed adverse effect of diabetes in the present study. Future molecular studies are needed to identify whether the arginase I isoform is upregulated or activated specifically in the vasculature to support previous observations using genetic tools.<sup>32</sup> Nonetheless, the present findings suggest that arginase activity limits the availability of L-arginine for endothelial NOS to produce NO in porcine retinal arterioles during 2 weeks of hyperglycemia.

Besides elevated arginase activity, increased superoxide anions in the vasculature can interact with NO and reduce its bioavailability. Although acute exposure to high glucose has been shown to increase superoxide in cultured retinal endothelial cells,<sup>48</sup> the impact of this oxidative stress on retinal vasodilator function during hyperglycemia/diabetes has not been evaluated. We found that treatment of the isolated vessels with superoxide dismutase mimetic TEMPOL did not improve endothelium-dependent dilations of retinal arterioles isolated from diabetic pigs (Fig. 4), suggesting that vascular superoxide is not contributing to the impaired endothelial vasodilator function during early diabetes. The concentration of 1 mmol/L TEMPOL used in this study was effective in blocking superoxide production by isolated porcine retinal arterioles during exposure to proinflammatory factor C-reactive protein in our previous study.<sup>38</sup> We also performed functional studies with coronary and retinal arterioles isolated from the same diabetic pigs, but surprisingly found that TEMPOL only restored NOS-mediated dilation of coronary arterioles.<sup>39</sup> Our findings from this previous work with coronary arterioles also suggest that upregulation of arginase I in these vessels leads to the production of superoxide. These disparate results related to the roles of superoxide and arginase in retinal and coronary arterioles underscore the apparent heterogeneity in mechanisms of diabetes-induced endothelial vasodilator dysfunction in the microcirculation. Furthermore, our present studies do not rule out the potential of superoxide-dependent signaling to initiate retinal endothelial dysfunction by augmenting the production of arginase. Previous evidence has shown that superoxide-derived hydrogen peroxide increases expression of arginase in porcine coronary arterioles leading to diminished endothelium-dependent NO-mediated dilation.<sup>28</sup> Additional studies are necessary to determine whether preventing excessive superoxide production in the very early stage of diabetes could preserve the endothelial NOS-mediated dilation of retinal arterioles during development and progression of diabetes. An alternative mechanism may involve upregulation of arginase by the stress-activated kinase c-Jun N-terminal kinase (JNK) because we have previously shown that acute and chronic hyperglycemia impair bradykinin-induced NOS-mediated dilation of retinal arterioles by activating the JNK pathway.<sup>22</sup> The potential link of JNK signaling to a specific arginase isoform in porcine retinal arterioles during diabetes warrants evaluation in future investigations.

In summary, we have demonstrated in pigs that early type 1 diabetes elevates arginase activity in retinal arterioles leading to selective impairment of endothelium-dependent NOS-mediated dilation. Because pharmacologic blockade of arginase activity fully restored arteriolar vasodilator function

after 2 weeks of hyperglycemia, this specific protein may be a beneficial clinical target to consider for retinal microvascular treatment to ameliorate retinal ischemia during the initial stages of diabetes.<sup>49,50</sup>

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