Vasoinhibins prevent retinal vasopermeability associated with diabetic retinopathy in rats via protein phosphatase 2A–dependent eNOS inactivation

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Increased retinal vasopermeability contributes to diabetic retinopathy, the leading cause of blindness in working-age adults. Despite clinical progress, effective therapy remains a major need. Vasoinhibins, a family of peptides derived from the protein hormone prolactin (and inclusive of the 16-kDa fragment of prolactin), antagonize the proangiogenic effects of VEGF, a primary mediator of retinal vasopermeability. Here, we demonstrate what we believe to be a novel function of vasoinhibins as inhibitors of the increased retinal vasopermeability associated with diabetic retinopathy. Vasoinhibins inhibited VEGF-induced vasopermeability in bovine aortic and rat retinal capillary endothelial cells in vitro. In vivo, vasoinhibins blocked retinal vasopermeability in diabetic rats and in response to intravitreous injection of VEGF or of vitreous from patients with diabetic retinopathy. Inhibition by vasoinhibins was similar to that achieved following immunodepletion of VEGF from human diabetic retinopathy vitreous or blockage of NO synthesis, suggesting that vasoinhibins inhibit VEGF-induced NOS activation. We further showed that vasoinhibins activate protein phosphatase 2A (PP2A), leading to eNOS dephosphorylation at Ser1179 and, thereby, eNOS inactivation. Moreover, intravitreous injection of okadaic acid, a PP2A inhibitor, blocked the vasoinhibin effect on endothelial cell permeability and retinal vasopermeability. These results suggest that vasoinhibins have the potential to be developed as new therapeutic agents to control the excessive retinal vasopermeability observed in diabetic retinopathy and other vasoproliferative retinopathies.

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Vasoinhibins prevent retinal vasopermeability associated with diabetic retinopathy in rats via protein phosphatase 2A–dependent eNOS inactivation

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Introduction
Diabetic retinopathy is a frequent complication in the eye of individuals with long-term diabetes and remains the leading cause of blindness in working-age adults throughout the world. Nearly 80% of patients develop diabetic retinopathy after the first 2 decades of diabetes (1). The primary pathogenic factor is believed to be chronic hyperglycemia, which causes apoptosis of pericytes and endothelial cells, thickening of vessel basement membranes, and increased retinal vasopermeability. Elevated retinal vasopermeability results in retinal hemorrhages, swelling, and formation of exudates that impair vision when the macula is affected (2, 3). Over time, intretinal hemorrhages and capillary closure create areas of nonperfusion that lead to localized hypoxia and increased production of proangiogenic factors in the retina. In advanced disease, the neovessels invade and bleed into the vitreous, producing a fibrovascular tissue that can cause retinal detachment and blindness. Laser photocoagulation is often effective to treat diabetic retinopathy but rarely improves visual acuity (4). Because laser photocoagulation damages neural tissue, vision can thereby even become worse (4). Thus, developing new strategies to oppose excessive retinal vasopermeability and angiogenic responses has become a major research focus.

VEGF is a prominent vasopermeability factor in diabetic retinopathy (5, 6). Intraocular injection of VEGF induces vasodilation, retinal hemorrhages, and vascular leakage in nondiabetic animals (7–10). Elevated concentrations of VEGF in diabetic retinas are detected in association with excessive retinal vasopermeability (11), and therapies based on the inhibition of VEGF expression and action have shown promising results as an effective and safe option for the treatment of diabetic macular edema (6). Both paracellular and transcellular pathways of vascular solute flux have been proposed for VEGF-induced vasopermeability: VEGF alters endothelial cell tight junctions (12) by redistributing occludin (13), but it can also induce formation of caveolae within endothelial cells (14). The latter action involves activation of eNOS and production of NO, a free radical known to be the main endothelium-derived relaxing factor and a mediator of vasopermeability (14–16). Notably, VEGF does not stimulate vasopermeability in

Nonstandard abbreviations used: BAEC, bovine aortic endothelial cell; NAME, Nω-nitro-ω-arginine methyl ester; OA, okadaic acid; PP2A, protein phosphatase 2A; PRL, prolactin; RRCEC, rat retinal capillary endothelial cell.

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Vasoinhibins (Vi) prevent VEGF-induced vasopermeability, eNOS activity, and eNOS phosphorylation in endothelial cells. (A) Line diagrams depicting the different protocols used in experiments represented in B, C, and D. (B) Vasopermeability was measured by HRP leakage in BAEC monolayers either untreated (ctl) or treated with 5 nM VEGF in the absence or presence of 0.1, 0.5, 1, 5, 20, or 50 nM vasoinhibins for 48 hours. BAECs were incubated with VEGF combined with 1 mM l-NAME. Values are mean ± SEM of 5 independent experiments. *P < 0.05 versus ctl; **P < 0.05 versus VEGF. (C) eNOS activity was determined by the [H]-citrulline assay in BAECs incubated or not with 5 nM VEGF, alone or combined with increasing concentrations of vasoinhibins for 1 hour. Values are mean ± SEM of 5 independent experiments. *P < 0.05 versus no treatment; **P < 0.05 versus VEGF. (D) Western blot analysis of eNOS-Ser1179 phosphorylation in BAECs preincubated or not with 20 nM vasoinhibins for 1 hour and then treated with 5 nM VEGF for 1, 5, or 10 minutes. Total eNOS served as loading control. (E) Quantification of eNOS phosphorylation by densitometry after normalization for total eNOS. Values are mean ± SEM of 3 independent experiments. *P < 0.05 versus no treatment; **P < 0.05 versus VEGF.

Vasoinhibins (Vi) prevent VEGF-induced vasopermeability by blocking NO production, the effect of vasoinhibins on eNOS activity was investigated using the [H]-citrulline assay in BAECs. Vasoinhibins prevented VEGF-induced activation of eNOS in a dose-dependent manner (Figure 1C). Because eNOS activation occurs through PI3K/Akt-mediated phosphorylation of its Ser1179 (24), we evaluated eNOS phosphorylation in BAECs by Western blot analysis. As shown in Figure 1D, VEGF treatment enhanced eNOS phosphorylation at Ser1179 as compared with no treatment, whereas preincubation with vasoinhibins for 1 hour prior to addition of VEGF blocked the VEGF-induced eNOS phosphorylation. In the presence of vasoinhibins, phosphorylation levels were slightly lower than those of controls (i.e., no treatment or vasoinhibins alone). Figure 1E shows quantification of phosphorylated eNOS after normalization for the amount of total eNOS on the gel. Phosphorylation of eNOS occurred within 1 minute after addition of VEGF, and the level of phosphorylation did not further increase with longer exposure to VEGF (5 and 10 minutes).

Figure 1

Vasoinhibins prevent VEGF-induced endothelial permeability and promote eNOS dephosphorylation at Ser1179. We investigated the effect of VEGF and vasoinhibins on vasopermeability by measuring leakage of HRP across BAEC monolayers. VEGF induced a 50% increase in permeability, and this increase was blocked in a dose-dependent manner by the coadministration of recombinant vasoinhibins (Figure 1B). Because NO is one of the main factors mediating the VEGF effect on vasopermeability (17, 18), we found that addition of the NOS inhibitor Nω-nitro-l-arginine methyl ester (l-NAME) (23) reduced VEGF-induced BAEC permeability to levels comparable to those of untreated or vasoinhibin-treated cells (Figure 1B). To determine whether vasoinhibins inhibited endothelial cell permeability by blocking NO production, the effect of vasoinhibins on eNOS activity was investigated using the [H]-citrulline assay in BAECs. Vasoinhibins prevented VEGF-induced activation of eNOS in a dose-dependent manner (Figure 1C). Because eNOS activation occurs through PI3K/Akt-mediated phosphorylation of its Ser1179 (24), we evaluated eNOS phosphorylation in BAECs by Western blot analysis. As shown in Figure 1D, VEGF treatment enhanced eNOS phosphorylation at Ser1179 as compared with no treatment, whereas preincubation with vasoinhibins for 1 hour prior to addition of VEGF blocked the VEGF-induced eNOS phosphorylation. In the presence of vasoinhibins, phosphorylation levels were slightly lower than those of controls (i.e., no treatment or vasoinhibins alone). Figure 1E shows quantification of phosphorylated eNOS after normalization for the amount of total eNOS on the gel. Phosphorylation of eNOS occurred within 1 minute after addition of VEGF, and the level of phosphorylation did not further increase with longer exposure to VEGF (5 and 10 minutes).

Vasoinhibins prevent VEGF stimulation of endothelial permeability via PP2A-induced eNOS dephosphorylation. We further examined...
whether vasoinhibins reduce VEGF-induced eNOS phosphorylation through protein phosphatase 2A (PP2A), which is known to dephosphorylate eNOS at Ser1179 (25). As shown in Figure 2, B and C, treatment of BAECs with vasoinhibins increased PP2A-like activity in a dose- and time-dependent manner. The maximal effect was observed after a 1-hour exposure of BAECs to 20 nM vasoinhibins; this time and dose were therefore used in subsequent experiments. We found that the vasoinhibin-stimulated phosphatase activity was prevented by okadaic acid (OA; 50 nM), a specific inhibitor of PP2A (26) (Figure 2D). Furthermore, the vasoinhibin effect on PP2A-like activity can be mimicked by rapamycin (20 nM), a known activator of PP2A (27), and OA also inhibited rapamycin-induced PP2A activation (Figure 2D). These results suggest that vasoinhibins activate PP2A.

We then determined whether inhibition of eNOS phosphorylation by vasoinhibins is mediated by their activation of PP2A. We found that the effect of vasoinhibins on eNOS dephosphorylation was inhibited by OA, thus implicating PP2A (Figure 3B).

The [3H]-citrulline assay, we showed that vasoinhibins inhibited VEGF-induced eNOS activation and that this effect was blocked by OA. OA alone, on the other hand, had no effect on either basal eNOS activation or VEGF-induced eNOS stimulation (Figure 3C). Next, we asked whether inactivation of PP2A by OA would abrogate the vasoinhibin effect on vasopermeability. We found that treatment of BAECs with OA blocked the ability of vasoinhibin to inhibit VEGF-induced vasopermeability (Figure 3D), and OA alone did not increase basal vasopermeability (Figure 3D). Together, our data showed that vasoinhibins blocked VEGF-induced vasopermeability via PP2A-dependent dephosphorylation and concomitant inactivation of eNOS.

Vasoinhibins inhibit VEGF-induced retinal hemorrhages and vasodilation in vivo. To assess the physiological relevance of our results, we next conducted in vivo studies. Injection of 300 ng VEGF intravitreally (resulting in 260 nM VEGF in the vitreous) in adult male Wistar rats caused multiple hemorrhage areas and vasodilation 24 hours after injection, as shown by staining flat mounts of rat retinas for peroxidase, a marker of red blood cells (Figure 4A, middle panel). Coinjection of 1 μg vasoinhibins (1 μM in the vitreous) with VEGF reduced the vascular changes induced by VEGF (Figure 4A, right panel).
A

Figure 4
Vasoinhibins prevent VEGF-induced retinal hemorrhages and vasodilation in vivo. (A) Representative flat mounts after the peroxidase reaction of a portion of the retina obtained from rats 24 hours after an intravitreal injection of PBS (ctl), 260 nM VEGF, or VEGF and 1 μM vasoinhibins. Each retina was digitized with a x20 objective and tiled to present the entire preparation. Arrows indicate hemorrhage areas. Original magnification, ×6. (B) Magnification of areas indicated by rectangles (×7; ×40 objective) in the flat mounts shown in A to illustrate the vasodilation of the microcirculation. Insets in B were magnified by an additional 2-fold. Arrows indicate the width of blood vessels. Quantification of hemorrhage areas (C) and averaged diameter of blood vessels (D) from retina flat mounts from rats injected intravitreally 24 hours earlier with PBS, 260 nM VEGF, or VEGF and 1 μM Vi. VEGF and vasoinhibin concentrations refer to the final, intravitreal levels. Values are mean ± SEM of 6 independent samples. *P < 0.05 versus control; **P < 0.05 versus VEGF.

Panel). No vascular alterations were observed in retinas from eyes that received a control intravitreal injection of PBS (Figure 4A, left panel). VEGF stimulated vasodilation of major, minor, and capillary vessels. Magnified areas of retinas in Figure 4A illustrate the retinal microcirculation, where VEGF-induced vasodilation allowed erythrocytes to adopt a round morphology (Figure 4B, middle panel versus left panel). Addition of vasoinhibins reduced VEGF-induced vasodilation of microvessels, thereby preventing the rounding of erythrocytes, which exhibited a shape that was more similar to that of the PBS-treated control (Figure 4B, left versus right panel). Quantification of hemorrhage areas and vessel diameter confirmed that coinjection of vasoinhibins with VEGF reduced both parameters to values similar to those of the controls (Figure 4, C and D). Thus, vasoinhibins inhibit VEGF-induced retinal hemorrhages and vasodilation in vivo.

Dephosphorylation of NOS by vasoinhibins prevents VEGF-induced retinal vasopermeability. Changes in retinal vasopermeability were further investigated using the Evans blue assay. VEGF injected intravitreally induced a 4-fold increase in retinal vasopermeability, which was totally blocked by coinjection of vasoinhibins (Figure 5A). Intravitreal injection of vasoinhibins alone, compared with PBS, did not significantly modify retinal vasopermeability (Figure 5A). The contribution of NOS to VEGF-induced retinal vasopermeability was confirmed by the fact that administration of l-NAME (1.8 mM) in the rats' drinking water for 15 days counteracted VEGF action, mimicking the vasoinhibin effect (Figure 5A). Next, OA (2 μM) administered intravitreally prevented vasoinhibins from inhibiting VEGF action and caused a stimulation significantly greater than VEGF alone (Figure 5A). Injection of OA alone or combined with VEGF had no significant effect with respect to untreated control or VEGF only, respectively. NOS activity in retinal extracts from rats previously injected intravitreally with the various reagents was then examined by the [3H]-citrulline assay. While injection of vitreous with VEGF activated retinal NOS relative to PBS injection, vasoinhibins abolished the VEGF-induced NOS activity (Figure 5B). To address whether the inhibition of NOS by vasoinhibins could occur in retinal endothelial cells, eNOS activity was determined in RRCECs. VEGF (5 nm) induced a 35% increase in permeability after 48 hours (Figure 5C), and this increase was blocked by the coadministration of l-NAME (1 mM) or of vasoinhibins in a dose-dependent manner (Figure 5C). While OA (50 nM) did not modify either basal or VEGF-induced eNOS activity, it blocked vasoinhibin inhibition of VEGF-induced eNOS activity in RRCECs (Figure 5D). As shown in Figure 5E, VEGF injection significantly increased retinal eNOS phosphorylation at Ser1179, while vasoinhibins blocked this effect. Quantification normalized to total eNOS content is presented in Figure 5F. Note that vasoinhibins alone did not affect eNOS phosphorylation at Ser1179. These findings established that vasoinhibins protect against VEGF-induced retinal vasopermeability in vivo by activating PP2A, which, in turn, dephosphorylates and inactivates eNOS.

Vasoinhibins block retinal vasopermeability associated with diabetes. We next sought to study the effect of vasoinhibins on retinal vasopermeability associated with diabetic retinopathy. As determined by the Evans blue assay, injection of rats with vitreous from patients with diabetic retinopathy induced an 8-fold increase in retinal vasopermeability, which was significantly higher than the 2-fold increase in retinal vasopermeability observed upon injection of nondiabetic vitreous (Figure 6A). These findings are in contrast to those of a previous report (28) showing a 2-fold increase in vasopermeability in response to vitreous from patients with diabetic retinopathy and no vasopermeability effect of nondiabetic vitreous. In that study, control vitreous was obtained from individuals undergoing vitrectomy, whereas we used vitreous from nondiabetic cadaveric donors. It is possible that postmortem changes in vitreous influence its vasopermeability of properties. Coinjection of vasoinhibins reduced the diabetic vitreous-induced effect on retinal vasopermeability to values comparable to those in eyes injected with the nondiabetic vitreous (Figure 6A). The effect of vasoinhibins on diabetic vitreous–induced retinal vasopermeability was similar to that observed with diabetic retinopathy vitreous in which VEGF was depleted by immunoprecipitation (Figure 6A). VEGF immunoassay showed a 60% reduction in VEGF in the diabetic and nondiabetic vitreous after immunoprecipitation.
The macula is affected (2, 3). These changes reflect an imbalance and formation of hard exudates that lead to reduced vision when between factors that stimulate and factors that inhibit retinal diabetic retinal vasopermeability have the potential to be developed for therapeutic application. The present study demonstrates for the first time to our knowledge that vasoinhibins prevent the diabetic retinopathy–associated increase in retinal vasopermeability. This effect involves the inhibition of VEGF-induced eNOS activation due to dephosphorylation of eNOS by PP2A. Our findings suggest that vasoinhibins could be developed as protective agents against diabetic macular edema.

Retinal vasopermeability: VEGF versus vasoinhibins. VEGF is one of the factors that lead to elevated retinal vasopermeability in diabetes. Vascular leakage is associated with upregulation of VEGF in ocular fluids of patients with diabetic retinopathy (29–31). Anti-VEGF treatments that were shown to reduce experimental retinal vasopermeability (32) are emerging therapies for the effective treatment of diabetic macular edema (6, 33). In this context, the present results unveil what we believe to be a novel action of vasoinhibins as inhibitors of diabetes-associated and VEGF-induced retinal vasopermeability. We demonstrate that intravitreal injection of vasoinhibins prevents excessive retinal vasopermeability in the streptozotocin-induced diabetes rat model, where VEGF plays a major role (34). Also, vasoinhibins block increased retinal vasopermeability induced by intravitreal injection of VEGF. However, the concentrations of VEGF required by us (260 nM) and by others (30 nM) (35) to increase retinal vasopermeability are much higher than the VEGF levels detected in the vitreous of diabetic patients (220 pM, ref. 29; and 5.8 pM, present study) or of streptozotocin-induced diabetic rats (225 pM) (34). Intravitreal delivery may limit...
the accessibility and tissue distribution of VEGF, which could account for such a discrepancy. Also, the composition of the vitreous proteome changes with diabetes (28), and molecular interactions with other vasopereability factors or cofactors could help potentiate the effect of the endogenous VEGF so that it is active at much lower concentrations. In any event, we also show that vasoinhibins counteract the effect of the vitreous from patients with diabetic retinopathy, resulting in vasopereability levels similar to those observed with diabetic vitreous in which VEGF was depleted by immunoprecipitation. These data indicate that vasoinhibins protect against the increased retinal vasopereability associated with diabetes by opposing the effects of endogenous VEGF. Consistent with this interpretation, we found that the small but significant effect of the vitreous from nondiabetic individuals on retinal vasopereability was independent of VEGF and was not affected by vasoinhibins. This may imply the presence of other factors, e.g., carbonic anhydrase (28), that also contribute to basal retinal vasopereability. Because control vitreous was obtained from cadaveric donors, upregulation of these factors may be due to possible postmortem changes, although such changes did not appear to modify VEGF levels. Finally, we report that vasoinhibins counteract VEGF-induced dilation of retinal blood vessels, a known effect of VEGF (36) that is among the earliest manifestations of diabetic retinopathy (37).

We believe the demonstration that vasoinhibins block the vasopereability and vasodilation effects of VEGF to be novel and potentially important. Vasoinhibins were previously shown to inhibit growth factor– (VEGF and bFGF) and IL-1β–induced endothelial cell migration (38, 39) and proliferation (40) and to promote endothelial cell apoptosis (41). Current studies extend vasoinhibin function to inhibition of increases in vascular permeability that accompany almost all states of angiogenesis (42), including those in areas of diseased tissue in angiogenesis-related disorders such as diabetic retinopathy. The role of vasoinhibins as physio(patho)logical regulators of retinal vasculature is supported by their presence in the normal rat retina (22) and in the subretinal fluid and fibrovascular membranes of patients with retinopathy of prematurity (20). Blockage of vasoinhibins by the intravitreal injection of small, interfering RNA or neutralizing antibodies stimulates vessel growth and dilation in the retina (22) and inhibits apoptosis-mediated regression of intraocular blood vessels during development (20). Notably, gene transfer of vasoinhibins via an adenoviral vector reduces angiogenesis in murine ischemia-induced retinopathy (43). These observations indicate that vasoinhibins are endogenous angiogenic, vasoconstrictive, and antivasopereability factors in retina. Thus, vasoinhibins can be used to ameliorate multiple features of diabetic retinopathy. In line with this, PRL levels increase in the circulation and in ocular fluids of patients with advanced retinopathy of prematurity, and this increase was proposed to result in higher levels of intraocular vasoinhibins that can counteract the progression of the disease (20).

Mechanism of vasoinhibin action. Vasoinhibin actions described here are consistent with their effect on inhibiting VEGF-induced NO synthesis (21), which is one of the mechanisms by which VEGF induces angiogenesis, vasodilation, and vasopereability (16–18). We show that L-NAME, a NOS inhibitor, mimicked the inhibitory effect of vasoinhibins on VEGF-induced vasopereability in vivo and in vitro. Moreover, L-NAME prevented elevated retinal vasopereability in response to the vitreous from patients with diabetic retinopathy, and it reduced retinal vasopereability to a level similar to that following VEGF depletion or vasoinhibin treatment. Vasoinhibins indeed impaired VEGF-induced NOS activity in the retina and, because vasoinhibins also inhibit eNOS activity in RCECs, this action can occur directly on retinal blood vessels. At present there are 2 mechanisms by which vasoinhibins might inhibit eNOS activation. We previously proposed that vasoinhibins, by interfering with the mobilization of intracellular calcium, could prevent the calcium-dependent activation of eNOS (21). Here, we show that vasoinhibins block eNOS activity by inducing its dephosphorylation at Ser1179 via PP2A stimulation. VEGF is known to stimulate eNOS activity via PI3K/Akt-dependent phosphorylation of eNOS at Ser1179 (bovine eNOS) or Ser1177 (human eNOS) (44). Phosphorylation of eNOS is an important mechanism that promotes eNOS-vascular functions,
vasoinhibins may not involve PP2A, since PP2A and vasoinhibins cells implicates an OA-sensitive phosphatase (26). OA had no significant induced vasopermeability in the retina and in cultured endothelial by reducing paracellular permeability at the level of occludin expression (48). By reducing eNOS phosphorylation via PP2A activation, endostatin was shown to impair endothelial cell migration and reduce excessive retinal vasopermeability in diabetic retinopathy and identify the relevance of eNOS dephosphorylation for the prevention of retinal toact VEGF action are necessary. Our findings strongly support the relevance of eNOS dephosphorylation for the prevention of retinal vasopermeability associated with diabetic retinopathy and identify a prominent association between eNOS-related VEGF signaling and vasoinhibins. Promoting vasoinhibin activity to counteract VEGF and reduce excessive retinal vasopermeability in diabetic retinopathy emerges from this study as a potentially powerful therapeutic approach for effective treatment of diabetic macular edema and the various vasoproliferative retinopathies.

Methods

Reagents. Rat vasoinhibin (corresponding to a 16-kDa fragment of PRL) used in in vivo experiments was generated by enzymatic proteolysis of rat PRL with a particulate fraction from rat mammary glands, followed by gel filtration and carbamidomethylation as previously described (61). Reagents

Table 1

Characteristics of the subjects studied

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<th>Duration of diabetes (yr)</th>
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<th>Mean blood pressure (mmHg)</th>
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aHbA1c, hemoglobin A1c blood test result. bRef. 68. cDonors died by accidental death, and samples were obtained 12.5 ± 0.5 hours postmortem. NA, not applicable.

i.e., vasodilation, vasopermeability, and angiogenesis (15–17), and it may represent a switch for their control under physiological and pathological conditions. Transgenic mice expressing only a phosphomimetic form of eNOS (due to point mutation of Ser1179 to Asp1179) respond better to vasorelaxing agents and display increased blood reperfusion during ischemia, reducing the severity of strokes (45). Also, shear stress and several vasoactive factors and hormones such as insulin (46) and estrogens (47) induced eNOS Ser1179 phosphorylation. Dephosphorylation of this residue may represent a common mechanism by which vasoinhibins could counteract the effects of several vasoactive substances.

Our work also demonstrates the prominent role of PP2A in the antipermeability effect of vasoinhibins in vivo and in vitro. The observation that OA, in the presence of vasoinhibins, permits VEGF-induced vasopermeability in the retina and in cultured endothelial cells implicates an OA-sensitive phosphatase (26). OA had no significant effect under unstimulated conditions or when combined with VEGF. This lack of effect implies that PP2A is not activated under basal conditions or in response to VEGF. The stimulatory effect of OA in the presence of VEGF and vasoinhibins suggests that activation of PP2A by vasoinhibins can reduce vasopermeability through additional pathways besides eNOS inactivation, such as dephosphorylation of occludin (48). By reducing eNOS phosphorylation via PP2A activation, endostatin was shown to impair endothelial cell migration and reduce blood pressure (mmHg)

recombinant human vasoinhibins (corresponding to a 14-kDa fragment of PRL) used in cell culture experiments were generated by site-directed mutagenesis as previously described (62). A vasoinhibin concentration of 1 μM was used in vitro because it fully blocked increased retinal vasopermeability elicited by VEGF and diabetic vitreous, as determined by a dose-response curve (data not shown). Human vitreous specimens were obtained after surgery on 6 subjects with diabetic retinopathy. The specimens were obtained with the approval of the Institutional Review Board of the Hospital “Dr. Luis Sánchez Bulnes,” and all subjects were treated in accordance with the tenets of the Declaration of Helsinki. All patients provided written informed consent before collection of samples. Vitreous samples from nondiabetic cadaveric donors were used as controls (n = 5). The Medical Forensic Service (SEMEO) of Mexico approved the collection and use of samples. All vitreous samples were tested individually. Table 1 summarizes the characteristics of all subjects studied. Recombinant human VEGF (rhVEGF165) was a gift from Genentech. The lowest doses of VEGF having a significant effect in vitro and in vivo were 5 nM and 260 nM, respectively. OA (Millipore) was used at 50 nM according to the manufacturer’s instructions; rapamycin and the rules and regulations of the Association for Research in Vision and Ophthalmo- logy (ARVO) were followed (21). For tissue assays, rats were euthanized 24 hours after intravitreal injections, and retinas were dissected and homogenized in extraction buffer (320 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM DTT, 10 μM leupeptin, and 2 μM/ml aprotinin, pH 7.2). Protein concentration was evaluated using the Bradford assay (Bio-Rad). The reaction was carried out in reaction buffer (50 mM HEPES, 1 mM NaCl, 1.25 mM CaCl2, 1 μM FAD, 1 μM FMN, and 10 μg/ml calmodulin) and initiated by the addition of 1 μCi/ml [3H]-arginine to 50 μg of total retinal extract protein in a final volume of 100 μl. After 25 minutes, ice-cold stop medium was added (50 mM HEPES, pH 5.5, and 4 mM EDTA). Retinal extracts in the extraction buffer described above were applied onto 1-ml columns of Dowex AG50WX8. [3H]-ciruilline was eluted with 1 ml water and quantified by liquid scintillation counting.

Phosphatase assay. The Serine/Threonine Phosphatase Assay was performed according to manufacturer’s instructions (Promega). After different treatments indicated in the legends, cells were lysed in a P2PA-specific buffer (25 mM Tris-HCl at pH 7.5, 10 mM β-mercaptoethanol, 2 mM EDTA, 0.1 mM PMFSF, 1 μM pepstatin A, and 10 μg/ml aprotinin). Endogenous phosphate was removed using spin columns, and the lysates were incubated with the serine/threonine phosphatase in PP2A buffer at 30°C for 15 minutes. P2PA-like activity, reported as free phosphate released, was calculated from the absorbance at 595 nm of a molybdate–malachite green–phosphate complex formed after addition of 1 ml molybdate dye solution and 10 μl of molybdate dye additive (Promega).

Statistics. Values are expressed as mean ± SEM. Statistical significance between groups was determined by ANOVA followed by the unpaired 2-tailed Student’s t test. The statistical analyses were performed using SigmaStat 7.0 software (SYSTAT). Differences in means with P < 0.05 were considered statistically significant.

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