Vasoinhibins: Novel inhibitors of ocular angiogenesis

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Abstract

Disruption of the quiescent state of blood vessels in the retina leads to aberrant vasopermeability and angiogenesis, the major causes of vision loss in diabetic retinopathy. Prolactin is expressed throughout the retina, where it is proteolytically cleaved to vasoinhibins, a family of peptides (including the 16 kDa fragment of prolactin) with potent antiangiogenic, vasoconstrictive, and antivasopermeability actions. Ocular vasoinhibins act directly on endothelial cells to block blood vessel growth, dilation, and to promote apoptosis-mediated vascular regression. Also, vasoinhibins prevent retinal angiogenesis and vasopermeability associated with diabetic retinopathy and inactivation of endothelial nitric oxide synthase via protein phosphatase 2A is among the various mechanisms mediating their actions. Here, we discuss the potential role of vasoinhibins both in the maintenance of normal retinal vasculature and in the cause and prevention of diabetic retinopathy and other vasoproliferative retinopathies.
Angiogenesis, the growth of new blood vessels from preexisting vessels, is highly restricted in most healthy tissues, in part by the action of naturally occurring antiangiogenic factors. Disruption of antiangiogenic environments underlies diseases such as cancer, rheumatoid arthritis, and diabetic retinopathies (33). Inhibiting angiogenesis therefore offers an opportunity to treat a wide variety of diseases characterized by the aberrant or excessive formation of blood vessels.

As in the majority of healthy adult tissues, the healthy adult eye shows no blood vessel growth, but it is distinct from most organs in that several of its compartments, i.e. the cornea, the lens, and the vitreous, lack blood vessels. Also, within the retina, blood vessels are confined to the inner half, since the outer half never becomes vascularized (89). Failure to inhibit ocular blood vessel growth can result in reduced visual acuity and underlies vasoproliferative retinopathies, such as retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration, the leading causes of blindness in infants, working-age adults, and the elderly population, respectively (12). Central to the pathogenesis of these diseases are increased vascular permeability leading to retinal edema and proliferation of blood vessels susceptible to hemorrhages. In the advanced stages, neovessels invade and bleed into the vitreous producing a fibrovascular tissue that can cause retinal detachment and blindness. The established therapy for retinal neovascularization, laser photocoagulation, is effective for delaying the progression of the disease but it is associated with retinal destruction that may worsen visual function (8). Therefore, developing new strategies for controlling retinal blood vessels, such as the use of angiogenesis inhibitors has become a major research focus (31).
Vasoinhibins are emerging as natural inhibitors of the angiogenesis process (16, 19, 45). They are generated by the proteolytic cleavage of prolactin (PRL), growth hormone, and placental lactogen (16). Recent data implicate the retina both as a source of and a target for vasoinhibins derived from PRL. Vasoinhibins interfere with ocular angiogenesis by blocking several endothelial functions, and their actions mediated by the inactivation of endothelial nitric oxide synthase (eNOS) are likely to affect the homeostasis of retinal blood vessels. Here, we briefly summarize the biology of vasoinhibins and focus on recent findings showing that these peptides may play a role in controlling the quiescent state of retinal blood vessels and be of value for the treatment of diabetic retinopathy and other vasoproliferative retinopathies.

Vasoinhibins

Vasoinhibins belong to a class of antiangiogenic factors that are derived from larger precursor proteins with no inhibitory effect on angiogenesis (27, 61). Due to a dearth of information on growth hormone and placental lactogen-derived vasoinhibins, the current review only addresses vasoinhibins generated from PRL. The following is a brief account of the structural and biological properties of these vasoinhibins (for more extended reviews see (16, 19)).

Proteolytic cleavage of PRL at specific sites near or within the long loop connecting the third and the fourth $\alpha$-helices (Figure 1) leads to N-terminal fragments of 14 to 18 kDa. Vasoinhibins can be generated by a variety of proteases, i.e., cathepsin D (6, 67), matrix metalloproteases (MMPs) (56), and bone morphogenic protein-1 (BMP-1) (41) (Figure 1). Proteolysis of human PRL by
cathepsin D generates vasoinhibins 1-132 (15 kDa), 1-147 (16.8 kDa), and 1-150 (17.2 kDa) (56, 67); whereas, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13 cleave the 156-157 peptide bond in human PRL to generate a 17.8 kDa vasoinhibin which can be further processed by MMPs to vasoinhibins of 16 and 14 kDa (56). In addition, BMP-1-like MMPs cleave human PRL at a single, conserved site 159-160 to generate a 18 kDa vasoinhibin (41). The defining examples of these peptides are 14 and 16 kDa and were produced by recombinant DNA (17) or by enzymatic proteolysis (15), respectively. The distribution of endogenous vasoinhibins has been recently reviewed (16, 19). These peptides occur naturally in the pituitary gland and in several extrapituitary tissues (16, 19), including avascular tissues (cartilage) (56) and tissues where blood vessels are highly restricted (retina) (4). In the retina, a 16 kDa vasoinhibin was identified by its binding to monoclonal antibodies against the N-terminal end of PRL in Western-blot but not to those recognizing the C-terminus of the hormone (4). Also a 16 kDa vasoinhibin was found in the subretinal fluid and intraocular fibrovascular membranes of patients with retinopathy of prematurity (29).

Vasoinhibins act in vivo and in vitro to block the stimulatory activity of various inducers of angiogenesis, i.e., VEGF, basic FGF, or IL-1β, on endothelial cell proliferation (17), migration (54), and permeability (39); and to inhibit endothelial cell survival (57). The underlying mechanisms are complex and require many signaling effectors. Endothelial cells were shown to have high affinity (Kd of 1-10 nM), saturable binding sites that are specific for vasoinhibins and do not bind PRL. Crosslinking studies indicated proteins of 52 and 32 kDa as the major
vasoinhibin-binding species (20), however these putative vasoinhibin receptors have not been identified. Vasoinhibins block endothelial cell proliferation by preventing the activation of the MAPK pathway induced by basic FGF and VEGF (25). Inhibition occurs at the level of Ras, distal to autophosphorylation of the receptors for both growth factors (24). Indeed, evidence suggests that vasoinhibins block the ability of Sos to activate Ras by inhibiting VEGF-induced disruption of the Grb2/Sos complex and Sos phosphorylation (24). In addition, nitric oxide (NO)-mediated pathways may also contribute to the inhibition of the MAPK activation by vasoinhibins. The eNOS pathway interacts with the MAPK pathway at the level of Raf in mediating the mitogenic effect of VEGF (90), and NO reverses the inhibition of VEGF-induced endothelial cell proliferation by vasoinhibins (42).

For endothelial cell migration, vasoinhibins inhibit the activity of urokinase plasminogen activator, by up-regulating the expression of plasminogen activator inhibitor type 1 (53). Also, they interfere with the Ras-Tiam1-Rac1-Pak1 signaling pathway activated by IL-1β to prevent endothelial cell migration (54). With respect to the apoptosis of endothelial cells, vasoinhibins promote the conversion of Bcl-X\textsubscript{L} to proapoptotic Bcl-X\textsubscript{s}, and they activate NF\textsubscript{KB}-mediated stimulation of initiator and effector caspases (57, 81). Finally, vasoinhibins block VEGF-induced vasopermeability and vasodilation by preventing the calcium-dependent activation of eNOS (42), and by activating protein phosphatase 2A, which dephosphorylates eNOS thereby inactivating it (39).

The diverse actions of vasoinhibins on endothelial cells and their production in the eye suggest a role for these peptides in the physiology and pathology of
ocular blood vessels. The eye is a good model system for studying regulators of vascular function because the retinal vasculature is highly accessible for both treatment and observation of subsequent effects. Furthermore, the study of ocular angiogenesis has enormous clinical significance, as retinal neovascularization is the most common cause of severe vision impairment and loss throughout the world (2).

**Ocular angiogenesis**

Ocular angiogenesis is under the control of multiple stimulatory and inhibitory factors (28) and is therefore promoted when angiogenesis inducers are up-regulated (26) and/or when angiogenesis inhibitors are down-regulated (4, 9, 30). Numerous experimental and clinical observations indicate that hypoxia is the driving force for retinal neovascularization (40). Metabolic demands and associated “physiological hypoxia” drive retinal vascular growth during development (14), whereas occlusion of retinal vessels leading to ischemia leads to pathological retinal angiogenesis (40). Hypoxia up-regulates and down-regulates the expression of angiogenic and antiangiogenic factors, respectively (38). Among these factors are proangiogenic VEGF and antiangiogenic pigment epithelium-derived factor (PEDF), both of which are strongly implicated in physiological and pathological retinal neovascularization (75). Unveiling retinal factors able to block or cooperate with VEGF and PEDF, respectively, or with other regulators of ocular angiogenesis, is a fruitful line of investigation.

**Vasoinhibins are natural inhibitors of ocular angiogenesis**

Evidence indicates that PRL and vasoinhibins occur naturally in the eye (Figure 2). PRL is found in the aqueous humor of the human eye (29, 68), and both
PRL and vasoinhibins are present in rat retina (4, 71). Some ocular PRL may originate from systemic PRL entering the eye, because iodinated PRL injected intracardially can be incorporated into the retina, choroids, and ciliary body (62). However, PRL and vasoinhibins can also be synthesized intraocularly. PRL mRNA and protein are localized in various retinal cell types, including photoreceptors, Müller cells, interneurons, ganglion cells, and astrocytes of rats and monkeys (4, 71). Also, cultures of rat retinal capillary endothelial cells synthesize and secrete PRL (63), and blood vessels from retrolental fibrovascular membranes of patients with retinopathy of prematurity express PRL mRNA, and the PRL protein is cleaved to vasoinhibins (29). Consistent with the presence of vasoinhibins, ocular fluids contain the PRL cleaving enzymes able to generate vasoinhibins from PRL (29).

The essential contribution of vasoinhibins to maintain the avascularity of ocular tissues was demonstrated by showing that implanting into the cornea a pellet containing antibodies that inactivate endogenous vasoinhibins (but not control antibodies) caused local blood vessels to invade the cornea (30). Also, intravitreal injection of antibodies against vasoinhibins stimulates vessel growth in the retina, and intraocular transfection of small interfering RNA to block the expression of PRL stimulates retinal angiogenesis and vasodilation (4). Furthermore, immnosquestering vasoinhibins in neonatal rats reduced the apoptosis of vascular cells in hyaloid vessels, suggesting that vasoinhibins stimulate the physiological regression of intraocular blood vessels after birth (29). These findings do not exclude other potent anti-angiogenic factors in the eye, but they strongly support the view that vasoinhibins are major ones in these locations.
Vasoinhibins have therapeutic potential to control excessive retinal vasopermeability and blood vessel growth. They are effective against various inducers of ocular angiogenesis. Local administration of vasoinhibins reduces the stimulation of corneal angiogenesis induced by basic FGF (30), and gene transfer of vasoinhibins via an adenoviral vector inhibits ischemia-induced retinal angiogenesis (66). Furthermore, vasoinhibins were very recently shown to block retinal vasopermeability in diabetic rats and in response to intravitreal injection of VEGF or of vitreous from patients with diabetic retinopathy (39). This action involved the activation of protein phosphatase 2A leading to blockage of VEGF-induced phosphorylation/activation of eNOS (39). In addition to VEGF, shear stress and several vasoactive factors and hormones such as insulin, IGF-I (69), and estrogens (46) induced eNOS Ser\(^{1179}\) phosphorylation. Dephosphorylation of this residue promoted by vasoinhibins may represent a common mechanism by which vasoinhibins could counteract the effects of several vasoactive substances.

Similarly to vasoinhibins, intravitreous delivery of PEDF, angiostatin, endostatin, and ephrinA1 has been shown to reduce VEGF-induced vasopermeability (55, 64, 74, 82). In these studies, the concentration of PEDF in the vitreous was 10-fold lower than those of ephrinA1, angiostatin, and vasoinhibins. However, comparison of the potential therapeutic advantages of these peptides would require information about their endogenous levels and a careful examination of their relative potencies. As for vasoinhibins, inhibition of NO production plays a role in the vascular effects of angiostatin (52) and endostatin, which also reduces eNOS phosphorylation by activating protein phosphatase 2A.
(87). Notably, eNOS-derived NO can be viewed both, as beneficial and detrimental to retinal angiogenesis. Because, endothelial NO promotes vasodilation, blocking retinal eNOS would worsen ischemia, and thereby enhance neovascularization. In this line, 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 (5). However, several studies support the clinical benefit of blocking NO production rather than promoting it. VEGF does not stimulate vasopermeability in eNOS-/- mice (37), NOS inhibitors prevent retinal hyperpermeability induced in rats by VEGF (84) and by the vitreous from patients with diabetic retinopathy (39). Also, in mice with ischemia-induced neovascularization, deficiency of eNOS, but not of inducible NOS (iNOS) or neuronal NOS, resulted in reduced retinal angiogenesis and decreased VEGF expression (3). Actually, there is some evidence that iNOS can be antiangiogenic in the retina (72) and vasoinhibins stimulate iNOS expression and NO production in pulmonary fibroblasts (22).

Because vasoinhibins are present in the healthy retina, it is possible that their protective effect gets lost under pathological conditions. As mentioned before, ocular angiogenesis in vasoproliferative retinopathies is driven by hypoxia. While there is no data regarding the effect of hypoxia on the generation of retinal vasoinhibins, hypoxia decreases PRL synthesis and suppresses its cathepsin-D-mediated conversion to vasoinhibins in the rat GH4C1 pituitary adenoma cell line (23). Furthermore, oxidative stress plays an important role in the onset and progression of diabetic retinopathy, and multiple biochemical pathways increase the production of reactive oxygen species, including glucose auto-oxidation, the
polyol pathway, and formation of advanced glycation end products (11, 88). Notably, oxidative stress induces the overproduction of vasoinhibins in postpartum cardiomyopathy (45) and, very likely, in preeclampsia (18, 43). On the other hand, systemic PRL may serve as a precursor of ocular vasoinhibins (29), so that lowering its levels would favor the progression of vasoproliferative retinopathies.

In this context, a functional connection has been established between PRL and diabetes, with PRL playing a protective role. Indeed, substantial evidence indicates that lactogens (PRL and placental lactogen) promote the function, proliferation, and survival of beta cells in normal physiology and in pregnancy (32, 34-36, 77). Notably, the pituitary secretion of PRL is influenced by diabetes, as the circulating level of PRL is often decreased in poorly controlled diabetic patients (50) or rats (10); suckling-induced PRL secretion is lower in diabetic rats (49), and the milk PRL level is reduced in women with insulin-dependent diabetes (65). Nevertheless, hyperprolactinemia has also been reported in diabetic patients (58) and either reduced (47) or normal (13) serum PRL levels have been found in association with diabetic retinopathy.

The relationship between circulating PRL and retinal neovascularization was investigated in patients with retinopathy of prematurity, a disease that originates as a side effect of the use of hyperoxia to improve the survival of premature neonates in respiratory distress. Hyperoxia destroys developing intraocular vessels so that when hyperoxia is terminated, hypoxia-driven vascular proliferation occurs (78). Higher PRL values were measured in serum and aqueous humor from patients with retinopathy of prematurity than from control patients with a non-neovascular eye disorder (congenital cataracts) (29). The concentration of PRL in the aqueous
humor correlated directly with that in subretinal fluid, suggesting that PRL incorporated from the circulation at the level of the ocular ciliary epithelium is transported through the posterior aqueous humor (29). Furthermore, vasoinhibins were detected in fibrovascular membranes and subretinal fluid of patients with retinopathy of prematurity, indicating the active cleavage of PRL in the eye of these patients (29). Indeed, vasoinhibins may relate to the progression of this disease as they promote the apoptosis of endothelial cells in vitro (57) and in vivo (29), and endothelial cell apoptosis is an important event mediating the regression of blood vessels in retinopathy of prematurity (70). It is well known that around 60% of infants who are born weighing 1.25 kg or less develop retinopathy of prematurity, but only about 6% of them progress to advanced stages (1). The low proportion of severe cases likely involves apoptosis-mediated vascular regression, since apoptosis of vascular cells actively occurs in stage 5 retinopathy of prematurity (29), and even the severe disease can undergo successful spontaneous involution (1). Notably, the observation that breast feeding reduces retinopathy of prematurity (48) can be explained on the basis of vasoinhibins actions; since PRL is found in milk (65), milk PRL could reach systemic circulation (44) and could be incorporated and processed to vasoinhibins in ocular fluids (29, 62).

Experiments addressing the endogenous levels of vasoinhibins and their proteolytic generation from PRL in diabetic patients and in experimental models of vasoproliferative retinopathies will help determine whether the down-regulation or up-regulation of vasoinhibins helps turn the “angiogenesis switch” on and off in the retina, respectively.

**Other diseases affected by vasoinhibins**
Vasoinhibins may be useful to treat other angiogenesis-related diseases such as cancer. Overproduction of vasoinhibins blocks tumor angiogenesis, growth, and metastasis (7, 51, 59). Also, vasoinhibins promote leukocyte infiltration in tumors (80), and they have proinflammatory effects in lung fibroblasts (22). Recently, the influence of vasoinhibins has been extended to reproductive disorders, such as postpartum cardiomyopathy, a disease where maternal heart failure between the last month of pregnancy and the early puerperium results in high mortality rates (76). A mouse model of postpartum cardiomyopathy was developed due to a cardiomyocyte-specific deletion of the transcription factor STAT-3. In this model, lack of STAT-3 in the myocardium promotes oxidative stress and consequently up-regulates cathepsin D. Cathepsin D cleaves circulating PRL to vasoinhibins, which in turn, cause heart failure by impairing coronary vasculature growth and function (45). Another example is preeclampsia, a disease in which defective placental angiogenesis results in substantial maternal and neonatal morbidity and mortality (73). A recent study showed that cathepsin D is activated in preeclamptic trophoblasts and that the levels of vasoinhibins are increased in the amniotic fluid, serum, and urine of preeclamptic patients (43).

**Other PRL variants with effects on angiogenesis**

Vasoinhibins of 16 and 14 kDa inhibit retinal angiogenesis and vasopermeability in vivo and in vitro (4, 39, 66). However, no studies have been conducted comparing the biological properties of the different vasoinhibin peptides, which is clearly an area for future research that could help identify molecules with similar actions but perhaps more stable, easier to produce, or with better pharmacokinetic properties. Along this line, the functional determinants of
vasoinhibins are unknown and structure-function studies are required to help identify smaller domains that retain activity and may be more effective for treatment than the whole molecule. Recently, a region of 14 hydrophobic aminoacids located in the N-terminal region of the second helix of PRL and growth hormone was shown to display antiangiogenic activity (60). This sequence is buried by the three other helices and the third loop of the hormones, and it remains to be determined how this functional determinant would be exposed to exert its actions.

There is much to learn regarding the structural features of PRL-derived peptides. S179D PRL, a molecular mimic of naturally occurring phosphorylated PRL, was recently shown to inhibit angiogenesis both in vivo and in vitro by interfering with endothelial cell migration, proliferation, survival, and growth factor signaling (85, 86). Phosphorylation of PRL at Ser\textsuperscript{179} alters the charge of the molecule, and it will be relevant to examine the structural properties of phosphorylated PRL for common features with vasoinhibins. Furthermore, there is evidence that the parental hormones, PRL, growth hormone, and placental lactogen, may be angiogenic (21, 79). Although little is known regarding the proangiogenic effects of PRL (for a recent review see (19)), the opposing actions would represent an efficient way to generate positive and negative signals required for maintaining the angiogenic balance. Accordingly, specific proteolytic cleavage of PRL provides an important target for angiogenesis regulation and may lead to new therapeutic strategies for treating angiogenesis-related disorders.

**Conclusions**

Vasoinhibins are natural inhibitors of ocular angiogenesis that are generated within the eye and may also be supplied from the circulation. Pathological
alterations of their levels may be linked to both the underlying causes and eventual remission of neovascular diseases such as retinopathy of prematurity (Figure 2). Identification of the mechanisms mediating inhibition of ocular vasopermeability and angiogenesis by vasoinhibins, under both physiological and pathological conditions, warrants further investigation.

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References


Figure legends

Figure 1. Schematic representation of the three-dimensional structure of PRL indicating the position of the proteolytic cleavage sites (dotted lines) in the human PRL molecule that generate vasoinhibins. Accordingly, cleavage of PRL by bone morphogenic protein 1 (BMP-1) occurs at position 159; by matrix metalloproteases (MMP) at position 155; and by cathepsin D (CD) at positions 132, 147 and 150. A representative vasoinhibin generated by cleavage with MMP is illustrated. The structures have been simplified and the positions of connecting loops distorted to enable annotation. Modified, with permission, from (16, 83).

Figure 2. PRL supplied from the circulation or produced in the retina is cleaved to vasoinhibins (Vi) that inhibit retinal angiogenesis and vasopermeability under normal and pathological conditions. Alteration of vasoinhibin levels in the eye may be linked to the causes and eventual remission of neovascular eye diseases such as diabetic retinopathy and retinopathy of prematurity.
Figure 1
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