High Levels of Serum Prolactin Protect Against Diabetic Retinopathy by Increasing Ocular Vasoinhibins

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OBJECTIVE—Increased retinal vasopermeability (RVP) occurs early in diabetes and is crucial for the development of sight-threatening proliferative diabetic retinopathy (DR). The hormone prolactin (PRL) is proteolytically processed to vasoinhibins, a family of peptides that inhibit the excessive RVP related to DR. Here, we investigate the circulating levels of PRL in association with DR in men and test whether increased circulating PRL, by serving as a source of ocular vasoinhibins, can reduce the pathological RVP in diabetes.

RESEARCH DESIGN AND METHODS—Serum PRL was evaluated in 40 nondiabetic and 181 diabetic men at various stages of DR. Retinal vasoinhibins were measured in rats rendered hyperprolactinemic by placing two anterior pituitary grafts under the kidney capsule and in PRL receptor–null mice. RVP was determined in hyperprolactinemic rats subjected to the intraocular injection of vascular endothelial growth factor (VEGF) or made diabetic with streptozotocin.

RESULTS—The circulating levels of PRL increased in diabetes and were higher in diabetic patients without retinopathy than in those with proliferative DR. In rodents, hyperprolactinemia led to vasoinhibin accumulation within the retina; genetic deletion of the PRL receptor prevented this effect, indicating receptor-mediated incorporation of systemic PRL into the eye. Hyperprolactinemia reduced both VEGF-induced and diabetes-induced increase of RVP. This reduction was blocked by bromocriptine, an inhibitor of pituitary PRL secretion, which lowers the levels of circulating PRL and retinal vasoinhibins.

CONCLUSIONS—Circulating PRL influences the progression of DR after its intraocular conversion to vasoinhibins. Inducing hyperprolactinemia may represent a novel therapy against DR.
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Diabetic retinopathy (DR) develops from a microangiopathy, in which the loss of pericytes and endothelial cells results in abnormally permeable retinal capillaries. In its early stages, elevated retinal vasopermeability causes intraretinal hemorrhages and exudates that, together with capillary closure, create nonperfusion areas. Over time, the resulting hypoxia stimulates the local production of proangiogenic factors, such as vascular endothelial growth factor (VEGF); the newly formed blood vessels extend and bleed into the vitreous, eventually causing detachment of the retina from the accompanying fibrous tissue as well as loss of vision (1). The current treatments for DR, laser photocoagulation and vitrectomy, are often effective but can be destructive and only treat the advanced disease (2). Thus, developing new strategies to oppose both excessive retinal vasopermeability and angiogenic responses has become a major research focus.

Vasoinhibins are a family of antiangiogenic prolactin (PRL) fragments (3) that inhibit ischemia-induced retinal angiogenesis (4) and prevent excessive retinal vasopermeability associated with diabetes (5). Vasoinhibins are present in the retina (6), and because radioactive PRL injected intracardially is incorporated into ocular tissues (ciliary body, choroid, and retina) (7), we reasoned that a portion of ocular vasoinhibins could originate from the intraocular cleavage of PRL coming from the circulation; therefore, high levels of serum PRL in diabetic patients may restrain DR progression.

RESEARCH DESIGN AND METHODS

The Institutional Review Board of the Hospital "Dr. Luis Sánchez Bulnes" approved the protocol for blood sample collection. All subjects were recruited in this hospital, provided written informed consent before collection of samples, and were treated in accord with the tenets of the Declaration of Helsinki. The cohort consisted of 181 male mestizo patients with type 1 or type 2 diabetes and an estimated mean time from disease onset of 13.6 ± 0.7 years. Forty healthy male, mestizo volunteers without diabetes served as control subjects. The mean age of the diabetic patients was 61.3 ± 1.2 years, and that of the control group was 57.0 ± 1.7 years. All participants underwent clinical evaluation and a comprehensive ophthalmologic examination including visual field testing, intraocular pressure evaluation, slit-lamp biomicroscopy, and indirect ophthalmoscopy. Also, fluorescein angiography was performed in all patients with DR. Patients with diabetes were under glycemic control and receiving either insulin or oral antidiabetic agents and an appropriate diet. Exclusion criteria included the treatment with common medications causing hyperprolactinemia (major tranquilizers and antipsychotics [chlorpromazine, haloperidol, risperidone, and amisulpride], prokinetics [metoclopramide and domperidone], or antihypertensive drugs [α-methyldopa, reserpine, and verapamil]), and having renal dysfunction (serum creatinine >1.5 mg/dl). Based on the international clinical DR severity scale (8), patients with diabetes were classified as having no DR (NDR) (n = 37), nonproliferative DR (NPDR) (n = 92), or proliferative DR (PDR) (n = 72). Table 1 summarizes the demographics and attributes of all patients studied. Blood samples were obtained by

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TABLE 1
Characteristics of the male subjects studied

	Control subjects*	Diabetic patients			Comparison among the three diabetic groups
		NDR	NPDR	PDR	*P*
Age (years)	57.7 ± 1.7	61.9 ± 1.8	61.8 ± 1.8	61.2 ± 1.0	NS
n	40	37	92	72	
Type 1 diabetes (*n*)	NA	0	16	7	NA
Type 2 diabetes (*n*)	NA	37	76	65	NA
Diabetes duration (years)	NA	14.3 ± 0.9	14.0 ± 0.6	13.9 ± 0.7	NS
n		37	92	50	
Glucose (mg/dl)	86.6 ± 3.2	156.9 ± 16.1†	177.7 ± 14.4†	165.6 ± 24.0†	NS
n	7	26	39	23	
Hb1Ac (%)	—	8.3 ± 0.5	9.8 ± 0.6	9.2 ± 1.4	NS
n		14	19	5	
Cholesterol (mg/dl)	—	194.8 ± 10.6	195.3 ± 9.8	235.3 ± 11.2	‡<0.05
n		23	35	20	
Creatinine (mg/dl)	—	0.85 ± 0.04	0.90 ± 0.03	0.84 ± 0.03	NS
n		37	92	52	
Systolic BP (mmHg)	120.6 ± 1.3	127.5 ± 3.3	126.6 ± 3.3	134.3 ± 4.1†	‡<0.05
n	40	25	36	29	
Diastolic BP (mmHg)	78.2 ± 0.7	74.4 ± 1.7	76.0 ± 2.1	79.0 ± 2.0	‡<0.05
n	40	25	36	29	
Prolactin (ng/ml)	16.3 ± 1.7	34.1 ± 3.6	32.8 ± 4.9	26.7 ± 2.7	§<0.005; ‖<0.05
n	40	37	92	72	

Data are means ± SEM or *n*. Diabetic patients were without diabetic retinopathy (NDR); with nonproliferative diabetic retinopathy (NPDR), and with proliferative diabetic retinopathy (PDR). *Volunteers with no diabetes-related disorders.†vs. control subjects $P < 0.001$. ‡NDR = NPDR < PDR. §Control subject < NDR. ‖NDR = NPDR > PDR. BP, blood pressure; NA, not applicable. NS, $P > 0.05$; —, not determined.

venipuncture from a peripheral vein after overnight fasting and prior to any treatment. Serum was collected and kept at −70°C until use.

Male Wistar rats (250–300 g) and PRL receptor–null and age-matched wild-type mice (9 weeks, 129SvJ background) were maintained and treated in accord with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The Bioethics Committee of the Institute of Neurobiology from the National University of Mexico (UNAM) approved all animal experiments. Hyperprolactinemia was induced by the implantation of two anterior pituitary glands (APs) under the kidney capsule as previously described (9), and sham rats were subjected to similar surgery without implantation. Ten days after surgery, AP-implanted and nonimplanted rats received a daily, intraperitoneal (i.p.) injection of vehicle or bromocriptine (5 mg/kg body wt); 5 days later, all animals were killed and the sera and retinas collected to evaluate PRL and vasoinhibin levels, respectively. In other experiments, rats with or without AP implants for 15 days were anesthetized with 70% ketamine and 30% xylazine (1 μl/g body wt, i.p.) and injected intravitreously with PBS or 9 μmol/l recombinant human VEGF ($rhVEGF_{165}$; gift from Genentech, South San Francisco, CA) as described (5). After 24 h, retinal vasopermeability was evaluated by fluoroangiography and the Evans blue method. Finally, to induce a diabetic state, rats with or without AP implants for 15 days were injected or not with a single i.p. dose of streptozotocin (60 mg/kg in 10 mmol/l citrate buffer, pH 4.5) (Sigma-Aldrich, St. Louis, MO) after overnight fasting. Rats with a blood glucose concentration ≥250 mg/dl were considered diabetic. After 75 days, animals were killed to determine the levels of serum PRL and retinal vasoinhibins or to evaluate the level of retinal vasopermeability by the Evans blue method. Some groups of diabetic and nondiabetic rats, with or without AP implants, were injected daily with bromocriptine 5 days before being killed.

Serum PRL. Serum PRL was measured in human subjects by a commercial ELISA kit (Genzyme Diagnostics, San Carlos, CA) or in rats and mice by the Nb2 cell bioassay, a standard procedure based on the proliferative response of the Nb2 lymphoma cells to PRL (10).

Western blot. Pools of rat or mouse retinas (four or six, respectively) were homogenized in 0.5% Nonidet P-40, 0.1% SDS, 50 mmol/l Tris, 150 mmol/l NaCl, 100 μg/ml phenylmethylsulnonyl fluoride, and 1 μg/ml aprotinin (pH 7) and centrifuged (9,600*g* for 10 min). Supernatant protein (50 μg) was resolved by 15% SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes, and probed with 1:500 monoclonal antibody INN-1 to rat PRL that reacts with the NH_2-terminus of PRL (6), or with 1:500 antiserum to mouse PRL (AFP-131078; National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Secondary antibodies conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA) were used. Optical density values were determined using the Quantity One, 1-D analysis software (Bio-Rad, Hercules, CA).

Immunohistochemistry. Rat eyes were fixed in 10% formalin and embedded in paraffin. Twelve-micrometer paraffin sections were then processed for immunohistochemistry using 1:100 monoclonal anti-PRL receptor U-5 antibody (Novus Biologicals, Littleton, CO) and an avidin-biotin-peroxidase reaction (Vector Laboratories, Burlingame, CA).

Fluorescein isothiocyanate-dextran angiography. Fluorescein isothiocyanate (FITC) angiography was carried out as reported (11). Blood vessel network was delineated and its area quantified using the Image Pro-Plus software (Media Cybernetics, Silver Spring, MD).

Retinal vasopermeability. Retinal vasopermeability was measured using a modification of the Evans blue assay as previously described (5).

Statistical analysis. Data are given as means ± SEM. The unpaired Student *t* test was used for all comparisons except for data with multiple groups using ANOVA.

RESULTS

Serum PRL levels are higher in diabetic patients with no retinopathy than in those with PDR. All diabetic patients showed higher levels of PRL than the control subjects (32.8 ± 4.9 vs. 16.3 ± 1.7 ng/ml, $P < 0.005$). However, patients with PDR had a reduced concentration of PRL (26.7 ± 2.7 ng/ml, $P < 0.05$) compared with diabetic patients without retinopathy (34.1 ± 3.6 ng/ml) (Fig. 1*A*). The pattern of circulating PRL levels (higher in diabetic patients with no retinopathy than in those with PDR) did not depend on the type of diabetes (Fig. 1*B*) or on other systemic complications associated with diabetes (hypertension and nephropathy). An inverse relationship between PRL levels and the progression of DR was observed when comparing only individuals having prehypertension (≥120/80 mmHg) or hypertension (≥140/90 mmHg) (Fig. 1*C*) and when a subset of all diabetic patients were matched for diabetes duration, glycemia, glycosylated hemoglobin, cholesterol, creatinine, and blood pressure (Fig. 1*D*).

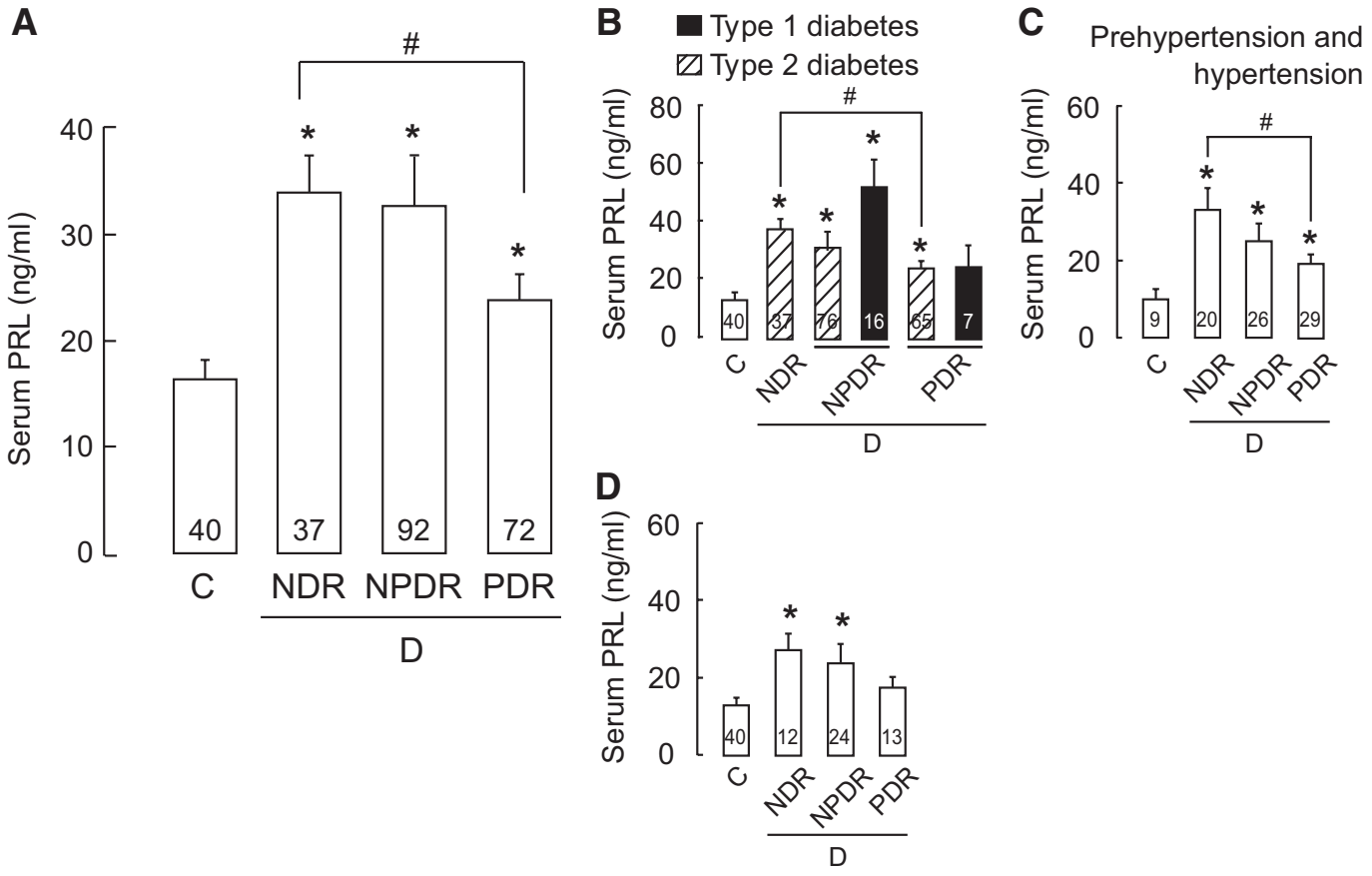

FIG. 1. Circulating levels of PRL are higher in diabetic patients than in nondiabetic control subjects and higher in diabetic subjects with no retinopathy than in those with PDR. *A–C*: Serum PRL levels measured by ELISA in the whole cohort of nondiabetic control subjects and diabetic patients with NDR, NPDR, or PDR (*A*); control subjects and patients having type 1 or type 2 diabetes (*B*); diabetic patients and control subjects having prehypertension (≥120/80 mmHg) or hypertension (≥140/90 mmHg) (*C*). *D*: Diabetic groups matched for diabetes duration (NDR, 14.1 ± 2.3 years; NPDR, 14.1 ± 1.3 years; and PDR, 15 ± 1.4 years), glycemia (NDR, 161.2 ± 20.5 mg/dl; NPDR, 176.3 ± 18.7 mg/dl; and PDR, 210.5 ± 34.7 mg/dl), glycosylated hemoglobin (NDR, 8.1 ± 0.5%; NPDR, 9.8 ± 0.8%; and PDR, 10.2 ± 1.05%), cholesterol (NDR, 176.9 ± 16.1 mg/dl; NPDR, 181.8 ± 9.3 mg/dl; and PDR, 208.8 ± 10.5 mg/dl), creatinine (NDR, 1 ± 0.04 mg/dl; NPDR, 0.9 ± 0.05 mg/dl; and PDR, 1.1 ± 0.1 mg/dl), and systolic blood pressure (NDR, 128.2 ± 4.7 mmHg; NPDR, 122.5 ± 3.5 mmHg; and PDR, 130 ± 4.8 mmHg). Data are means ± SEM. Numbers inside bars correspond to *n* values. *$P \leq 0.001$ vs. control; #$P < 0.05$ vs. NDR. C, control subjects; D, diabetic subjects.

Serum PRL is incorporated into the eye and converted to vasoinhibins. To investigate whether serum PRL could serve as a source of retinal vasoinhibins we used a rat model of hyperprolactinemia induced by placing two AP grafts under the kidney capsule for 15 days (9). As demonstrated by the Nb2 cell bioassay and Western blot, AP-grafted rats showed a sixfold increase of serum PRL (Fig. 2*A*, $n = 20$) and threefold higher levels of vasoinhibins in the retina (Fig. 2*B* and *C*, $n = 3$) compared with the nongrafted controls. Injection of grafted rats with the dopamine D2 receptor agonist bromocriptine, an inhibitor of PRL secretion (12), reduced both PRL in the circulation and vasoinhibins in the retina to basal levels (Fig. 2*A–C*).

The ciliary body is responsible for the active transport of plasma proteins to intraocular fluids (13), and we detected that the PRL receptor is localized in this structure (Fig. 2*D*, $n = 3$). To examine if the PRL receptor in the ciliary body could mediate uptake of circulating PRL and its transfer into the vitreous, we analyzed retinal vasoinhibins in PRL receptor–null mice, which are known to be hyperprolactinemic (12). The mice displayed 700-fold higher levels of circulating PRL (Fig. 2*E*, $n = 8$), but the levels of retinal vasoinhibins were similar to those of their wild-type littermates (Fig. 2*F* and *G*, $n = 3$).

High levels of circulating prolactin mitigate increased retinal vasopermeability in diabetic rats. We next investigated whether hyperprolactinemia, by raising intraocular vasoinhibins, could lower diabetes-induced retinal hypervasopermeability. To examine this issue, AP-grafted rats were challenged or not with intravitreously injected VEGF, which is one of the prominent vasopermeability factors in DR (1). VEGF caused multiple hemorrhage areas (Fig. 3*A* and *B*, $n = 3$) and increased vasopermeability (Fig. 3*C*, $n = 3$) in the retina. These VEGF-induced vascular alterations in the retina were greatly diminished in hyperprolactinemic animals (Fig. 3). Next, retinal vasopermeability was assessed in nongrafted and AP-grafted rats made diabetic by streptozotocin injection. Serum PRL levels were similar in diabetic rats compared with nondiabetic controls (Fig. 3*D*, $n = 4$). Hyperprolactinemia was higher in nondiabetic than in diabetic animals (13-fold vs. 8.5-fold, respectively) and was blocked by treatment with bromocriptine (Fig. 3*D*). Consistent with these changes, the levels of vasoinhibins were elevated in the retina of grafted rats, and bromocriptine reduced this increase (Fig. 3*E*), supporting that hyperprolactinemia raises vasoinhibins in the retina. Then, we confirmed that retinal vasopermeability increases in dia-

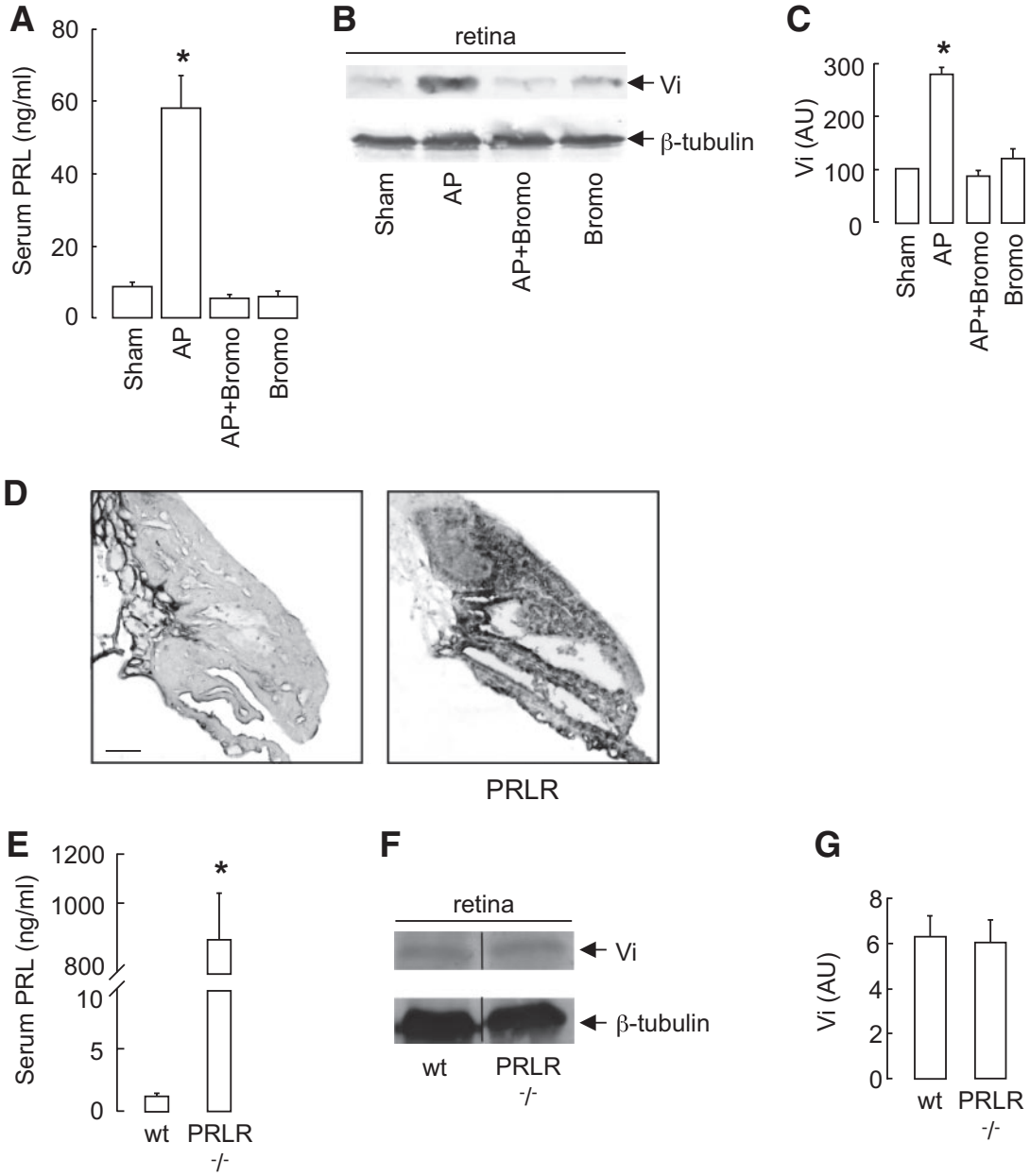

FIG. 2. Hyperprolactinemia results in higher levels of retinal vasoinhibins via PRL receptor–mediated PRL internalization into the eye. *A–C*: Rats implanted (AP) or not (Sham) with two APs under the renal capsule for 15 days were injected (Bromo) or not with bromocriptine. *A*: Serum PRL levels as measured by the Nb2 cell bioassay. *$P < 0.05$ vs. Sham ($n = 20$ rats per group). Western blot analysis of vasoinhibin levels in the retina (*B*) and corresponding densitometric analysis normalized to β-tubulin (*C*). *$P < 0.05$ vs. Sham ($n = 3$, each a pool of four retinas). *D*: Representative immunohistochemistry of rat ciliary body sections stained with monoclonal anti-PRL receptor (PRLR) antibody or without primary antibody (*left*) ($n = 3$ independent experiments). Scale bar, 50 μm. *E*: Serum PRL levels assessed with the Nb2 cell bioassay in wild-type (wt) and PRLR-null ($PRL^{-}/^{-}$) mice. *$P < 0.05$ vs. wild-type mice ($n = 8$ mice per group). Representative Western blot analysis of retinal vasoinhibins in wild-type and $PRLR^{-}/^{-}$ mice (different lanes from the same gel) (*F*) and corresponding evaluation of retinal vasoinhibins by densitometry normalized to β-tubulin (*G*) ($n = 3$, each a pool of six retinas). Data in *A*, *C*, *E*, and *G* are means ± SEM. Vi, vasoinhibins.

betic animals ($P \leq 0.011$) and found that the magnitude of this increase was significantly smaller ($P \leq 0.05$) in hyperprolactinemic diabetic rats compared with the normoprolactinemic counterparts (Fig. 3*F*, $n = 4$). Reduced retinal vasopermeability is likely due to the hyperprolactinemia-induced elevation of intraocular vasoinhibins because it was prevented by bromocriptine (Fig. 3*F*). In the absence of diabetes, neither hyperprolactinemia nor administration of bromocriptine affected retinal vasopermeability compared with untreated animals (Fig. 3*F*).

DISCUSSION

The association between circulating PRL levels and DR has long been controversial. Studies performed over 2 decades ago reported increased (14), decreased (15), or normal (16,17) PRL levels in patients with DR. Here, we show that the circulating concentration of PRL is higher in diabetic patients without retinopathy than in those with PDR. These findings indicate an inverse relationship between systemic PRL and the severity of DR and suggest that previous contradictory findings are due to the lack of DR grading and the small number of patients reported (14–17).

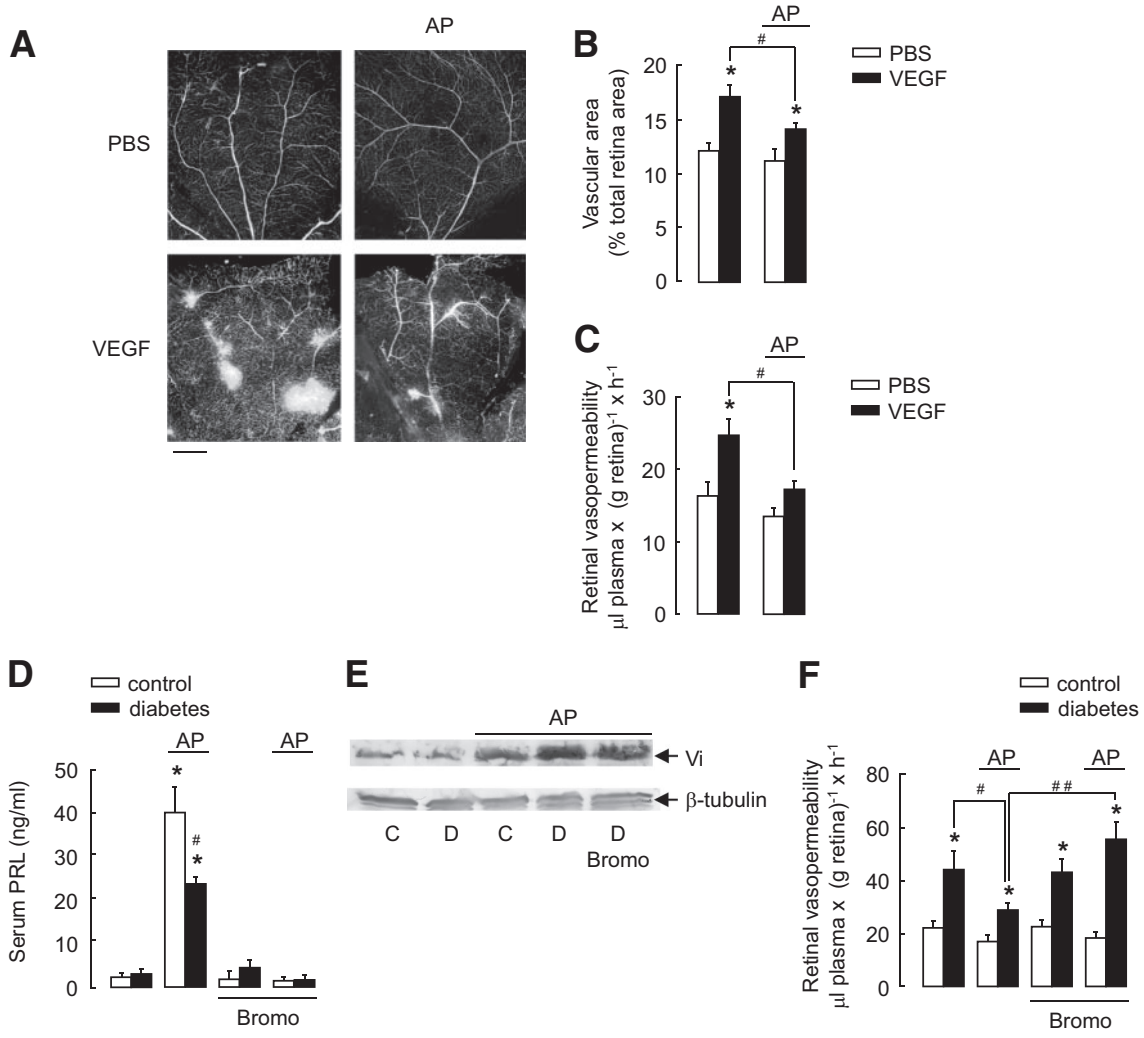

FIG. 3. Hyperprolactinemia mitigates excessive retinal vasopermeability in VEGF-treated or diabetic rats. *A–C*: Rats implanted (AP) or not with two APs under the kidney capsule were injected intravitreally with PBS or 9 µmol/l VEGF and examined 24 h later. *A*: Representative images of fluorescein-labeled retinas. *B*: Quantification of vascular area from 10 flat-mounted retinas in each group. Scale bar = 500 µm. *C*: Retinal vasopermeability determined by the Evans blue assay. **P* < 0.05 vs. PBS, #*P* < 0.05 vs. VEGF-treated nonimplanted rats (*n* = 3 independent experiments). *D–F*: Nonimplanted and AP-implanted rats made diabetic for 75 days by streptozotocin were injected (Bromo) or not with bromocriptine and evaluated for serum PRL levels using the Nb2 cell bioassay (P* < 0.05 vs. nondiabetic control and nonimplanted control and diabetic rats; *n* = 4 rats per group) (*D*), retinal vasoinhibins by Western blot (pool from four retinas) (*E*), and retinal vasopermeability by the Evans blue method (*n* = 4 rats per group) (*F*). **P* < 0.05 vs. nondiabetic control, #*P* < 0.05 vs. diabetic nonimplanted control, and ##*P* < 0.05 vs. AP diabetic without bromocriptine. Data in *B–D* and *F* are means ± SEM. Vi, vasoinhibins.**

The inverse correlation between PRL and DR suggests that systemic PRL, after its conversion to vasoinhibins, influences the progression of DR. It was reported recently that patients with DR have lower levels of circulating vasoinhibins than nondiabetic patients (18). This result, together with our finding of higher circulating levels of PRL in diabetic patients without retinopathy, suggests that one way to upregulate intraocular vasoinhibins would be to further increase the systemic levels of PRL, thereby favoring its ocular incorporation and cleavage. Supporting this notion, hyperprolactinemia led to the accumulation of vasoinhibins within the retina of rodents. Notably, genetic deletion of the PRL receptor prevented this effect, indicating receptor-mediated incorporation of systemic PRL into the eye in a manner similar to that described for PRL transport across the choroid plexus to the cerebral spinal fluid (19) or across the mammary epithelium to the milk (20). These results suggest that the PRL receptor mediates the ocular incorporation of systemic PRL, which can then be cleaved to vasoinhibins.

Furthermore, the observation that higher levels of serum PRL mitigate excessive retinal vasopermeability in diabetic and VEGF-injected rats is consistent with hyperprolactinemia producing higher levels of ocular vasoinhibins and suggests hyperprolactinemia as a therapeutic strategy against diabetes-induced retinal hypervasopermeability. A broadly protective role of PRL in diabetes is supported by studies showing that PRL stimulates β-cell proliferation, insulin gene transcription, and insulin secretion in normal physiology and in pregnancy (21,22). Notably, the risk of DR development and progression increases during pregnancy, followed by a high regression rate during the postpartum period (23). The pathogenic mechanisms of DR in pregnancy are not fully understood, but PRL may play a protective role. There is evidence that PRL levels are lower in diabetic than in healthy pregnant women (24) and high, sustained PRL levels occur during lactation, when DR improves significantly (25). However, serum PRL levels showed no correlation with DR during pregnancy (24), and larger studies analyzing various stages

of retinopathy are required to evaluate this relationship conclusively.

Here, we reveal PRL to be an important systemic inhibitor of diabetes-induced retinal hypervasopermeability after its intraocular conversion to vasoinhibins, which act directly on endothelial cells to block blood vessel growth, dilation, and permeability and to promote apoptosis-mediated vascular regression (5). Thus, we propose that PRL/vasoinhibins are endogenous regulators of the development and progression of DR and that current medications known to induce hyperprolactinemia constitute novel therapeutic options to treat DR and other vasoproliferative retinopathies.

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E.A. performed most experiments and contributed to postulating the hypothesis, designing the experiments, and writing the manuscript. J.C.R. performed measurements on human serum, participated in animal experiments, and contributed to postulating the hypothesis. S.T. analyzed patient data and contributed to designing the experiments and writing the manuscript. D.M.-P. and H.Q.-M. enrolled patients, collected human serum samples, and carried out the ophthalmologic examination. A.Q.-S. provided hyperprolactinemic rats. N.B. provided PRL receptor–null mice. G.M.E. gave scientific advice, supervised analysis, and edited the manuscript. C.C. conceived, coordinated, and supervised the study and contributed to postulating the hypothesis, designing the experiments, and writing the manuscript.

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