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. Diabetes 59:3192–3197, 2010

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 ± 7.0 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 opthalmologic 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 [amlodipine, 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
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).

**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. 1A). 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. 1B) 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. 1C) and when a subset of all diabetic patients were matched for diabetes duration, glycemia, glycosylated hemoglobin, cholesterol, creatinine, and blood pressure (Fig. 1D).

### TABLE 1
Characteristics of the male subjects studied

| Age (years) | Control subjects | NDR | NPDR | PDR | Comparison among the three diabetic groups |
|-------------|------------------|-----|------|-----|------------------------------------------|
| n           | 40               | 37  | 25   | 36  | 40                                       | P |
| Type 1 diabetes (n) | NA             | 0   | 16   | 7   | NS                                       |   |
| Type 2 diabetes (n) | NA             | 37  | 76   | 65  | NS                                       |   |
| Diabetes duration (years) | NA             | 14.3| 14.0| 13.9| NS                                       |
| Glucose (mg/dl) | 86.6 ± 3.2      | 156.9 ± 16.1† | 177.7 ± 14.4† | 165.6 ± 24.0† | NS |
| Hb1Ac (%) | 8.3 ± 0.5       | 9.8 ± 0.6 | 9.2 ± 1.4 | NS |
| Cholesterol (mg/dl) | —              | 194.8 ± 10.6 | 195.3 ± 9.8 | 235.3 ± 11.2 | <0.05 |
| Creatinine (mg/dl) | —              | 0.85 ± 0.04 | 0.90 ± 0.03 | 0.84 ± 0.03 | NS |
| Systolic BP (mmHg) | 120.6 ± 1.3     | 127.5 ± 3.3 | 126.6 ± 3.3 | 134.3 ± 4.1† | <0.05 |
| Diastolic BP (mmHg) | 78.2 ± 0.7      | 74.4 ± 1.7 | 76.0 ± 2.1 | 79.0 ± 2.0 | <0.05 |
| Prolactin (ng/ml) | 16.3 ± 1.7      | 34.1 ± 3.6 | 32.8 ± 4.9 | 26.7 ± 2.7 | <0.005; <0.05 |

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.
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. 2A, n = 20) and threefold higher levels of vasoinhibins in the retina (Fig. 2B 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. 2A-C).

The ciliary body is responsible for the active transport of plasma proteins to intraocular fluids (13), and we confirmed that retinal vasopermeability increases in diabetic patients 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 ≤ 0.001 vs. control; #P < 0.05 vs. NDR. C, control subjects; D, diabetic subjects.

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. 3A and B, n = 3) and increased vasopermeability (Fig. 3C, 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. 3D, 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. 3D). Consistent with these changes, the levels of vasoinhibins were elevated in the retina of grafted rats, and bromocriptine reduced this increase (Fig. 3E), supporting that hyperprolactinemia raises vasoinhibins in the retina. Then, we confirmed that retinal vasopermeability increases in dia-
betic animals (P ≤ 0.011) and found that the magnitude of this increase was significantly smaller (P ≤ 0.05) in hyperprolactinemic diabetic rats compared with the normoprolactinemic counterparts (Fig. 3F, n = 4). Reduced retinal vasopermeability is likely due to the hyperprolactinemia-induced elevation of intraocular vasoinhibins because it was prevented by bromocriptine (Fig. 3F). In the absence of diabetes, neither hyperprolactinemia nor administration of bromocriptine affected retinal vasopermeability compared with untreated animals (Fig. 3F).

**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. This finding is likely due to the hyperprolactinemia-induced elevation of intraocular vasoinhibins because it was prevented by bromocriptine (Fig. 3F). In the absence of diabetes, neither hyperprolactinemia nor administration of bromocriptine affected retinal vasopermeability compared with untreated animals (Fig. 3F).
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 H9252-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 pregnancy are needed to confirm these observations.
of retinopathy are required to evaluate this relationship conclusively.

Here, we reveal PRL to be an important systemic inhibitor of diabetes-induced retinal hypervasoproliferability 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 wrote the manuscript. D.D. Pless, Neurobiology Institute, UNAM, Mondragón, A. Prado, and M. García for their technical assistance, and D.D. Pless, Neurobiology Institute, UNAM, for critically editing the manuscript.

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