tvst
Article

Levosulpiride Increases the Levels of Prolactin and Antiangiogenic Vasoinhibin in the Vitreous of Patients with Proliferative Diabetic Retinopathy

Carlos D. Nuñez-Amaro[1,*], Aura Ileana Moreno-Vega[2,*], Elva Adan-Castro[2,*], Magdalena Zamora[2,*], Renata Garcia-Franco[3], Paulina Ramirez-Neria[4], Marlon Garcia-Roa[4], Yolanda Villalpando[4], Juan Pablo Robles[2], Gabriela Ramirez-Hernandez[2], Mariana Lopez[4], Jorge Sanchez[3], Ellery Lopez-Star[4], Thomas Bertsch[5], Gonzalo Martinez de la Escalera[2], Ma. Ludivina Robles-Osorio[1], Jakob Triebel[5,†], and Carmen Clapp[2,†]

[1] Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro, Querétaro, México
[2] Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla, Querétaro, México
[3] Instituto de la Retina del Bajío, Querétaro, México
[4] Instituto Mexicano de Oftalmología, Querétaro, México
[5] Institute for Clinical Chemistry, Laboratory Medicine and Transfusion Medicine, Nuremberg General Hospital and Paracelsus Medical University, Nuremberg, Germany

Correspondence: Carmen Clapp, Instituto de Neurobiología, Universidad Nacional Autónoma de México, Campus Juriquilla, Querétaro, 76230 México.
e-mail: clapp@unam.mx
Ludivina Robles-Osorio, Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro, Querétaro, 76230 México.
e-mail: ludirobles7@yahoo.com

Received: June 16, 2020
Accepted: July 16, 2020
Published: August 17, 2020

Keywords: proliferative diabetic retinopathy; antiangiogenic factor; dopamine D2 receptor blocker; 16K prolactin; hyperprolactinemia

Citation: Nuñez-Amaro CD, Moreno-Vega AI, Adan-Castro E, Zamora M, Garcia-Franco R, Ramirez-Neria P, Garcia-Roa M, Villalpando Y, Robles JP, Ramirez-Hernandez G, Lopez M, Sanchez J, Lopez-Star E, Bertsch T, Martinez de la Escalera G,

Purpose: High circulating levels of the hormone prolactin (PRL) protect against experimental diabetic retinopathy (DR) due to the retinal accumulation of vasoinhibin, a PRL fragment that inhibits blood vessel permeability and growth. A phase 2 clinical trial is investigating a new therapy for DR based on elevating serum PRL levels with levosulpiride, a prokinetic dopamine D2 receptor blocker. Here, we tested whether levosulpiride-induced hyperprolactinemia elevates PRL and vasoinhibin in the vitreous of volunteer patients with proliferative DR (PDR) undergoing elective pars plana vitrectomy.

Methods: Patients were randomized to receive placebo (lactose pill, orally TID; $n = 19$) or levosulpiride (25 mg orally TID; $n = 18$) for the 7 days before vitrectomy. Vitreous samples from untreated non-diabetic ($n = 10$) and PDR ($n = 17$) patients were also studied.

Results: Levosulpiride elevated the systemic (101 $\pm$ 13 [SEM] vs. 9.2 $\pm$ 1.3 ng/mL, $P < 0.0001$) and vitreous (3.2 $\pm$ 0.4 vs. 1.5 $\pm$ 0.2 ng/mL, $P < 0.0001$) levels of PRL, and both levels were directly correlated ($r = 0.58$, $P < 0.0002$). The vitreous from non-diabetic patients or from PDR patients treated with levosulpiride, but not from placebo-treated PDR patients, inhibited the basic fibroblast growth factor (bFGF)- and vascular endothelial growth factor (VEGF)-induced proliferation of endothelial cells in culture. Vasoinhibin-neutralizing antibodies reduced the vitreous antiangiogenic effect. Matrix metalloproteases (MMPs) in the vitreous cleaved PRL to vasoinhibin, and their activity was higher in non-diabetic than in PDR patients.

Conclusions: Levosulpiride increases the levels of PRL in the vitreous of PDR patients and promotes its MMP-mediated conversion to vasoinhibin, which can inhibit angiogenesis in DR.

Translational Relevance: These findings support the potential therapeutic benefit of levosulpiride against vision loss in diabetes.

Robles-Osorio ML, Triebel J, Clapp C. Levosulpiride increases the levels of prolactin and antiangiogenic vasoinhibin in the vitreous of patients with proliferative diabetic retinopathy. Trans Vis Sci Tech. 2020;9(9):27, https://doi.org/10.1167/tvst.9.9.27

Introduction

Diabetic retinopathy (DR) and diabetic macular edema (DME) are microvascular diseases and major causes of vision loss in adults throughout the world.[1,2] Approximately 28 million persons with diabetes have sight-threatening stages of DR and DME,[3] and, as the number of people with diabetes increases, the global incidence of DR and DME is predicted to rise to 55% by 2030.[4] Current treatments—laser photocoagulation, intravitreal injections, and vitrectomy—are invasive, not always effective, temporary, and costly, thus emphasizing the need for additional therapeutic options.[5,6] An ongoing clinical trial is investigating a new therapy for DR and DME based on elevating the circulating levels of the hormone prolactin (PRL) with the prokinetic dopamine D2 receptor blocker, levosulpiride (ClinicalTrials.gov ID: NCT03161652).

PRL, the pituitary hormone essential for lactation, acquires antiangiogenic properties upon its proteolytic cleavage to vasoinhibin, a PRL fragment that inhibits angiogenesis, vasopermeability, and vasodilation.[7] The regulation of vasoinhibin generation and action occurs at the hypothalamus, the pituitary, and the target tissue levels defining the PRL–vasoinhibin axis.[8] This axis helps preserve corneal avascularity[9] and normal retinal vasculature[10] and is altered in retinopathy of prematurity[11] and DR.[12] The circulating levels of vasoinhibin are reduced in patients with DR,[13] and studies have shown that the elevation of systemic PRL results in the accumulation of vasoinhibin in the retina capable of inhibiting vascular endothelial growth factor (VEGF)- and diabetes-induced retinal vasopermeability,[14,15] as well as ischemia-induced retinal angiogenesis.[16] These findings have led to the hypothesis that medications causing hyperprolactinemia are beneficial in DR and DME. Levosulpiride, a prokinetic frequently used to treat diabetic gastroparesis,[17] is effective for inducing hyperprolactinemia[18] and acts by blocking dopamine D2 receptors at the gastrointestinal and anterior pituitary levels.[19]

Here, we report results from our ongoing clinical trial showing that levosulpiride increases the levels of PRL and its conversion to bioactive vasoinhibin in the vitreous of PDR patients, which may help counteract disease progression.

Methods

Study Design and Participants

The design of the clinical trial was reported previously[20] and is summarized briefly here. This trial is a prospective, randomized, double-blind, placebo-controlled study in male and female PDR patients with type 2 diabetes who underwent elective primary pars plana vitrectomy at Instituto Mexicano de Oftalmología (IMO), Querétaro, México, from May 2017 to August 2019. Written informed consent was obtained from all participants prior to study enrollment. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Bioethics Committees of the Instituto de Neurobiología, Universidad Nacional Autónoma de México (UNAM) and the IMO. Exclusion criteria included age below 40 years and above 69 years, history of prior vitrectomy, PRL serum levels $>$ 20 ng/mL, glomerular filtration rate $<$ 30 mL/minute, contraindications for the use of levosulpiride (Parkinson's disease, epilepsy, breast cancer), and pathologies (prolactinoma, hypothyroidism, hepatic dysfunction) and medications (antipsychotics, antidepressants, prokinetics, estrogens) inducing hyperprolactinemia. The cohort consisted of 37 mestizo patients (16 females and 21 males) randomized to receive placebo (lactose pill orally TID, $n = 19$) or levosulpiride (25 mg orally TID, $n = 18$) for 1 week before vitrectomy. Blood samples were withdrawn after the informed consent was signed to evaluate whether clinical parameters, including PRL levels, avoided the exclusion criteria. Blood samples were also obtained immediately prior to surgery but before induction of anesthesia. Before fluid infusion, 1 mL of non-dilute vitreous sample was collected using 25- or 27-gauge vitrectomy systems.

Serum and vitreous samples were assayed for PRL levels on the day of the surgery, and the remaining sample was stored at –80°C.

In addition, a non-interventional (observational) study, independent of the clinical trial, was carried out to acquire vitreous samples from elective vitrectomies that would otherwise have been discarded. These samples allowed us to compare PRL and vasoinhibin levels in the vitreous of non-diabetic mestizo males scheduled to undergo vitrectomy ($n = 10$) for rhegmatogenous retinal detachment or epiretinal membrane to those of male mestizo subjects undergoing elective vitrectomy for the treatment of PDR ($n = 17$). The absence of diabetes was defined as having no diabetes symptoms, no medical history of hyperglycemia, a lack of antihyperglycemic medications, and glycosylated hemoglobin (HbA1C) levels of <6.5%. Patients were recruited after their written informed consent in accordance with the tenets of the Declaration of Helsinki and the approval of the IMO institutional review board. Blood was collected before the induction of anesthesia, immediately before surgery.

PRL and Vasoinhibin Standards

Recombinant human PRL produced in *Escherichia coli* was provided by Michael E. Hodsdon (Yale University, New Haven, CT), and recombinant vasoinhibin (corresponding to the first 123 amino acids of human PRL) was generated by site-directed mutagenesis in insect cells as previously described.[21]

PRL and Vasoinhibin Analyses

PRL levels in serum and vitreous samples were quantified using the IMMULITE 2000 XPi immunoassay system (Siemens, Munich, Germany). The intra-assay and inter-assay coefficients of variance were less than 1%. Vasoinhibin has low immunoreactivity (<10%) in this PRL immunoassay (data not shown) and in other quantitative PRL immunoassays[22] and is frequently determined semiquantitatively by immunoprecipitation–western blot (IP-WB). The IP-WB technique previously reported[11] analyzed vasoinhibin levels in the vitreous. Briefly, 200 µL of vitreous was immunoprecipitated overnight with 2 µL of antihuman PRL polyclonal antibodies (anti-hPRL-IC5; National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) followed by a 2-hour incubation with protein A Sepharose beads (35 µL; Sigma-Aldrich, St. Louis, MO). The sample was centrifuged and washed, and the final pellet was subjected to reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15% acrylamide) and blotted. Blots were probed overnight at 4°C with a 1:500 dilution of an anti-human PRL antiserum, either anti-human PRL antiserum (HC1), obtained locally and characterized as reported,[23] or anti-human PRL monoclonal antibodies against the N-terminus of PRL (mAb5602; Diagnostics Biochem Canada, Inc., London, ON, Canada). Detection used alkaline phosphatase-coupled goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and a colorimetric kit (Bio-Rad Laboratories, Hercules, CA).

Endothelial Cell Proliferation

Human umbilical vein endothelial cells (HUVECs) and bovine umbilical vein endothelial cells (BUVECs) were obtained as described.[24,25] HUVECs were cultured in 20% fetal bovine serum (FBS)-F12K medium (Thermo Fisher Scientific, Waltham, MA) containing 100 µg/mL Endothelial Cell Growth Supplement (Corning, Inc., Corning, NY) and 100 -µg/mL heparin. HUVECs were seeded at 14,000 cells/cm^2 and starved 24 hours later with 0.5% FBS-F12K medium for 12 hours. Medium was then replaced by 20% FBS-F12K and 100-µg/mL heparin in the absence or presence of 25 ng/mL VEGF (provided by Genentech, South San Francisco, CA) plus 20 ng/mL of the angiogenic factor basic fibroblast growth factor (bFGF, provided by Scios, Inc., Mountain View, CA), alone or together with 125 ng/µL of vitreous protein from a pool of vitreous obtained from six placebo- or levosulpiride-treated PDR patients. In some experiments, a 1:100 dilution of HC1 or normal rabbit serum (NRS) was added to the VEGF plus bFGF combination treatment. Cells were allowed to proliferate in the presence of 10 µM of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) (Sigma-Aldrich), and the number of cells with newly synthesized DNA was evaluated after 24 hours by the click reaction as reported.[26] Images obtained in a fluorescence inverted microscope were quantified using CellProfiler software.[27] BUVECs were cultured and seeded in 10% FBS-F12K at 5000 cells/cm^2, allowed to attach for 3 to 4 hours, and serum-starved (0.5% FBS) for 12 to 16 hours. The medium was then replaced with 10% FBS-F12K containing or not 10-ng/mL bFGF alone or together with 125-ng/µL protein from individual vitreous samples of non-diabetic and PDR patients alone or together with a 1:100 dilution of HC1 or NRS. BUVECs were allowed to proliferate in the presence of 0.1 µCi ^{3}H-thymidine, and DNA synthesis was evaluated after 24 hours as described.[28] The intra-assay and

inter-assay coefficients of variance of the bioassays were 6% and 10%, respectively.

PRL Cleavage by Vitreous Proteases

The activity of proteases that cleave PRL to vasoinhibin was assessed in vitreous from non-diabetic and PDR patients by the incubation of 200 ng of human PRL with 15 µg of vitreous protein in a final volume of 20 µL of incubation buffer (0.05-M Tris-HCl, 0.15-M NaCl, and 0.01-M CaCl2; pH 7) for 24 hours at 37°C under agitation (600 rpm). In separate experiments, the aspartyl protease inhibitor pepstatin-A (1.4 µM), thrombin inhibitor PPACK (1 µM), serine protease inhibitor aprotinin (10 µg/mL), or matrix metalloprotease (MMP) inhibitors 1,10-phenanthroline (10 mM), ethylenediaminetetraacetic acid (EDTA, 5 mM), and Galardin (Gal, 10 µM) were preincubated for 30 minutes at room temperature with 15 µg protein of vitreous from non-diabetic patients before adding 200 ng of human PRL and adjusting to a final volume of 20 µL of incubation buffer. In all cases, the reaction was stopped by adding reducing Laemmli buffer and boiling the samples for 5 minutes. PRL-cleaved products were investigated by western blot performed as described above.

Statistical Analyses

Sigma Stat 7.0 software (Systat Software, San Jose, CA) was used. Statistical differences between two groups were determined by Student's *t*-test, whereas one-way analysis of variance (ANOVA) followed by Bonferroni's or Tukey's post hoc tests were used to compare means of multiple groups. The χ^2 test was used to determine if there was an association between categorical variables, and Pearson's correlation test was used to evaluate the level of association between numerical variables. All results are expressed as means ± SEM. The threshold for significance was set at $P < 0.05$.

Results

Levosulpiride-Induced Hyperprolactinemia Increases PRL Levels in the Vitreous of PDR Patients

The interventional clinical trial study consisted of 37 patients with type 2 diabetes and PDR undergoing elective pars plana vitrectomy due to vitreous hemorrhage or tractional retinal detachment. Patients were randomized to receive placebo ($n = 19$) or levosulpiride ($n = 18$) orally TID for 1 week before vitrectomy. Before treatment, both groups were similar in age, sex, body mass index, diabetes duration, HbA1c, kidney function (serum creatinine and glomerular filtration rate), and serum PRL levels (Table 1).

The levels of serum PRL before treatment and after placebo were similar and did not differ between males and females (Figs. 1A, 1B). PRL levels in serum were 11-fold higher in patients receiving levosulpiride (101 ± 13 [SEM] vs. 9.2 ± 1.3 ng/mL; $P < 0.0001$), and hyperprolactinemia was significantly higher in females than males (152.8 ± 14 vs. 78.7 ± 8.4 ng/mL; $P < 0.0001$) (Figs. 1A, 1B). PRL was measured in the vitreous from placebo-treated patients at levels that were similar ($P < 0.99$) between the males and females (1.5 ± 0.3 vs. 1.6 ± 0.4 ng/mL, respectively) (Figs. 1C, 1D). PRL vitreous levels increased twofold after treatment with levosulpiride (3.2 ± 0.4 vs. 1.5 ± 0.2 ng/mL, $P < 0.001$) (Fig. 1C) and were higher in treated females than males (4.5 ± 0.3 vs. 2.7 ± 0.3 ng/mL, $P < 0.02$) (Fig. 1D), implying that the increase in vitreous PRL relates to the level of hypeprolactinemia. In agreement, there was a direct association ($r = 0.58$, $P < 0.0002$) between serum and vitreous PRL levels when considering all patients (placebo and levosulpiride) (Fig. 1E), suggesting that high levels of circulating PRL favor the ocular incorporation of PRL.

To determine whether levosulpiride-induced hyperprolactinemia also elevates vasoinhibin in the vitreous, we investigated the presence of vasoinhibin in vitreous samples from placebo and levosulpiride-treated PDR patients by IP-WB (Fig. 2). IP recovered nearly all of the PRL and vasoinhibin standards added to the assay buffer as revealed by WB probed with HC1 anti-human PRL antiserum (Fig. 2A, lanes 1–4) or monoclonal anti-human PRL antibodies directed against the N-terminal end of PRL (Fig. 2B, lanes 1 and 2). The vitreous from levosulpiride-treated patients contained higher levels of a 23-kDa immunoreactive protein compared to placebo-treated patients. The protein has the molecular mass of full-length PRL and was specific in that its increment was detected by both polyclonal and monoclonal anti-PRL antibodies, confirming the levosulpiride-induced elevation of vitreous PRL. The anti-PRL antiserum detected smaller immunoreactive proteins that were not specific, as they were found in samples with and without vitreous (Fig. 2A, lanes 5, 7, and 8). The non-PRL nature of the smaller proteins was confirmed by their absence in blots probed with anti-PRL monoclonal antibodies (Fig. 2B, lanes 3, 5, and 6). Lack of vasoinhibin detection may imply that vitreous vasoinhibin is under the detection limit of the assay (5 ng).

Table 1. Basal Demographic and Clinical Characteristics of the Interventional Study Population

Characteristic	Placebo ($n = 19$)	95% CI	Levosulpiride ($n = 18$)	95% CI	*P*
Age (y), mean ± SEM	56.6 ± 1.3	53.9–59.3	57.4 ± 1.8	53.7–61.1	0.69[a]
Sex F, *n* (%)	8 (42.1)	—	8 (44.4)	—	0.89[b]
BMI (kg/m^2), mean ± SEM	28.1 ± 1.2	25.6–30.5	26.1 ± 1.2	23.6–28.6	0.24[a]
DM2 (y), mean ± SEM	17.5 ± 1.2	15.1–19.9	15.3 ± 1.6	11.9–18.6	0.26[a]
HbA1c (%), mean ± SEM	7.5 ± 0.3	6.8–8.2	7.8 ± 0.4	6.9–8.7	0.55[a]
SCr (mg/dL), mean ± SEM	1.3 ± 0.1	1.0–1.5	1.0 ± 0.08	0.9–1.2	0.08[a]
eGFR (mL/min), mean ± SEM	64 ± 5.7	52–75.9	76.4 ± 4.5	66.9–85.9	0.09[a]
SPRL (ng/mL), mean ± SEM	6.9 ± 0.8	5.2–8.6	9.3 ± 1.4	6.3–12.3	0.15[a]

All patients had proliferative diabetic retinopathy before undergoing elective pars plana vitrectomy and oral treatment with placebo or levosulpiride. *P* values compare basal values of groups receiving placebo and levosulpiride. BMI, body mass index; CI, confidence interval; DM2, type 2 diabetes mellitus; HbA1c, glycosylated hemoglobin; SCr, serum creatinine; eGFR, estimated glomerular filtration rate (CKD-EPI equation); SPRL, serum PRL.

[a]Based on Student's *t*-test.

[b]Based on χ^2 test.

Levosulpiride Increases Bioactive Vasoinhibin in the Vitreous of Patients with PDR

Vasoinhibin was then investigated in the vitreous by means of its bioactive properties. We explored whether the vitreous from levosulpiride-treated PDR patients contained an endogenous PRL-like protein that inhibits endothelial cell proliferation, a well-known effect of vasoinhibin.[7] HUVECs were cultured in the absence and presence of a combination of the angiogenic factors VEGF and bFGF with or without the vitreous from levosulpiride- or placebo-treated PDR patients. PRL and vasoinhibin standards were used as negative and positive controls, respectively. Proliferation was evaluated through the incorporation of the thymidine analog EdU into DNA detected by the click reaction. HUVEC proliferation was significantly increased by VEGF and bFGF (Figs. 3A, 3B). Vasoinhibin or the vitreous from levosulpiride-treated patients inhibited the VEGF- and bFGF-induced endothelial cell proliferation, whereas PRL or the vitreous from placebo-treated patients had no effect. PRL, vasoinhibin, or vitreous did not alter basal proliferation (Figs. 3A, 3B). To investigate whether endogenous vasoinhibin could be responsible for the antiangiogenic action of the vitreous from levosulpiride-treated PDR patients, we used anti-PRL antibodies (HC1 anti-PRL antiserum) that neutralize vasoinhibin action.[29] The anti-PRL antiserum, but not NRS, blocked the inhibition of VEGF- and bFGF-induced proliferation in response to the vitreous from levosulpiride-treated patients or vasoinhibin (Fig. 3C). None of the sera modified the lack of effect of PRL or the vitreous from placebo-treated patients. These results suggest that levosulpiride increases the antiangiogenic properties of the vitreous from PDR patients by elevating PRL and, thereby, vasoinhibin levels.

The Levels of Bioactive Vasoinhibin Are Reduced in PDR Vitreous Compared to Non-Diabetic Vitreous

To investigate whether lower levels of vasoinhibin in PDR vitreous may relate to the angiogenesis in DR, we compared the vasoinhibin-related antiangiogenic properties of the vitreous from male patients who underwent vitrectomy for the treatment of PDR ($n = 10$) to those of non-diabetic men undergoing vitrectomy for a non-vascular cause (i.e., rhegmatogenous retinal detachment or epiretinal membrane; $n = 17$). The antiangiogenic properties of the non-diabetic patient vitreous are closer to those of a normal eye than to those of PDR patient vitreous.[30] The non-diabetic and PDR patients were similar in age, body mass index, and serum and vitreous PRL levels, but differed in HbA1c and kidney function (serum creatinine and glomerular filtration rate) values, which were higher and lower, respectively, in the PDR group (Table 2).

BUVECs were cultured in the absence and presence of bFGF with or without the vitreous from patients without diabetes (control) or PDR patients. Proliferation was evaluated through the incorporation of ^{3}H-thymidine into DNA. Basic FGF stimulated the proliferation of BUVECs ($P < 0.001$), and the vitreous from control patients, but not from PDR patients, reduced this effect ($P < 0.01$) (Fig. 4). The antiangiogenic action

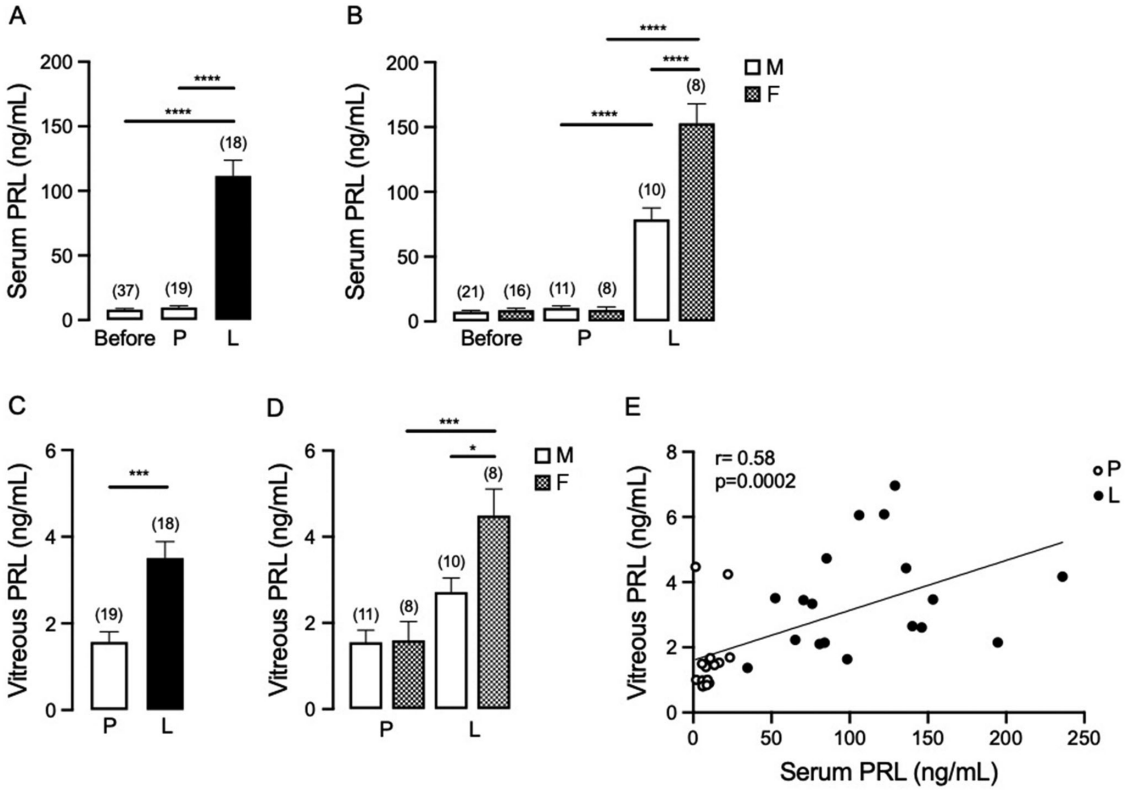

Figure 1. Levosulpiride-induced hyperprolactinemia increases PRL levels in the vitreous of PDR patients. Serum PRL levels in (**A**) all PDR patients and (**B**) in male (M) and female (W) PDR patients before and after undergoing vitrectomy and treatment with placebo (P) or levosulpiride (L). Vitreous PRL levels in (**C**) all PDR patients and (**D**) in male and female PDR patients before and after undergoing vitrectomy and treatment with P or L. Parentheses indicate the number of patients. Results are mean ± SEM. $^{*}P < 0.02$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (**E**) Correlation between vitreous and serum PRL levels in the whole P- and L-treated study population.

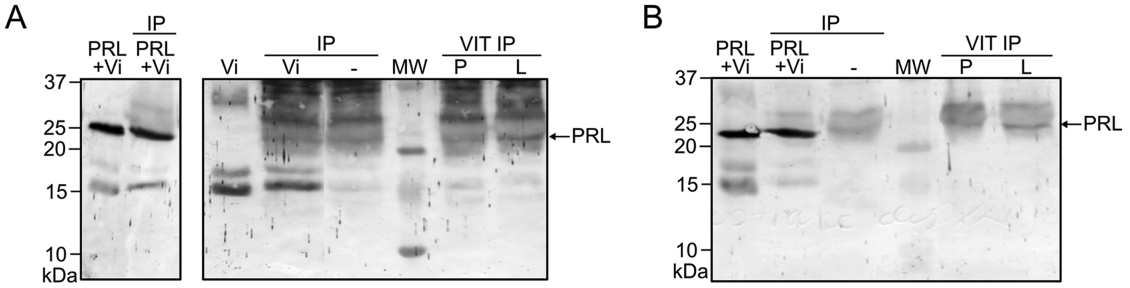

Figure 2. Immunoprecipitation and western blot evaluation of PRL and vasoinhibin in the vitreous of PDR patients treated with placebo (P) or levosulpiride (L). Representative IP-WB probed with (**A**) anti-human PRL antiserum or (**B**) monoclonal anti-human PRL antibodies. A combination of PRL and vasoinhibin standards (PRL+Vi) or only a vasoinhibin standard (Vi) was subjected (IP) or not subjected to IP. Negative controls were without vitreous (−). Vitreous (VIT) IP from P- and L- treated patients. Numbers on the left side indicate the molecular weights (MW) of marker proteins run in the MW lane of blots.

of control vitreous was prevented by co-incubation with anti-PRL antiserum but not NRS (Fig. 4). These findings confirmed the antiangiogenic properties of normal vitreous[31,32] and suggested endogenous vasoinhibin as a substantial contributor. Because the vitreous from PDR patients is not antiangiogenic (present findings), vasoinhibin levels may be reduced in the PDR patient vitreous.

Matrix Metalloproteases Generate Less Vasoinhibin in the Vitreous of PDR Patients than Non-Diabetic Patients

To investigate whether the generation of vasoinhibin is reduced in the vitreous from PDR patients, we evaluated the activity of neutral PRL-cleaving proteases in the vitreous of non-diabetic patients

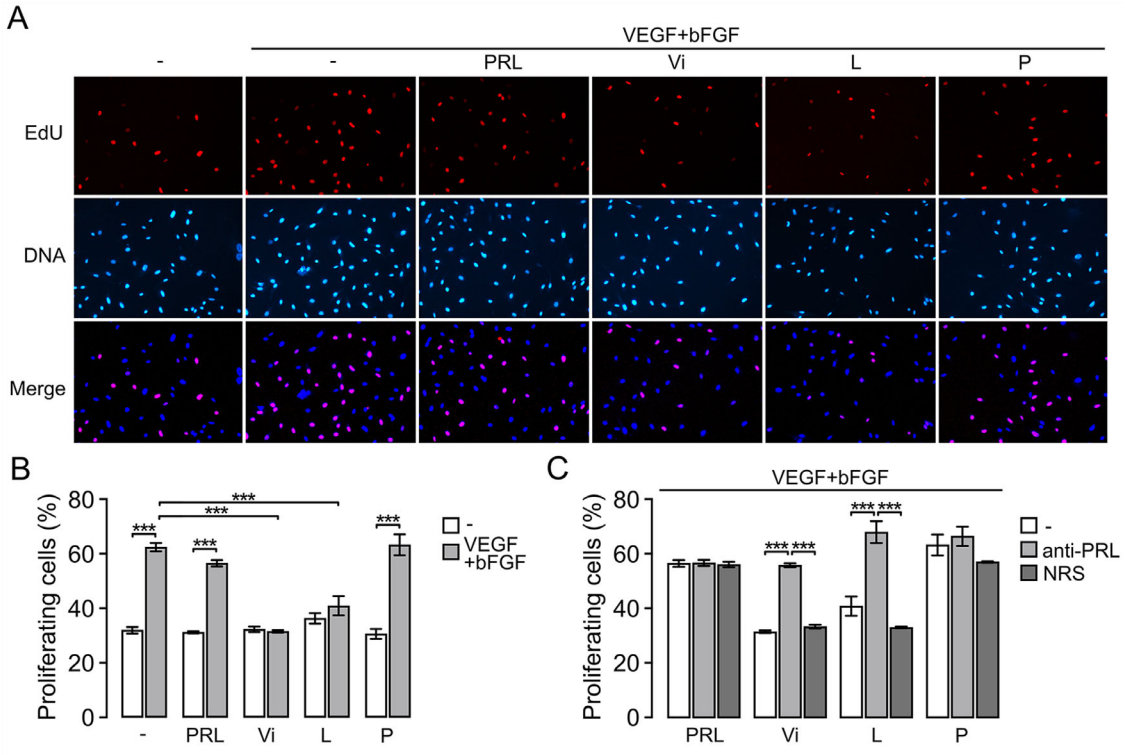

Figure 3. Levosulpiride increased bioactive vasoinhibin in the vitreous of patients with PDR. (**A**) Representative fields of HUVECs stained for DNA synthesis (EdU), DNA (Hoechst), and the overlay (merge) of both reactions. Cells were incubated for 24 hours in the absence (–) or presence of a combination of VEGF and bFGF (VEGF+bFGF) with or without (–) PRL, vasoinhibin (Vi), or a vitreous pool from six placebo (P) or six levosulpiride (L)-treated PDR patients. (**B**) Cell proliferation was quantified by the EdU click reaction and expressed relative to the total number of cells in the field. (**C**) HUVECs were incubated for 24 hours in the presence of VEGF+bFGF with PRL, Vi, or the vitreous pool from placebo- or levosulpiride-treated PDR patients with or without (–) anti-PRL antiserum (anti-PRL) or NRS. Values are means ± SEM of at least three independent experiments. $^{***}P < 0.001$.

Table 2. Demographic and Clinical Characteristics of the Non-Interventional Study Population

Characteristic	Non-Diabetic ($n = 10$) Mean ± SEM	95% CI	PDR ($n = 17$) Mean ± SEM	95% CI	*P*
Age (y)	44.8 ± 6.5	30.1–59.5	55.4 ± 2.5	50.0–60.8	0.08[a]
BMI (kg/m^2)	27.5 ± 2.1	22.3–32.7	25.6 ± 0.9	23.7–27.6	0.36[a]
DM2 (y)	—	—	15.4 ± 1.9	11.4–19.4	—
HbA1c (%)	4.7 ± 0.7	2.9–6.4	7.9 ± 0.7	6.5–9.4	0.009[a]
SCr (mg/dL)	0.9 ± 0.06	0.7–1.0	1.5 ± 0.2	1.1–1.9	0.01[a]
eGFR (mL/min)	103.5 ± 9.9	81.0–126.0	64.4 ± 8.1	47.2–81.6	0.006[a]
SPRL (ng/mL)	8.6 ± 1.3	5.6–11.6	8.5 ± 0.9	6.5–10.5	0.94[a]
VPRL (ng/mL)	1.8 ± 1.0	–1.0 to 4.7	1.2 ± 0.09	1.0–1.4	0.32[a]

Non-diabetic patients underwent vitrectomy due to rhegmatogenous retinal detachment or epiretinal membrane. Patients with PDR underwent vitrectomy due to vitreous hemorrhage and/or retinal detachment. *P* values compare non-diabetic and PDR groups. eGFR, glomerular filtration rate (CKD-EPI equation); VPRL, vitreous PRL.

[a]Based on Student's *t*-test.

compared to PDR patients. Recombinant PRL was incubated with the respective vitreous at a neutral pH (Fig. 5A). Western blots probed with N-terminal anti-PRL monoclonal antibodies revealed that incubation of PRL resulted in its partial conversion to vasoinhibin when the vitreous was harvested from control non-diabetic patients, and that conversion was reduced when the vitreous was derived from PDR patients

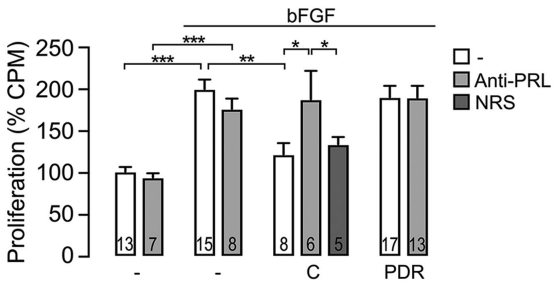

Figure 4. The level of bioactive vasoinhibin was higher in non-diabetic than PDR vitreous. BUVECs were incubated for 24 hours in the absence or presence of bFGF with or without (–) independent vitreous samples from non-diabetic control (C) or PDR patients with or without (–) anti-PRL antiserum (anti-PRL) or NRS. Cell proliferation was quantified by the incorporation of ^{3}H-thymidine into DNA expressed relative to untreated basal values. Results are mean ± SEM. Number of vitreous samples (*n*) is indicated inside bars. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

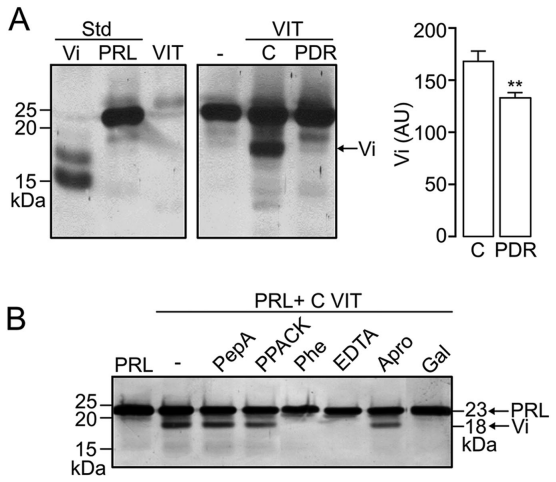

Figure 5. Matrix metalloproteases in the vitreous of PDR patients generate less vasoinhibin than in non-diabetic patients. (**A**) Representative western blot probed with monoclonal anti-human PRL antibodies showing the cleavage of human PRL by vitreous (VIT) from non-diabetic controls (C) and PDR patients. PRL proteolytic products were obtained at pH 7 after incubating 200 ng of human PRL with and without (–) 15 µg of protein from C or PDR VIT. Human vasoinhibin (Vi) and PRL standards and VIT without PRL (VIT) are shown. Densitometric values are in arbitrary units (AU) of Vi by six different vitreous samples. Bars are means ± SEM. $^{**}P < 0.01$. (**B**) Cleavage of human PRL by VIT from control patients in the absence (–) or presence of aspartyl protease inhibitor pepstatin A (Pep A); thrombin inhibitor (PPACK); MMP inhibitors EDTA, 1,10-phenanthroline (Phe), and Galardin (Gal); and serine protease inhibitor aprotinin (Apro). Numbers on the left and right indicate the position of molecular weight markers and PRL and Vi, respectively.

(Fig. 5A). The cleavage of PRL to vasoinhibin was prevented by the addition of three different MMP inhibitors (1,10-phenanthroline, EDTA, and Galardin) but not by the aspartyl protease inhibitor pepstatin-A, the thrombin inhibitor PPACK, or the serine protease inhibitor aprotinin (Fig. 5B). These results identified MMPs as the neutral PRL cleaving enzymes present in vitreous (Fig. 5B) and suggest that MMP activity results in lower vasoinhibin levels in PDR than in normal vitreous.

Discussion

Angiogenesis is highly restricted in the healthy adult eye where several compartments (i.e., cornea, lens, vitreous, and outer retina) lack blood vessels. The failure to inhibit ocular angiogenesis underlies vasoproliferative retinopathies, including DR.[33] The pathogenesis of DR involves glucose-mediated vascular damage leading to the leakage and occlusion of retinal vessels and macular edema in the non-proliferative phase and angiogenesis and retinal detachment in the proliferative phase. Laser photocoagulation is the standard treatment for PDR, whereas intravitreal injections of anti-VEGF medications are the first-line therapy in patients whose vision is threatened by DME.[5,6] However, suboptimal and temporal responses, adverse effects, and high socioeconomic costs have stimulated the search for additional treatment options. Our ongoing clinical trial in patients with PDR and DME is investigating the therapeutic value of elevating the circulating levels of PRL by the oral administration of levosulpiride, the prokinetic dopamine D2 receptor blocker. The present work supports the beneficial outcome of this treatment by showing that levosulpiride-induced hyperprolactinemia increases the levels of bioactive vasoinhibin in the vitreous of patients with PDR.

Levosulpiride induces hyperprolactinemia by blocking the hypothalamic dopaminergic inhibition of PRL synthesis and secretion by the anterior pituitary gland.[34] The mean circulating PRL levels were elevated over 100 ng/mL after treatment with levosulpiride, and the hyperprolactinemia was higher in females than males. The higher effect of levosulpiride in females has been reported previously[18,34] and may relate to the increased activity and responsiveness of tuberoinfundibular dopaminergic neurons in females compared to males.[35]

To the best of our knowledge, our report shows for the first time the presence of PRL in the vitreous and the ability of levosulpiride to increase vitreous PRL levels. The level of vitreous PRL correlated directly with its systemic concentration, implying that high levels of circulating PRL in response to levosulpiride favored incorporation of the hormone into the eye. PRL crosses the blood–ocular barrier.

Radioautographic studies have shown that iodinated PRL injected intracardially is incorporated into ocular tissues, including the retina, choroid, and ciliary bodies.[36] Ocular access may involve receptor-mediated transport, as PRL receptors are expressed in elements of the blood–ocular barrier (i.e., retinal pigment epithelium[37] and ciliary epithelium).[14] Supporting this notion, systemic PRL enters the cerebrospinal fluid via its receptors in the choroid plexus,[38,39] and the dopamine D2 receptor antagonist domperidone induces an increase in circulating PRL that is followed by a rise in the cerebrospinal fluid levels of the hormone.[40] However, breakdown of the blood–ocular barrier in PDR[41,42] could cause an influx of the circulating hormone into the eye, although similar levels of PRL were detected in the vitreous of patients without diabetes and PDR patients in the absence of levosulpiride.

The levosulpiride-induced increase in ocular PRL was minor relative to that in serum, yet this minor change may help counteract disease progression by increasing the levels of ocular vasoinhibin. In rodents, hyperprolactinemia leads to vasoinhibin accumulation in the retina which, in turn, reduces both VEGF-induced and diabetes-induced increases in retinal vasopermeability.[14] There is no quantitative immunoassay for vasoinhibin, and its endogenous levels are commonly determined semiquantitatively by IP-WB.[11,29] This method confirmed the increase in vitreous PRL after treatment of PDR patients with levosulpiride but was unable to detect vasoinhibin in vitreous. Lack of detection implied that, if present, vasoinhibin was below the detection limit of the assay. Because the potency of vasoinhibin to inhibit endothelial cell proliferation is high (EC_{50} = 1 nM),[43] we reasoned that this bioassay could be a more sensitive method for detecting vasoinhibin in the vitreous.

The vitreous from PDR patients treated with levosulpiride, but not with placebo, inhibited VEGF- and bFGF-induced endothelial cell proliferation, and this action was blocked by anti-PRL antibodies that are known to immunoneutralize vasoinhibin.[29] Consistent with this observation, vasoinhibin, but not PRL, inhibited VEGF- and bFGF-induced endothelial cell proliferation, and the anti-PRL antibodies prevented the vasoinhibin effect. These findings suggest that levosulpiride-induced hyperprolactinemia causes the accumulation of vasoinhibin in the vitreous of PDR patients, which inhibits endothelial cell proliferation (Fig. 6). Vasoinhibin inhibits proliferation, as well as migration, survival, and permeability, of endothelial cells by binding to a multicomponent complex formed by plasminogen activator inhibitor 1, urokinase, and the urokinase receptor on endothelial cell membranes.[44] These and other unidentified binding partners or receptors[45] mediate the vasoinhibin blockage of various signaling pathways (Ras–Raf–MAPK; Ras–Tiam1–Pak1; PI3K–Akt, and PLCγ–IP3–eNOS) activated by different vasoactive substances (VEGF, bFGF, bradykinin, IL-1β).[7,8]

A major question is whether endogenous vasoinhibin plays a role in the progression of DR. There is evidence that vasoinhibin is a natural inhibitor of angiogenesis in the adult retina[10] and cornea[9] and that it contributes to the regression of the hyaloid vascular system that nourishes the immature lens, retina, and vitreous during development.[46] Moreover, the healthy adult vitreous is avascular and has antiangiogenic properties[31,47] due to antiangiogenic factors[48] that may include vasoinhibin. Here, we show that the vitreous from patients without diabetes but not vitreous from PDR patients inhibited the bFGF-induced proliferation of endothelial cells and that immunoneutralization of vasoinhibin abrogated the antiangiogenic action. These findings suggest that vasoinhibin is a major antiangiogenic factor in the vitreous of patients without diabetes and that its levels are reduced in PDR.

To investigate the lower generation of vasoinhibin in the vitreous of PDR patients, we evaluated the activity of neutral proteases that cleave PRL to vasoinhibin. We found that the vitreous contains MMPs that convert PRL to vasoinhibin and that the amount of vasoinhibin generated by the MMPs in the vitreous of non-diabetic patients is higher than in the PDR counterpart. These findings suggest that MMP activity capable of generating vasoinhibin is reduced in PDR. This assumption makes sense when considering that the levels of antiangiogenic factors decrease in the PDR vitreous.[48] Different MMPs (MMP-1, -2, -3, -8, -9, and -13) cleave PRL to generate vasoinhibin.[49] MMP-2, -8, and -9 are found in the vitreous from non-diseased human eyes,[50] MMP-2 and MMP-9 have been detected in the vitreous from controls without diabetes (with macular hole or epiretinal membranes) and PDR patients,[51] and the vitreous levels of MMP-9 are frequently associated with vasoproliferative changes.[52] The regulation of MMP activity is complex. MMPs are secreted as inactive pro-enzymes that are activated upon proteolysis and inhibited by tissue inhibitors of metalloproteinases, which are present in human vitreous,[53] and the reduced MMP-mediated generation of vasoinhibin in PDR may thereby reflect an imbalance between the type and concentration of active MMPs and their inhibitors.[54]

Altogether, the present work helps identify the mechanism by which levosulpiride could limit the progression of DR and DME (i.e., induced hyperprolactinemia promoting the ocular incorporation of

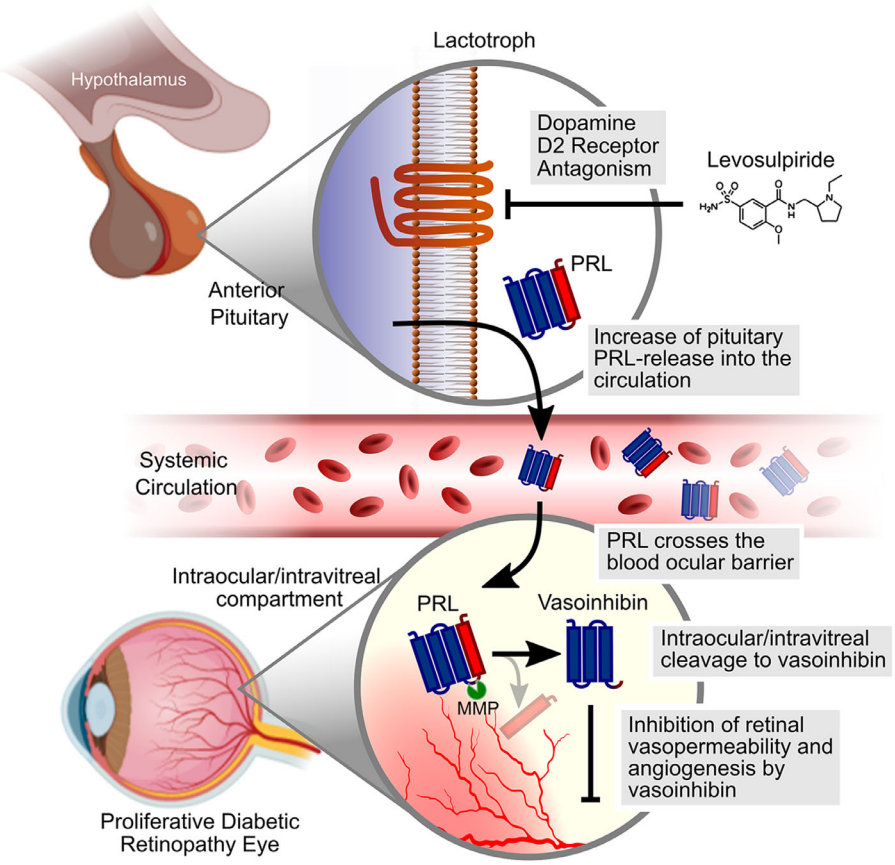

Figure 6. Schematic representation of the study findings supporting the mechanism by which levosulpiride therapy could limit the progression of DME and DR. Levosulpiride, a dopamine D2 receptor antagonist, blocks dopamine D2 receptors located in the membrane of anterior pituitary cells that produce PRL (lactotrophs). Given that hypothalamic dopamine inhibits the release of PRL, levosulpiride leads to high levels of PRL in the circulation (hyperprolactinemia) which, in turn, favor PRL penetration across the blood–ocular barrier. MMPs in the intraocular/vitreous compartment cleave PRL to vasoinhibin, which can reduce retinal vasopermeability and angiogenesis in DME and DR. Scheme was partly created with Biorender.com.

PRL and its proteolytic conversion to vasoinhibin) (Fig. 6). Because levosulpiride represents a potential therapy, general drug information and safety considerations pertain. Levosulpiride is the levo enantiomer of sulpiride, a benzamide substitute of established use as prokinetic and antipsychotic medication. It acts by blocking enteric and central dopamine D2 receptors.[19] It is used at low doses (75 mg/day) to stimulate gastrointestinal motility,[55] whereas higher doses (400–3200 mg/day) are required for the treatment of psychotic illnesses,[56] as it penetrates the blood–brain barrier poorly.[57] Because the pituitary gland is outside the blood–brain barrier, low doses of levosulpiride increase serum PRL.[34] Adverse drug effects may occur that are independent of hyperprolactinemia (e.g., sedation, fatigue, drowsiness, headache, extrapyramidal effects such as acute muscular dystonia and tardive dyskinesia) or dependent on hyperprolactinemia (e.g., decreased libido, amenorrhea, and infertility; breast engorgement and galactorrhea; gynecomastia).[17,56,58] However, levosulpiride is well tolerated during both short-term (2 to 16 weeks) and long-term (4 to 42 months) administration.[17,56,58] A multicenter study in 342 dyspeptic female and male patients receiving oral levosulpiride (25 mg TID for 4 weeks) reported 40 patients (11%) with adverse effects (26.7% galactorrhea, 17.8% somnolence, 11% fatigue, and 11.5% headache), none of whom abandoned the study due to the side effects.[59] Using the same oral dose of levosulpiride, a 6-month, randomized, placebo-controlled trial of 40 patients with insulin-dependent diabetes and delayed gastric emptying reported two levosulpiride-treated patients (5%) and one patient on placebo (2.5%) with minor adverse events (breast tenderness, loss of libido, and/or drowsiness), but they did not withdraw from the study.[60]

Attention should also be given to the fact that dopamine plays a role in visual function. The importance of the dopaminergic system and dopamine receptor subtypes in the eye has been recently

highlighted.[61,62] Dopamine and dopamine D2 receptors have been localized in retinal amacrine cells and photoreceptors (rods and cones), respectively, and it has been suggested that they play a role in the regulation of the flow of visual information and light-adaptation circuits.[63] Also, dopamine may influence intraocular blood pressure by modulating ciliary blood flow and aqueous humor production.[64] Thus, levosulpiride therapy may impact compromised vision in patients with DR and DME by mechanisms independent of the vascular actions of the PRL–vasoinhibin axis. Of note, complete eye examination, including slit-lamp evaluation, did not reveal any ophthalmological changes in 16 severely ill schizophrenic patients treated with sulpiride for 12 weeks.[65]

Proof for the beneficial outcome of the use of levosulpiride in DME and DR awaits completion of the ongoing randomized, placebo-controlled clinical trial. If shown to be effective, it will have the potential to impact the loss of vision in people with diabetes.

Acknowledgments

The authors thank Fernando López Barrera and Xarubet Ruiz-Herrera for their excellent technical assistance and the Laboratorio de Ensayos de Desarrollo Farmacéutico, Facultad de Estudios Superiores Cuautitlán, UNAM, for producing the placebo. We thank Jessica Gonzalez Norris for correcting the English style of the manuscript.

Supported by grants from the National Council of Science and Technology of Mexico (CONACYT; 247164, 289568) and UNAM (UR405PC) to C.C. C.D.N.-A. is a doctoral student from Programa de Doctorado en Ciencias de la Nutrición Humana, Universidad Autónoma de Querétaro, and received a fellowship from CONACYT (370956).

Disclosure: **C.D. Nuñez-Amaro,** None; **A.I. Moreno-Vega,** None; **E. Adan-Castro,** None; **M. Zamora,** None; **R. Garcia-Franco,** None; **P. Ramirez-Neria,** None; **M. Garcia-Roa,** None; **Y. Villalpando,** None; **J.P. Robles,** None; **G. Ramirez-Hernandez,** None; **M. Lopez,** None; **J. Sanchez,** None; **E. Lopez-Star,** None; **T. Bertsch,** None; **G. Martinez de la Escalera,** None; **M.L. Robles-Osorio,** None; **J. Triebel,** None; **C. Clapp,** None

[*] CDN-A, AIM-V, EA-C, and MZ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

[†] JT and CC share senior author position.

References

1. Ting DSW, Cheung GCM, Wong TY. Diabetic retinopathy: global prevalence, major risk factors, screening practices and public health challenges: a review. *Clin Exp Ophthalmol*. 2016;44:260–277.
2. Gundogan FC, Yolcu U, Akay F, Ilhan A, Ozge G, Uzun S. Diabetic macular edema. *Pak J Med Sci*. 2016;32:505–510.
3. Yau JWY, Rogers SL, Kawasaki R, et al. Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care*. 2012;35:556–564.
4. International Diabetes Federation. *IDF Diabetes Atlas*. 9th ed. Brussels, Belgium: International Diabetes Federation; 2019.
5. Cohen SR, Gardner TW. Diabetic Retinopathy and diabetic macular edema. *Dev Ophthalmol*. 2015;55:137–146.
6. Kim EJ, Lin WV, Rodriguez SM, Chen A, Loya A, Weng CY. Treatment of diabetic macular edema. *Curr Diab Rep*. 2019;19:68.
7. Clapp C, Thebault S, Macotela Y, Moreno-Carranza B, Triebel J, Martinez de la Escalera G. Regulation of blood vessels by prolactin and vasoinhibins. *Adv Exp Med Biol*. 2015;846:83–95.
8. Triebel J, Bertsch T, Bollheimer C, et al. Principles of the prolactin/vasoinhibin axis. *Am J Physiol Regul Integr Comp Physiol*. 2015;309:R1193–R1203.
9. Dueñas Z, Torner L, Corbacho AM, et al. Inhibition of rat corneal angiogenesis by 16-kDa prolactin and by endogenous prolactin-like molecules. *Invest Ophthalmol Vis Sci*. 1999;40:2498–2505.
10. Aranda J, Rivera JC, Jeziorski MC, et al. Prolactins are natural inhibitors of angiogenesis in the retina. *Invest Ophthalmol Vis Sci*. 2005;46:2947–2953.
11. Zepeda-Romero LC, Vazquez-Membrillo M, Adan-Castro E, et al. Higher prolactin and vasoinhibin serum levels associated with incidence and progression of retinopathy of prematurity. *Pediatr Res*. 2017;81:473–479.
12. Triebel J, Macotela Y, de la Escalera GM, Clapp C. Prolactin and vasoinhibins: endogenous players in diabetic retinopathy. *IUBMB Life*. 2011;63:806–810.
13. Triebel J, Huefner M, Ramadori G. Investigation of prolactin-related vasoinhibin in sera from patients with diabetic retinopathy. *Eur J Endocrinol*. 2009;161:345–353.
14. Arnold E, Rivera JC, Thebault S, et al. High levels of serum prolactin protect against diabetic retinopathy by increasing ocular vasoinhibins. *Diabetes*. 2010;59:3192–3197.

15. Ramírez M, Wu Z, Moreno-Carranza B, et al. Vasoinhibin gene transfer by adenoassociated virus type 2 protects against VEGF- and diabetes-induced retinal vasopermeability. *Invest Ophthalmol Vis Sci*. 2011;52:8944–8950.
16. Pan H, Nguyen NQ, Yoshida H, et al. Molecular targeting of antiangiogenic factor 16K hPRL inhibits oxygen-induced retinopathy in mice. *Invest Ophthalmol Vis Sci*. 2004;45:2413–2419.
17. Ratnani I, Panchal B, Gandhi R, Vala A, Mandal K. Role of levosulpiride in the management of functional dyspepsia. *J Fam Med*. 2015;2:1034.
18. Andrade C. Low-dose amisulpride and elevation in serum prolactin. *J Clin Psychiatry*. 2013;74:e558–e560.
19. Tonini M, Cipollina L, Poluzzi E, Crema F, Corazza GR, De Ponti F. Review article: clinical implications of enteric and central D2 receptor blockade by antidopaminergic gastrointestinal prokinetics. *Aliment Pharmacol Ther*. 2004;19:379–390.
20. Robles-Osorio ML, García-Franco R, Núñez-Amaro CD, et al. Basis and design of a randomized clinical trial to evaluate the effect of levosulpiride on retinal alterations in patients with diabetic retinopathy and diabetic macular edema. *Front Endocrinol*. 2018;9:242.
21. Galfione M, Luo W, Kim J, et al. Expression and purification of the angiogenesis inhibitor 16-kDa prolactin fragment from insect cells. *Protein Expr Purif*. 2003;28:252–258.
22. Clapp C, Sears PS, Russell DH, Richards J, Levay-Young BK, Nicoll CS. Biological and immunological characterization of cleaved and 16K forms of rat prolactin. *Endocrinology*. 1988;122:2892–2898.
23. Corbacho AM, Macotela Y, Nava G, et al. Human umbilical vein endothelial cells express multiple prolactin isoforms. *J Endocrinol*. 2000;166:53–62.
24. Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat Protoc*. 2007;2:481–485.
25. Cajero-Juarez M, Avila B, Ochoa A, et al. Immortalization of bovine umbilical vein endothelial cells: a model for the study of vascular endothelium. *Eur J Cell Biol*. 2002;81:1–8.
26. Robles JP, Zamora M, Velasco-Bolom JL, et al. Vasoinhibin comprises a three-helix bundle and its antiangiogenic domain is located within the first 79 residues. *Sci Rep*. 2018;8:17111.
27. Carpenter AE, Jones TR, Lamprecht MR, et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol*. 2006;7:R100.
28. Ferrara N, Clapp C, Weiner R. The 16K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells. *Endocrinology*. 1991;129:896–900.
29. Gonzalez C, Parra A, Ramirez-Peredo J, et al. Elevated vasoinhibins may contribute to endothelial cell dysfunction and low birth weight in preeclampsia. *Lab Invest*. 2007;87:1009–1017.
30. Ogata N, Tombran-Tink J, Nishikawa M, et al. Pigment epithelium-derived factor in the vitreous is low in diabetic retinopathy and high in rhegmatogenous retinal detachment. *Am J Ophthalmol*. 2001;132:378–382.
31. Preis I, Langer R, Brem H, Folkman J. Inhibition of neovascularization by an extract derived from vitreous. *Am J Ophthalmol*. 1977;84:323–328.
32. Lutty GA, Mello RJ, Chandler C, Fait C, Bennett A, Patz A. Regulation of cell growth by vitreous humour. *J Cell Sci*. 1985;76:53–65.
33. Zhang SX, Ma J. Ocular neovascularization: Implication of endogenous angiogenic inhibitors and potential therapy. *Prog Retin Eye Res*. 2007;26:1–37.
34. Kuchay MS, Mithal A. Levosulpiride and serum prolactin levels. *Indian J Endocrinol Metab*. 2017;21:355–358.
35. Manzanares J, Wagner EJ, LaVigne SD, Lookingland KJ, Moore KE. Sexual differences in kappa opioid receptor-mediated regulation of tuberoinfundibular dopaminergic neurons. *Neuroendocrinology*. 1992;55:301–307.
36. O'Steen WK, Sundberg DK. Patterns of radioactivity in the eyes of rats after injection of iodinated prolactin. *Ophthalmic Res*. 1982;14:54–62.
37. Melendez Garcia R, Arredondo Zamarripa D, Arnold E, et al. Prolactin protects retinal pigment epithelium by inhibiting sirtuin 2-dependent cell death. *EBioMedicine*. 2016;7:35–49.
38. Walsh RJ, Slaby FJ, Posner BI. A receptor-mediated mechanism for the transport of prolactin from blood to cerebrospinal fluid. *Endocrinology*. 1987;120:1846–1850.
39. Mangurian LP, Walsh RJ, Posner BI. Prolactin enhancement of its own uptake at the choroid plexus. *Endocrinology*. 1992;131:698–702.
40. Felicio LF, Bridges RS. Domperidone induces a probenecid-sensitive rise in immunoreactive prolactin in cerebroventricular perfusates in female rats. *Brain Res*. 1992;573:133–138.
41. Zhang C, Wang H, Nie J, Wang F. Protective factors in diabetic retinopathy: focus on blood-retinal barrier. *Discov Med*. 2014;18:105–112.

42. Moriarty AP, Spalton DJ, Moriarty BJ, Shilling JS, Ffytche TJ, Bulsara M. Studies of the blood-aqueous barrier in diabetes mellitus. *Am J Ophthalmol*. 1994;117:768–771.
43. Clapp C, Martial JA, Guzman RC, Rentier-Delure F, Weiner RI. The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. *Endocrinology*. 1993;133:1292–1299.
44. Bajou K, Herkenne S, Thijssen VL, et al. PAI-1 mediates the antiangiogenic and profibrinolytic effects of 16K prolactin. *Nat Med*. 2014;20:741–747.
45. Clapp C, Weiner RI. A specific, high affinity, saturable binding site for the 16-kilodalton fragment of prolactin on capillary endothelial cells. *Endocrinology*. 1992;130:1380–1386.
46. Dueñas Z, Rivera JC, Quiróz-Mercado H, et al. Prolactin in eyes of patients with retinopathy of prematurity: implications for vascular regression. *Invest Ophthalmol Vis Sci*. 2004;45:2049–2055.
47. Lutty GA, Thompson DC, Gallup JY, Mello RJ, Patz A, Fenselau A. Vitreous: an inhibitor of retinal extract-induced neovascularization. *Invest Ophthalmol Vis Sci*. 1983;24:52–56.
48. Nawaz IM, Rezzola S, Cancarini A, et al. Human vitreous in proliferative diabetic retinopathy: characterization and translational implications. *Prog Retin Eye Res*. 2019;72:100756.
49. Macotela Y, Aguilar MB, Guzman-Morales J, et al. Matrix metalloproteases from chondrocytes generate an antiangiogenic 16 kDa prolactin. *J Cell Sci*. 2006;119:1790–1800.
50. Skeie JM, Roybal CN, Mahajan VB. Proteomic insight into the molecular function of the vitreous. *PLoS One*. 2015;10:e0127567.
51. Sánchez MC, Luna JD, Barcelona PF, et al. Effect of retinal laser photocoagulation on the activity of metalloproteinases and the alpha(2)-macroglobulin proteolytic state in the vitreous of eyes with proliferative diabetic retinopathy. *Exp Eye Res*. 2007;85:644–650.
52. Loukovaara S, Robciuc A, Holopainen JM, et al. Ang-2 upregulation correlates with increased levels of MMP-9, VEGF, EPO and TGFβ1 in diabetic eyes undergoing vitrectomy. *Acta Ophthalmol*. 2013;91:531–539.
53. De La Paz MA, Itoh Y, Toth CA, Nagase H. Matrix metalloproteinases and their inhibitors in human vitreous. *Invest Ophthalmol Vis Sci*. 1998;39:1256–1260.
54. Singh M, Tyagi SC. Metalloproteinases as mediators of inflammation and the eyes: molecular genetic underpinnings governing ocular pathophysiology. *Int J Ophthalmol*. 2017;10:1308–1318.
55. Guslandi M. The clinical use of levosulpiride. *Curr Ther Res*. 1993;53:484–501.
56. Mauri MC, Bravin S, Bitetto A, Rudelli R, Invernizzi G. A risk-benefit assessment of sulpiride in the treatment of schizophrenia. *Drug Saf*. 1996;14:288–298.
57. Kapur S, Langlois X, Vinken P, Megens AAHP, De Coster R, Andrews JS. The differential effects of atypical antipsychotics on prolactin elevation are explained by their differential blood-brain disposition: a pharmacological analysis in rats. *J Pharmacol Exp Ther*. 2002;302:1129–1134.
58. Wagstaff AJ, Fitton A, Benfield P. Sulpiride. *CNS Drugs*. 1994;2:313–333.
59. Lozano R, Concha MP, Montealegre A, et al. Effectiveness and safety of levosulpiride in the treatment of dysmotility-like functional dyspepsia. *Ther Clin Risk Manag*. 2007;3:149–155.
60. Melga P, Mansi C, Ciuchi E, Giusti R, Sciaba L, Prando R. Chronic administration of levosulpiride and glycemic control in IDDM patients with gastroparesis. *Diabetes Care*. 1997;20:55–58.
61. Bucolo C, Leggio GM, Drago F, Salomone S. Dopamine outside the brain: the eye, cardiovascular system and endocrine pancreas. *Pharmacol Ther*. 2019;203:107392.
62. Leggio GM, Bucolo C, Platania CBM, Salomone S, Drago F. Current drug treatments targeting dopamine D3 receptor. *Pharmacol Ther*. 2016;165:164–177.
63. Archibald NK, Clarke MP, Mosimann UP, Burn DJ. The retina in Parkinson's disease. *Brain J Neurol*. 2009;132:1128–1145.
64. Reitsamer HA, Kiel JW. Effects of dopamine on ciliary blood flow, aqueous production, and intraocular pressure in rabbits. *Invest Ophthalmol Vis Sci*. 2002;43:2697–2703.
65. Mielke DH, Gallant DM, Kessler C. An evaluation of a unique new antipsychotic agent, sulpiride: effects on serum prolactin and growth hormone levels. *Am J Psychiatry*. 1977;134:1371–1375.