



# Monosodium Urate Contributes to Retinal Inflammation and Progression of Diabetic Retinopathy

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**We have investigated the contributing role of monosodium urate (MSU) to the pathological processes associated with the induction of diabetic retinopathy (DR). In human postmortem retinas and vitreous from donors with DR, we have found a significant increase in MSU levels that correlated with the presence of inflammatory markers and enhanced expression of xanthine oxidase. The same elevation in MSU levels was also detected in serum and vitreous of streptozotocin-induced diabetic rats (STZ-rats) analyzed at 8 weeks of hyperglycemia. Furthermore, treatments of STZ-rats with the hypouricemic drugs allopurinol (50 mg/kg) and benzbromarone (10 mg/kg) given every other day resulted in a significant decrease of retinal and plasma levels of inflammatory cytokines and adhesion factors, a marked reduction of hyperglycemia-induced retinal leukostasis, and restoration of retinal blood-barrier function. These results were associated with effects of the hypouricemic drugs on downregulating diabetes-induced levels of oxidative stress markers as well as expression of components of the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome such as NLRP3, Toll-like receptor 4, and interleukin-1 $\beta$ . The outcomes of these studies support a contributing role of MSU in diabetes-induced retinal inflammation and suggest that**

**asymptomatic hyperuricemia should be considered as a risk factor for DR induction and progression.**

Diabetic retinopathy (DR) is a progressive complication of type 1 and type 2 diabetes and the leading cause of legal blindness in adults (1). The identification of specific risk factors for DR is crucial to establish early therapeutic intervention and ultimately prevent vision loss. Poor glycemic control, hypertension, and hyperlipidemia are considered primary risk factors for the development and progression of DR (2). However, new evidence suggests that monitoring circulating levels of proinflammatory factors may hold better diagnostic value for the identification of patients at risk and/or for predicting disease progression (3,4).

Uric acid (UA) is a by-product of the purine metabolism (5), resulting from the oxidative catabolism of nucleic acids by xanthine oxidoreductase (XOD) (5). In normal physiological conditions, relatively high levels of UA are present in cells and in serum (6–11); however, when these levels reach and/or exceed 356  $\mu\text{mol/L}$  (6 mg/dL) at physiological pH, UA undergoes nucleation in crystals of monosodium urate (MSU) (6,7,9). UA plasma levels >476  $\mu\text{mol/L}$  (>8 mg/dL) cause gout, a human metabolic disorder and

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systemic inflammatory disease particularly affecting joints and kidneys (11,12).

Clinical studies have recently suggested that moderate “asymptomatic” hyperuricemia, defined as an elevation in serum UA levels  $\geq 356 \mu\text{mol/L}$ , represents a risk factor for the development of cardiovascular disease, metabolic syndrome, and diabetic complications (8,13–15). The potential contributing role of UA in the induction and progression of these disease conditions has been linked to MSU function as an “alarmin” to activate the immune response and to promote auto (sterile) inflammation (16,17).

MSU has shown to be an activator of sterile inflammation through the induction of the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome (17–20). Formation of this macromolecular complex in competent cells leads to cleavage/activation of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 (21). Toll-like receptor 4 (TLR4) and other TLRs functionally contribute to the inflammasome by promoting pro-IL-1 $\beta$  expression in a nuclear factor- $\kappa$ B-dependent manner (22,23).

In patients with diabetes, augmented UA serum levels have been correlated with the development of diabetic macroangiopathy (24), nephropathy (25–27), and neuropathy (28,29). To date, little is known on the specific contribution/correlation of UA to DR pathogenesis (30). However, evidence is provided that sterile inflammation is involved in DR pathogenesis and that this may implicate MSU activity (31).

In this study, we investigated the specific role of MSU in hyperglycemia-induced inflammatory processes in human and experimental DR by monitoring its levels in serum, vitreous, and retina of diabetic rodents and patients and by assessing the effects of UA-lowering drugs in preventing diabetes-induced retinal vessels inflammation and activation of the NLRP3 inflammasome.

## RESEARCH DESIGN AND METHODS

### Postmortem Human Samples

Deidentified postmortem human vitreous and retina samples were obtained from Georgia Eye Bank (Atlanta, GA) through its approved research program and by Augusta University Biosafety Committee. Supplementary Table 1 summarizes the demographics and clinical history available of the donors whose samples we used in our experiments.

### Patients

The procedures in patients were conducted in compliance with the Declaration of Helsinki and according to protocols approved by the Ethical Committees of Clinica San Domenico, Ospedale San Giovanni dell' Addolorata (Rome, Italy) and Istituto Dermopatico dell'Immacolata, Istituto di Ricovero e Cura a Carattere Scientifico San Carlo, Rome Italy. Patients provided preoperative informed written consent and approved the use of the excised vitreous fluids for the

presented studies. Diagnosis and staging of DR were made after complete ophthalmologic examination that included measurements of visual acuity (Early Treatment of Diabetic Retinopathy Study [ETDRS]), fluorescein angiography (FA), and optical coherence tomography.

In addition to the ophthalmological examination, the patients were asked to complete a questionnaire comprehensive of present and past comorbidities and treatments as well as questions pertaining to lifestyles, as summarized in Supplementary Table 2.

All patients were candidates for vitrectomy as a consequence of tractional retinal detachment or a nonclearing vitreous hemorrhage. Importantly, no technical changes to the surgical procedures were made to accommodate in any way the research protocol.

### Human Vitreous Processing

Postmortem human vitreous samples were diluted (1:3) with PBS without calcium and magnesium and resuspended using a 26-gauge needle. Vitreous samples were briefly centrifuged at 2,000 rpm at 4°C, and supernatants were assessed for UA concentration as described below.

Undiluted vitreous samples (0.3–0.6  $\mu\text{L}$ ) were obtained from 18 patients undergoing pars plana vitrectomy. The control group consisted of nine patients who had undergone vitrectomy for the treatment of pucker or retinal detachment consequent to trauma. Vitreouses were collected undiluted by manual suction into a syringe through the aspiration line of the vitrectomy unit before the infusion line was opened. The samples were frozen at  $-80^\circ\text{C}$  until processed for the different analyses.

### Animals

All animal procedures were performed in agreement with the statement of the Association for Research in Vision and Ophthalmology for the humane use of animals in vision science and in compliance with approved institutional protocols. Animals were kept with a 12-h day/night light cycle and fed ad libitum. Adult male Sprague-Dawley rats (250–300 g), obtained from Envigo (Dublin, VA), were made diabetic by one intraperitoneal injection of streptozotocin (STZ) (65 mg/kg dissolved in 0.1 mol/L sodium citrate, pH 4.5) (Sigma-Aldrich, St. Louis, MO). Age-matched control rats received vehicle alone. Rats were considered to be diabetic (STZ-rats) when fasting blood glucose levels were  $\geq 300 \text{ mg/dL}$ . All animals were sacrificed after 8 weeks of hyperglycemia with an overdose of anesthesia, followed by thoracotomy.

Some STZ-rats received the hypouricemic drugs, allopurinol (50 mg/kg) or benzbromarone (10 mg/kg), which were administered every other day orally with sugar-/fat-free Jell-O mix for the 8-week duration of diabetes. The drugs' effective doses were established based on previously published protocols (32,33). Untreated STZ-rats received Jell-O mix alone. Blood glucose levels, body weights, and

several metabolic parameters for each experimental group are summarized in Supplementary Table 3.

### UA and Uricase Activity Measurements

UA levels in vitreous of postmortem donors were assessed using a commercially available assay kit (BioAssay Systems, Hayward, CA) following the manufacturer's instructions. UA levels in vitreous from patients undergoing vitrectomy were measured using a kit (donated by Roche Diagnostic S.p.a., Milano, Italy) for the cobas 6000 analyzer (Roche Diagnostics). UA and uricase activity in rat vitreous and serum was measured using the Amplex Red Uric Acid/Uricase Assay Kit (Life Technologies, Carlsbad, CA).

### Leukostasis

Leukocyte adhesion was determined as previously described (3). Rats were perfused with 10 mL PBS, followed by 10 mL FITC-labeled concanavalin A (ConA) lectin (40  $\mu$ g/mL in PBS, pH 7.4) (Vector Laboratories, Burlingame, CA). Residual unbound ConA was removed by perfusion with PBS only. Eye globes were fixed with 4% paraformaldehyde. Flat-mounted retinas were observed by fluorescence microscopy, using a Zeiss Axioplan-2 microscope (Carl Zeiss, Göttingen, Germany) equipped with the Axiovision 4.7 software. The total number of adherent leukocytes per retina was counted in blind fashion.

### Assessment of Blood-Retinal Barrier Integrity

Retinal vascular permeability in living animals was assessed as described before (34). Briefly, rats were anesthetized (ketamine, 100 mg/kg; xylazine, 30 mg/kg; and acepromazine, 10 mg/kg). Pupils were dilated using 1% tropicamide (Bausch & Lomb, Rochester, NY), and Goniovisc 2.5% (hypromellose; Sigma Pharmaceuticals, LLC, Monticello, IA) was applied liberally to retain surface moisture during imaging. Each animal was placed on the imaging platform of the Phoenix Micron III retinal imaging microscope (Phoenix Research Laboratories, Pleasanton, CA), and an intraperitoneal injection (80–100  $\mu$ L) of fluorescein sodium (10% Lite) (Apollo Ophthalmics, Newport Beach, CA) was administered. Rapid acquisition of fluorescent images ensued for  $\sim$ 5 min. For a quantitative evaluation of blood-retinal barrier (BRB) integrity, we assessed albumin extravasation in rats after perfusion (34,35). Serum albumin levels were measured in the perfused retinal tissue by Western blot using anti-rat albumin antibody (Cell Signaling Technology, Danvers, MA).

### Immunohistochemistry

Frozen retinal sections were fixed in 4% paraformaldehyde, followed by incubation at 4°C overnight with the following primary antibodies: anti-UA (Abcam, Cambridge, MA), anti-XOD (Novus Biologicals, Littleton, CO), anti-gial fibrillary acidic protein (GFAP) (Cayman Chemical, Ann Arbor, MI), and anti-4-hydroxynonenal (4-HNE) (Abcam). After washing, the slides were incubated

for 1 h with IgG-conjugated Alexa Fluor-488 secondary antibodies (Molecular Probes-Life Technologies, Grand Island, NY). Some sections were colabeled with isolectin B4 to identify the retinal vasculature. Mounted sections were examined by epifluorescence using a Zeiss Axioplan-2 microscope.

### Protein Analysis

Immunoblotting was performed using the following antibodies: anti-NLRP3 (LifeSpan BioSciences, Seattle, WA), anti-TLR4 (Santa Cruz Biotechnology), anti-XOD (Novus Biologicals), and anti-intercellular adhesion molecule-1 (ICAM-1) (Abcam). Dot blot analysis was conducted to analyze immunoreactivity to 4-HNE (Abcam). After incubation with horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Pittsburgh, PA), bands were detected using the enzymatic chemiluminescence reagent (ECL; GE Healthcare) or clarity ECL-Blotting substrate (Bio-Rad). Assessment of vascular endothelial growth factor (VEGF) protein levels was done using heparin affinity columns (Sigma-Aldrich) and Western blot analysis, as previously described (34).

### Cytokines Assay

Tissue and plasma cytokine levels were determined using a customized Rat Mix and Match Cytokine ELISA strip assay (Signosis, Santa Clara, CA). Retinal tissue samples from different experimental groups were homogenized using 1 $\times$  cell lysis buffer (Signosis), and total protein concentration was quantified using the Coomassie Plus (Bradford) Assay Kit. Samples containing equivalent amount of proteins were added to different wells individually coated with primary antibodies (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], transforming growth factor- $\beta$  [TGF- $\beta$ ], IL-1 $\beta$ , IL-10, IL-17, and IL-6). Plates were processed according to the manufacturer's instructions and were read at 450 nm. Protein standards provided by the manufacturer were used to calculate each cytokine concentration and expressed as ng/mg of protein. Quantitative determination of IL-1 $\beta$  in human retinal extracts was performed using Human IL-1 $\beta$  Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

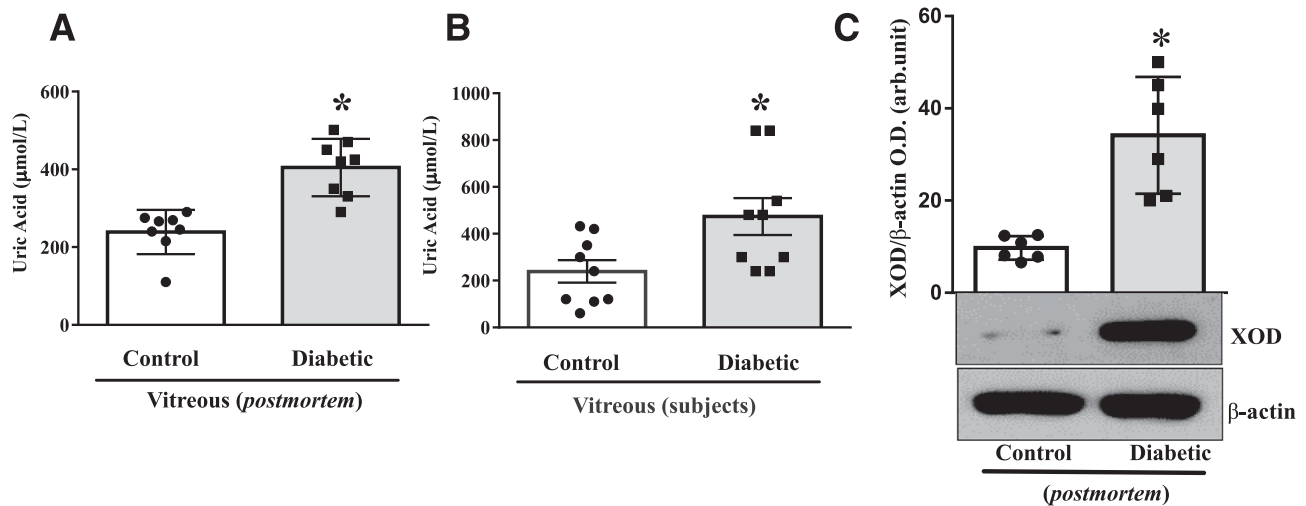
### Statistical Analysis

Graphs were prepared using Graph Pad Prism 3.0 software for Windows (Graph Pad Software, San Diego, CA). Data are shown as means  $\pm$  SD. Statistical significance among experimental groups was established using one-way ANOVA, followed by the Bonferroni multiple-comparison test. Differences were considered significant when  $P$  was  $<0.05$ .

## RESULTS

### Increased Levels of UA in Retinal Extracts and Vitreous of Human DR Donors and Patients

We first analyzed UA levels in vitreous of human post-mortem donors (Fig. 1A) and in patients undergoing pars



**Figure 1**—Assessment of UA and XOD levels in human retinas. **A:** UA levels in the vitreous of postmortem normoglycemic donors (Control) and donors with DR (Diabetic) are represented as a bar histogram. Data are means  $\pm$  SD;  $n = 8$ . \* $P < 0.001$  vs. control. **B:** UA levels in the vitreous of patients with DR (Diabetic) or control subjects without diabetes (Control) who underwent pars plana vitrectomy for retinal detachment or nonclearing vitreous hemorrhage. Data are means  $\pm$  SD;  $n = 9$ . \* $P < 0.01$  vs. Control. **C:** Western blot analysis of XOD-specific immunoreactivity in retinas of postmortem donors. Bar histograms represent measures of optical density (O.D.) of normalized vs.  $\beta$ -actin. Data are means  $\pm$  SD;  $n = 6$ . \* $P < 0.01$  vs. Control.

plana vitrectomy (Fig. 1B). In vitreous of human postmortem control donors without diabetes, UA was found at nonpathological concentration ( $237.5 \pm 63.52 \mu\text{mol/L}$ ), whereas in donors with DR, UA vitreous levels were significantly higher than the control donors ( $P < 0.001$ ;  $n = 8$ ) and at nucleation threshold levels ( $404.5 \pm 73.64 \mu\text{mol/L}$ ).

In vitreous of patients undergoing pars plana vitrectomy, UA levels were found at nonpathological concentration in control patients without diabetes ( $239.1 \pm 142.80 \mu\text{mol/L}$ ), whereas in vitreous of patients with DR, UA levels exceeded the nucleation threshold ( $473.3 \pm 235.2 \mu\text{mol/L}$ ) and were significantly higher than in control donors ( $P < 0.01$ ;  $n = 9$ ) (Fig. 1B).

Furthermore, we assessed the expression pattern of XOD, the rate-limiting enzyme leading to UA production. As shown in Fig. 1C, XOD protein levels, measured by immunoblotting, were significantly upregulated in retinal lysates of human postmortem donors with diabetes compared with control donors without diabetes ( $P < 0.01$ ;  $n = 6$ ).

#### Increased UA Levels in Serum, Vitreous, and Retina of STZ-Rats

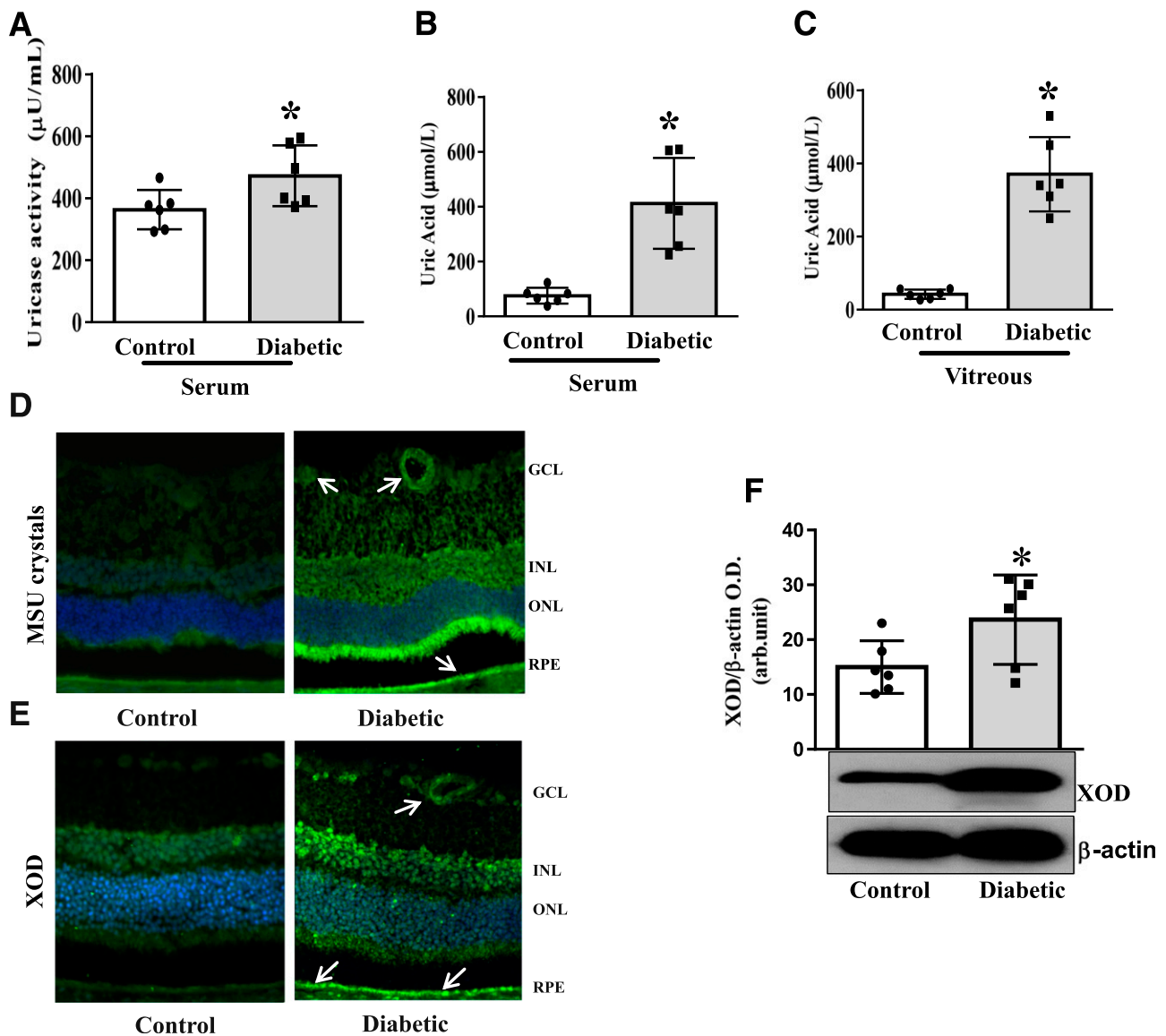
In nonprimate mammals, UA undergoes the conversion to allantoin by uricase (urate oxidase). Because this enzyme can prevent the accumulation of UA in diabetic rats (36), we first assessed serum uricase activity in control and STZ-rats at 8 weeks of diabetes. We found that hyperglycemia increased uricase activity in STZ-rats compared with control normoglycemic rats ( $P < 0.05$ ;  $n = 6$ ) (Fig. 2A). However, despite this increase, UA serum levels were significantly augmented in STZ-rats compared with

normoglycemic rats ( $412.4 \pm 67.55 \mu\text{mol/L}$  vs.  $75.5 \pm 11.84 \mu\text{mol/L}$ ;  $P < 0.01$ ;  $n = 6$ ) (Fig. 2B). Moreover, UA concentration measured in vitreous was also significantly upregulated in STZ-rats compared with control ( $370.6 \pm 5 \mu\text{mol/L}$  vs.  $42.3 \pm 9.57 \mu\text{mol/L}$ ;  $P < 0.01$ ;  $n = 6$ ) (Fig. 2C). Increased MSU deposition in diabetic retinas was also confirmed by immunohistochemical analysis using antibodies specifically recognizing MSU, which is the crystal form of UA. As shown in Fig. 2D, immunoreactivity to MSU was higher in retinas of STZ-rats compared with control rats. MSU accumulation was evident throughout all retinal layers and was also found around the retinal blood vessels and retinal pigmented epithelium (RPE) (Fig. 2D). Retinal UA was also determined in 6-week-old Akita mice, a genetic model of type 1 diabetes, in cryoslides, offered by A.T. (Supplementary Fig. 1).

Western blot and immunohistochemical analyses were conducted to assess the expression and immunolocalization of XOD in the different treatment groups. XOD-specific immunoreactivity was increased throughout the STZ-rat retinas and also localized in the retinal vasculature and the RPE (Fig. 2E). Immunoblotting analysis confirmed increased XOD protein levels in the diabetic compared with control retinas ( $P < 0.05$ ;  $n = 6$ ) (Fig. 2F).

#### Hypouricemic Drugs Diminish Hyperglycemia-Induced Retinal and Systemic Inflammation

To further establish a direct influence of UA on DR induction and progression, we determined the effects of hypouricemic drugs in preventing hyperglycemia-induced retinal inflammation and tissue damage during the early stages of DR. STZ-rats were treated with allopurinol,

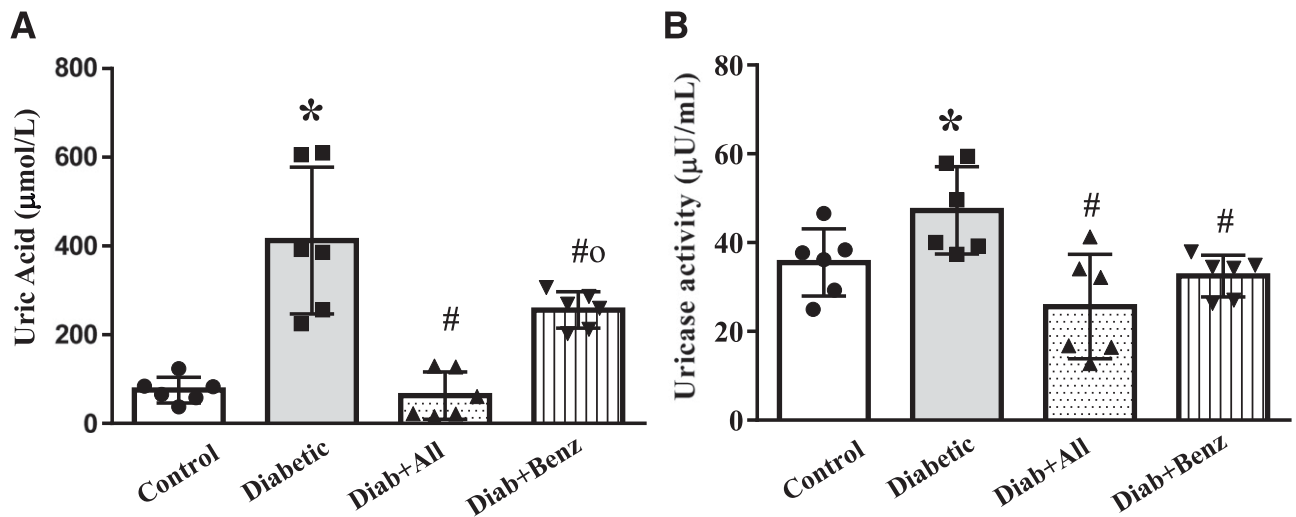


**Figure 2**—Assessment of UA and XOD levels in STZ-rats (Diabetic). **A**: Uricase activity in serum in control and STZ-rats. \* $P < 0.05$  vs. Control. UA levels in serum (**B**) and vitreous (**C**) in control and STZ-rats. \* $P < 0.01$  vs. Control. **D**: Representative images of immunohistochemical analysis of UA (green) in retinas from control and STZ-rats. **E**: Representative images of immunohistochemical analysis of XOD-specific immunoreactivity (green) in retinal vascular and RPE (white arrows) sections from control and STZ-rats. Nuclei were labeled with Hoechst 33342 (blue). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. **F**: XOD protein levels determined by Western blot analysis. Bar histograms are representative of measurements of the optical density (O.D.) of XOD-specific immunoreactivity of normalized vs.  $\beta$ -actin. Data are mean  $\pm$  SD;  $n = 6$ . \* $P < 0.05$  vs. Control.

a specific inhibitor of XOD (32), and benzbromarone, which enhances UA urinary excretion (33). As shown in Fig. 3A, both drugs significantly reduced serum levels of UA in STZ-rats ( $P < 0.001$  for allopurinol and  $P < 0.05$  benzbromarone;  $n = 6$ ). Allopurinol had a more pronounced effect by decreasing serum UA as low as control rats (Fig. 3A), and differences between the two drugs were statistically significant ( $P < 0.001$ ;  $n = 6$ ). The reduction in UA levels in STZ-rats treated with these hypouricemic drugs also normalized uricase activity ( $P < 0.01$ ;  $n = 6$ ) (Fig. 3B). Treatments with allopurinol and benzbromarone, however, did not modify blood glucose and glycated

hemoglobin (HbA<sub>1c</sub>) levels in STZ-rats (Supplementary Table 3) but did not normalize the levels of several metabolic parameters in STZ-rats, including alanine aminotransferase, aspartate transaminase, and cholesterol. Furthermore, treatment with allopurinol but not benzbromarone significantly reduced body weight loss in STZ-rats ( $P < 0.05$ ) (Supplementary Table 3).

Using custom cytokine ELISA plate arrays, we further assessed the levels of several inflammatory cytokines in retinal extracts and plasma in response to the different treatments. Levels of IL-1 $\beta$ , IL-6, IL-17, TNF- $\alpha$ , and TGF- $\beta$  were significantly elevated in retinal extracts ( $P < 0.05$ ;

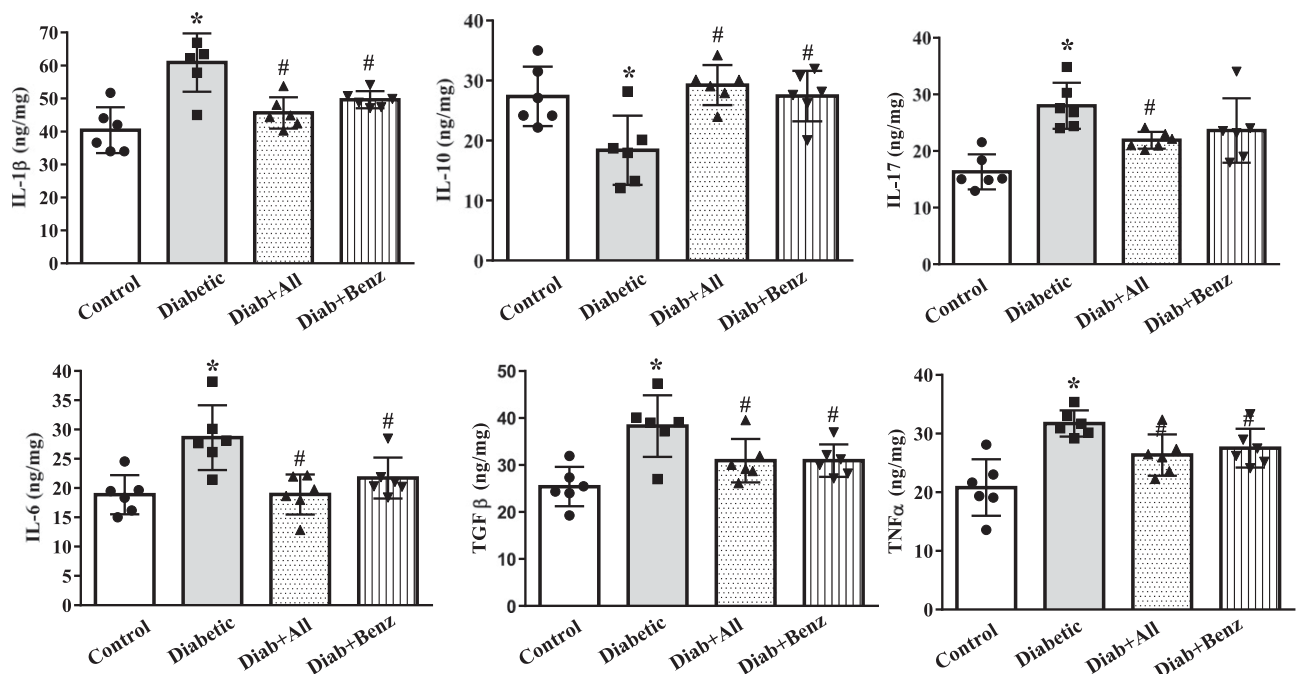


**Figure 3**—Effects of UA-lowering drugs on levels of UA and uricase activity. *A*: Levels of UA in serum of control rats (Control), STZ-rats (Diabetic), and STZ-rats receiving allopurinol (Diab+All) (#*P* < 0.001 vs. Diabetic) or benzbromarone (Diab+Benz) (#*P* < 0.05 vs. Diabetic). \**P* < 0.001 vs. Control; o*P* < 0.001 Diab+Benz vs. Diab+All. *B*: Uricase activity measured in serum of STZ-rats treated with allopurinol or benzbromarone (#*P* < 0.01 vs. Diabetic). Data are mean ± SD; *n* = 6.

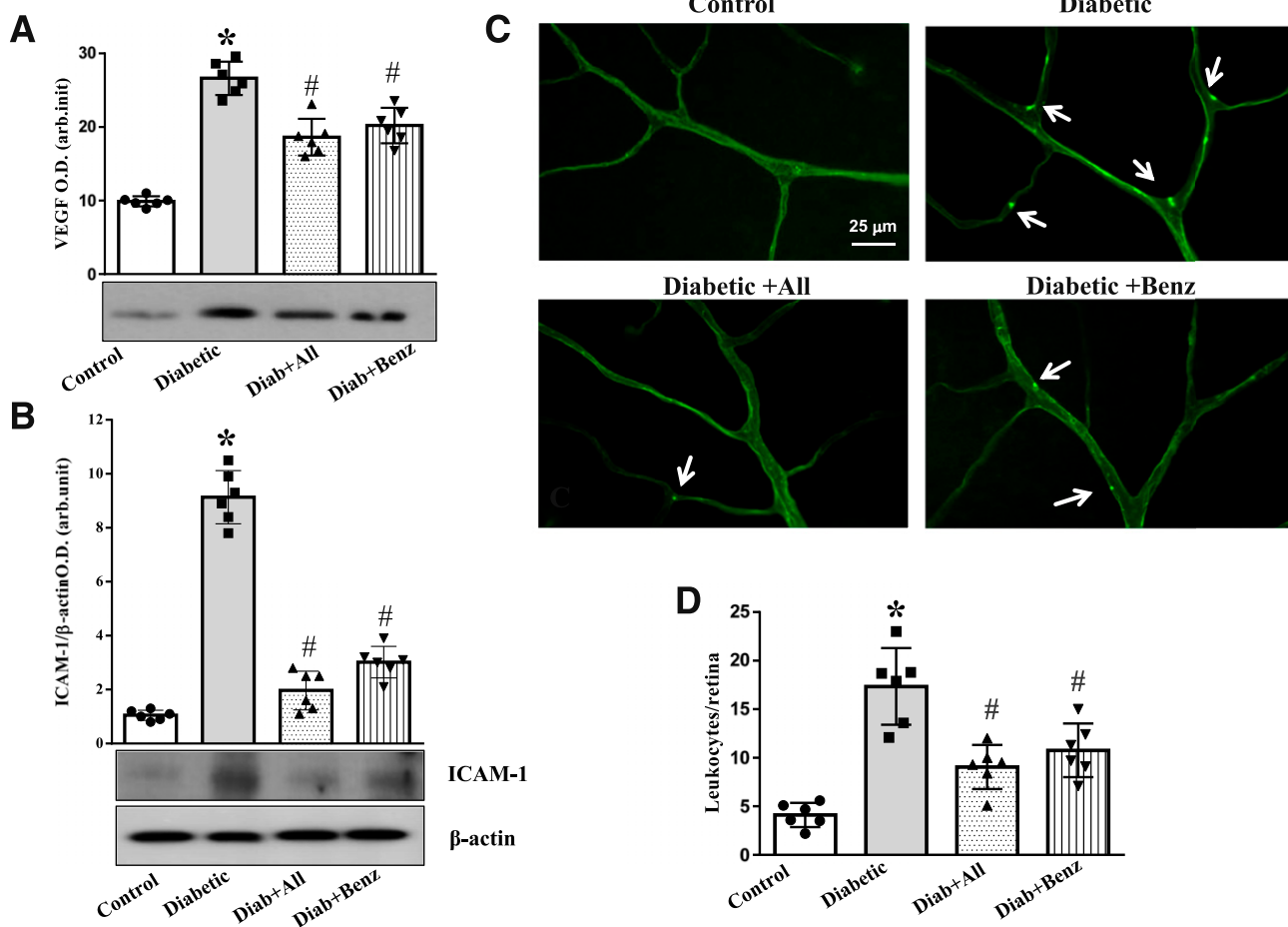
*n* = 6) (Fig. 4) and plasma (*P* < 0.05; *n* = 6) (Supplementary Fig. 2) of STZ-rats compared with controls. This effect was blocked by treatment of STZ-rats with allopurinol or benzbromarone (*P* < 0.05; *n* = 6) (Fig. 4 and Supplementary Fig. 2). However, IL-10 protein levels were significantly downregulated in retina and plasma of STZ-rats compared with controls (*P* < 0.05; *n* = 6), and treatments

with allopurinol or benzbromarone significantly increased IL-10 levels to control values (Fig. 4 and Supplementary Fig. 2).

Next, we determined the effect of UA-lowering drugs on hyperglycemia-induced retinal expression of VEGF and ICAM-1. VEGF expression was significantly increased in diabetic retinas compared with controls (*P* < 0.001; *n* = 6)



**Figure 4**—Effects of UA inhibition on cytokine profile in rat retinas and serum. Expression of IL-1β, IL-6, IL-17, IL-10, TNF-α, and TGF-β were evaluated in retinal tissue in control rats (Control), STZ-rats (Diabetic), and STZ-rats receiving allopurinol (Diab+All) or benzbromarone (Diab+Benz) using a customized ELISA kit. Data are mean ± SD; *n* = 6. \**P* < 0.05 vs. Control; #*P* < 0.05 vs. Diabetic.



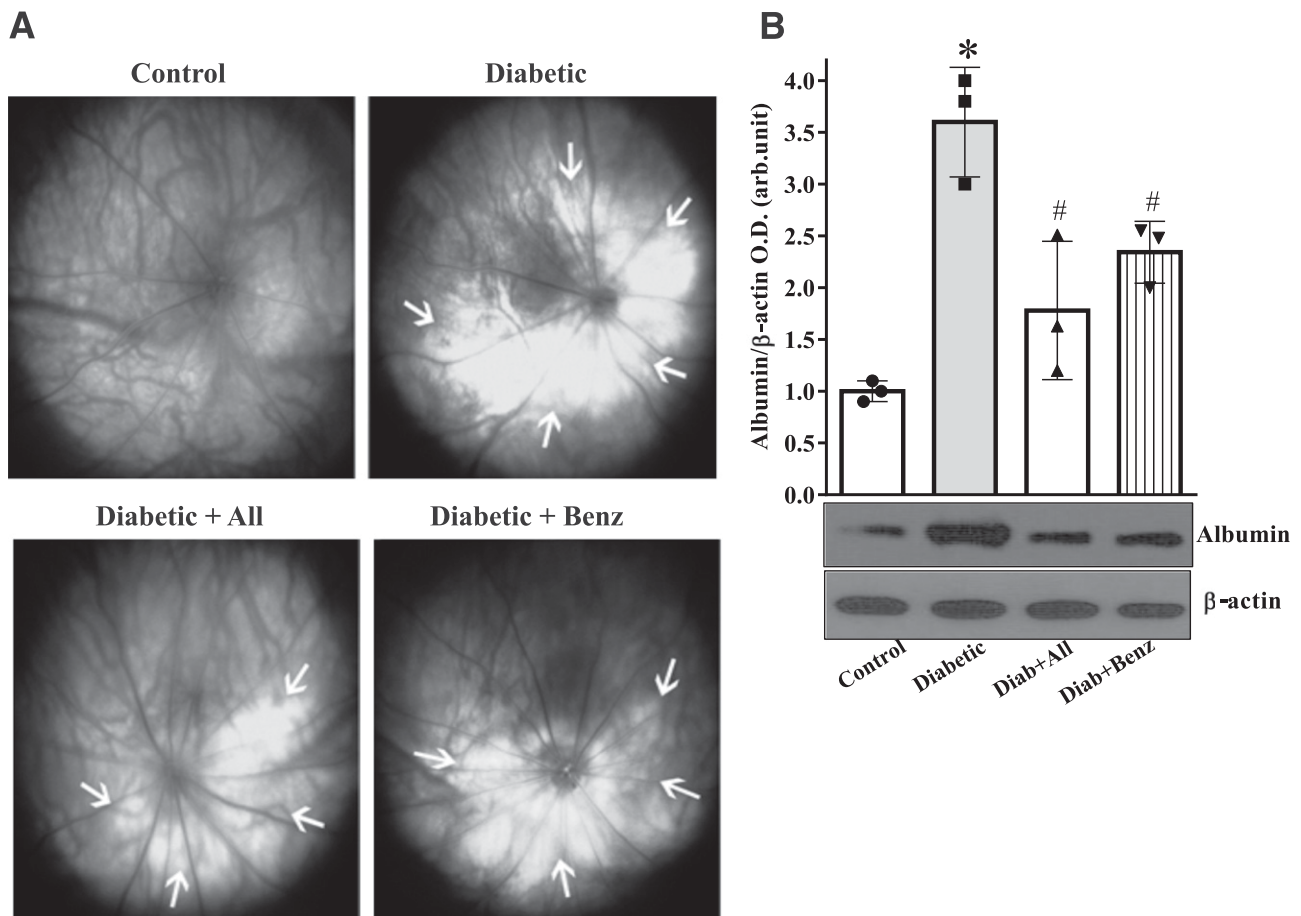
**Figure 5**—Effects of hypouricemic drugs on diabetes-induced VEGF and ICAM-1 expression and retinal leukostasis. **A:** VEGF protein levels determined by Western blot analysis in retinal tissue in control rats (Control), STZ-rats (Diabetic), and STZ-rats receiving allopurinol or benzbromarone (Diab+All and Diab+Benz, respectively). Bar histograms are representative of measurements of the optical density (O.D.) of VEGF-specific immunoreactivity. \* $P < 0.001$  vs. Control; # $P < 0.001$  vs. Diabetic. **B:** ICAM-1 protein levels determined by Western blot analysis in retinal tissue in control rats (Control), STZ-rats (Diabetic), and STZ-rats receiving allopurinol (Diab+All) or benzbromarone (Diab+Benz). Bar histograms are representative of measurements of the optical density of ICAM-1-specific immunoreactivity normalized vs.  $\beta$ -actin. \* $P < 0.001$  vs. Control; # $P < 0.001$  vs. Diabetic. **C:** Representative images of flat-mounted retinas stained with ConA to identify leukocytes adherent to retinal microvessels (white arrows). Scale bar = 25  $\mu$ m. **D:** Quantification of number of adherent leukocytes per retina quantified in the different experimental groups. Data are mean  $\pm$  SD;  $n = 6$ . \* $P < 0.01$  vs. Control; # $P < 0.01$  vs. Diabetic.

(Fig. 5A). However, expression of VEGF in STZ-rats was significantly suppressed by treatments with allopurinol or benzbromarone ( $P < 0.001$ ;  $n = 6$ ) (Fig. 5A). ICAM-1 is one of key mediators of diabetes-induced retinal inflammation (37), and ICAM-1 upregulation leads to leukocyte adhesion (leukostasis) in the diabetic retina, an inflammatory event involved in progression of DR (38). In STZ-rat retinas, ICAM-1 expression was significantly upregulated compared with normoglycemic rats ( $P < 0.001$ ;  $n = 6$ ) (Fig. 5B); however, treatments with allopurinol or benzbromarone significantly reduced ICAM-1 expression in diabetic retinas ( $P < 0.001$ ;  $n = 6$ ) (Fig. 5A). In agreement with these data, the number of adherent leukocytes in the STZ-rat microvasculature was 4.8-fold higher than in normoglycemic rats ( $P < 0.001$ ;  $n = 6$ ) (Fig. 5C and D). Treatments of STZ-rats with allopurinol or benzbromarone significantly reduced the number of adherent leukocytes by

44.24% and 34.5%, respectively ( $P < 0.01$ ;  $n = 6$ ) (Fig. 5D), thus suggesting that UA contributes to leukostasis in the diabetic retina.

#### UA-Lowering Drugs Diminish Hyperglycemia-Induced BRB Breakdown in STZ-Rats

Breakdown of the BRB is an important pathological feature of DR (39,40). To assess the effects of UA-lowering drugs on the BRB, we performed FA in all the treatment groups. The obtained results show that allopurinol or benzbromarone significantly reduced fluorescein extravasation induced by hyperglycemia (Fig. 6A). This effect was also confirmed by measurements of albumin protein levels by immunoblotting in retinal extracts obtained from rats after perfusion. As shown in Fig. 6B, albumin levels were significantly elevated in retinas of STZ-rats compared with control rats ( $P < 0.001$ ;  $n = 6$ ). Allopurinol and



**Figure 6**—Effects of hypouricemic drugs on diabetes-induced BRB breakdown. *A*: Representative images of FA of control normoglycemic age-matched rats (Control), STZ-rats (Diabetic), and STZ-rats treated with allopurinol (Diab+All) or benzbromarone (Diab+Benz). Photographs were taken at constant intervals for every rat studied in each experimental group ( $n = 6$ ). White arrows indicate the areas of vascular leakage. *B*: Western blot analysis assessing albumin protein levels in retinal extracts of perfused rats from the different experimental groups. The data are expressed as arbitrary units of optical density (O.D.) and normalized for the loading control  $\beta$ -actin. Data are mean  $\pm$  SD;  $n = 6$ . \* $P < 0.001$  vs. Control; # $P < 0.05$  vs. Diabetic.

benzbromarone both significantly decreased albumin levels in retinal extracts of STZ-rats ( $P < 0.05$ ;  $n = 6$ ), thus suggesting that limitation of UA production and accumulation in the diabetic retina, while halting retinal inflammation, also prevents hyperglycemia-induced breakdown of the BRB.

#### Hypouricemic Drugs Diminish Hyperglycemia-Induced Retinal Stress Markers

We further sought to determine the potential mechanism of action of UA in the diabetic retina by examining the effects of hypouricemic drugs on hyperglycemia-induced retinal oxidative stress, a major contributing factor for diabetes-induced retinal inflammation and progression to DR (3,41).

Detection of the lipid peroxidation by-product 4-HNE is used as an indicator of dysregulated lipid metabolism due to aberrant reactions between lipids and free radicals (42,43). Immunohistochemical analysis revealed that 4-HNE-specific immunoreactivity was significantly enhanced in the diabetic rat retina (Fig. 7A). Treatments

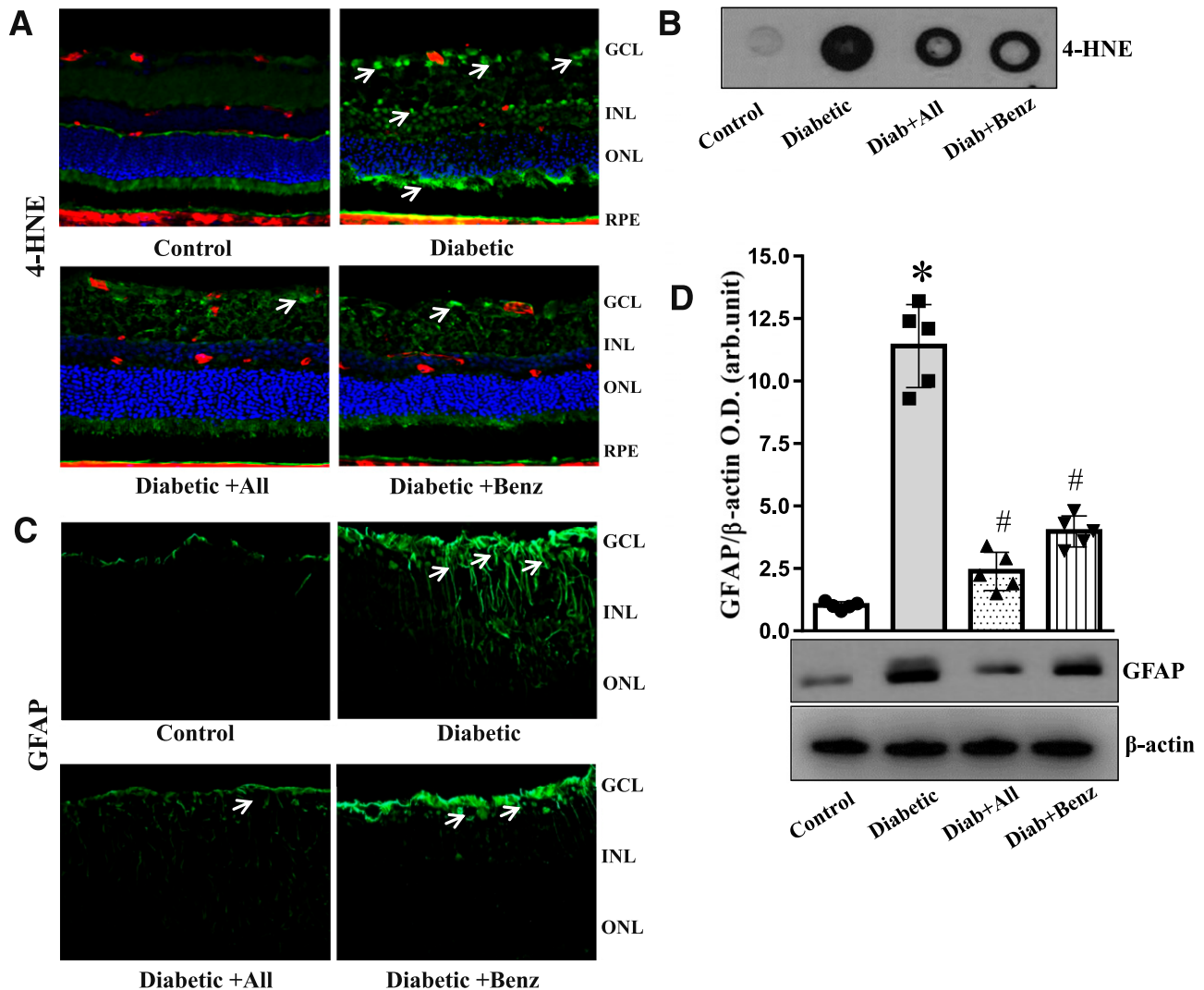
with allopurinol or benzbromarone lowered 4-HNE-specific immunoreactivity (Fig. 7A). Dot blot analysis measuring 4-HNE levels confirmed the immunohistochemical data (Fig. 7B).

Finally, UA-lowering drugs decreased hyperglycemia-induced reactive gliosis as assessed by measures of GFAP immunoreactivity in STZ-rat retina compared with age-matched normoglycemic rat retina (Fig. 7C). Western blot analysis further confirmed these findings and showed that treatments of STZ-rats with allopurinol and benzbromarone significantly lowered GFAP expression in comparison with untreated STZ-rats ( $P < 0.001$ ;  $n = 6$ ) (Fig. 7D).

#### UA-Lowering Drugs Diminish Hyperglycemia-Induced NLRP3 Inflammasome Activation

Previous studies suggest that MSU-induced proinflammatory responses involve the activation of the sterile inflammation via NLRP3 inflammasome (17–20). To further investigate the specific contribution of UA to hyperglycemia-induced metabolic (sterile) inflammation, we

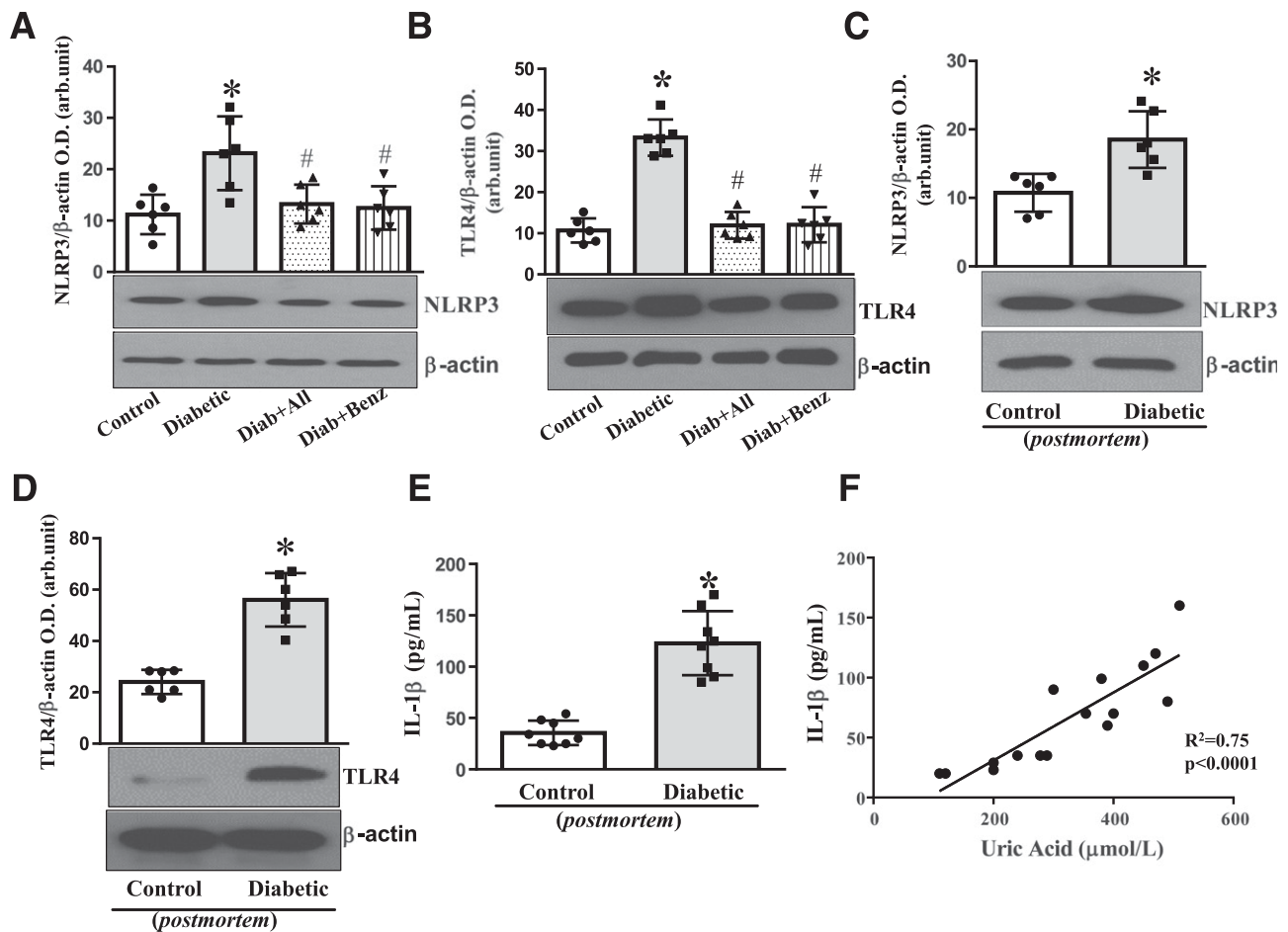




**Figure 7**—Effects of hypouricemic drugs on 4-HNE and GFAP expression. **A:** Immunohistochemical analysis of 4-HNE (green) in retinas of control rats (Control), STZ-rats (Diabetic), and STZ-rats treated with allopurinol (Diabetic+All) or benzbromarone (Diabetic+Benz). Sections were colabeled with isolectin B4 (red) for detection of vascular structures. Hoechst staining was used for nuclear counterstain (blue). White arrows indicate 4-HNE-positive cells. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. **B:** Dot blot analysis of 4-HNE in retinal tissue of control rats, STZ-rats, and STZ-rats treated with allopurinol (Diab+All) or benzbromarone (Diab+Benz). **C:** Representative images for GFAP immunoreactivity in control rats, STZ-rats, and STZ-rats treated with allopurinol and benzbromarone. White arrows indicate GFAP-positive cells. **D:** GFAP protein levels determined by Western blot analysis in retinal tissue in control rats, STZ-rats, and STZ-rats receiving allopurinol or benzbromarone. Bar histograms are representative of measurements of the optical density (O.D.) of GFAP-specific immunoreactivity normalized vs.  $\beta$ -actin. Data are mean  $\pm$  SD;  $n = 6$ ; \* $P < 0.001$  vs. Control; # $P < 0.001$  vs. Diabetic.

examined the effects of diabetes and hypouricemic drugs on diabetes-induced activation of the NLRP3 inflammasome by assessing the expression levels of its constituents. As shown in Fig. 8, administration of allopurinol and benzbromarone effectively reduced hyperglycemia-induced expression of NLRP3 (Fig. 8A) and TLR4 (Fig. 8B) in rat retinas after 8 weeks of diabetes ( $P < 0.01$ ;  $n = 6$ ). In all experimental conditions, changes in TLR4 and NLRP3 retinal expression levels positively correlated with levels of IL-1 $\beta$  that we have measured in retinal extracts (Fig. 4), further confirming that specific relationship between UA levels and induction of sterile inflammation.

Furthermore, we analyzed the expression of NLRP3 inflammasome constituents in postmortem retinas of donors with and without diabetes. Immunoblotting analysis showed a significant upregulation in protein levels of NLRP3 ( $P < 0.01$ ;  $n = 8$ ) (Fig. 8D) and TLR4 ( $P < 0.01$ ;  $n = 8$ ) (Fig. 8E) in donors with DR compared with control donors without diabetes. In particular, ELISA assay measuring IL-1 $\beta$  in retinal lysates of donors with DR showed a 4.1-fold increase in levels of this cytokine ( $P < 0.05$ ;  $n = 8$ ) (Fig. 8F), and correlation analysis demonstrated a positive relationship of these values with UA vitreous levels ( $R^2 = 0.75$ ,  $P < 0.01$ ;  $n = 8$ ) (Fig. 8F), thus confirming that



**Figure 8**—Effects of UA-lowering drugs on inflammasome activation. *A* and *B*: Western blot analysis showing NLRP3 (*A*) and TLR4 (*B*) specific immunoreactivity in retinal extracts of control rats (Control), STZ-rats (Diabetic), and STZ-rats receiving allopurinol (Diab+All) or benzbromarone (Diab+Benz). Optical density (O.D.) values of NLRP3 and TLR4 protein levels relative to  $\beta$ -actin are represented in bar histograms. Data are mean  $\pm$  SD;  $n = 6$ . \* $P < 0.01$  vs. Control; # $P < 0.05$  vs. Diabetic. *C* and *D*: Western blot analysis of NLRP3-specific (*C*) and TLR4-specific (*D*) immunoreactivity in retinas of postmortem normoglycemic donors (Control) and donors with DR (Diabetic). Bar histograms are representing measures of optical density normalized vs.  $\beta$ -actin. \* $P < 0.01$  vs. Control. *E*: Quantification of IL-1 $\beta$  production in vitreous samples of control donors and donors with DR. Concentrations are represented as a bar histogram. Data are mean  $\pm$  SD;  $n = 8$ . \* $P < 0.001$  vs. Control. *F*: The correlation analysis between serum UA levels and IL-1 $\beta$  in postmortem samples of DR and control donors ( $R^2 = 0.75$ ;  $P < 0.001$ ;  $n = 16$ ).

activation of autoinflammatory processes (sterile inflammation) in the retina of individuals with diabetes is directly associated with elevated vitreous levels of UA.

## DISCUSSION

We investigated the contribution of MSU, the crystal form of UA, to retinal inflammation and progression to DR. To date, hyperglycemia, hypertension, and hyperlipidemia have been considered main risk factors for DR, and their clinical management represents a main therapeutic goal (44). New evidence, however, has suggested that monitoring inflammatory mediators in patients with diabetes may hold important adjunctive value as predictors of occurrence and progression of diabetic complications, including retinopathy (3,38).

UA is a by-product of purine oxidative catabolism by XOD (45). Excessive UA levels cause the human disease of

gout, which is characterized by systemic inflammatory processes (18). Recent clinical studies have pointed out that a modest elevation in the “normal-high” range (6–8 mg/dL [357–476  $\mu$ mol/L]) of UA blood levels is strongly associated with adverse cardiovascular outcomes, metabolic syndrome, diabetes, and progression of diabetic nephropathy (8,13,14,24–27). Despite few studies reporting enhanced UA levels in the vitreous of patients affected by diabetic macular edema (30,46), no further clinical investigation has been conducted to determine the potential contribution of UA to DR.

Here, we report that levels of UA were elevated in vitreous of postmortem human donors with DR and also in freshly isolated vitreous samples from patients with DR undergoing pars plana vitrectomy. Although preliminary, these results in human DR further confirm previous observations (30,47) and indicate the need for much

larger clinical studies. Furthermore, in an experimental model of type 1 diabetes, STZ-rats, we found that hyperglycemia promotes an elevation in serum and vitreous levels of UA compared with age-matched normoglycemic controls.

The presence of UA in the normal vitreous may exert beneficial effects through its antioxidant ability (48). However, in the diabetic condition, the UA concentration arises above the nucleation threshold value of 354  $\mu\text{mol/L}$  (6 mg/dL), thus implying that most of the UA in these biological fluids exists in the form of MSU crystals (6). These are known irritants, which exert adverse biological activities by promoting pro-oxidative and proinflammatory effects (18,45).

Our data showed that allopurinol and benzbromarone both prevented retinal vascular permeability and leukostasis. These effects were associated with the ability of the hypouricemic drugs to reduce the expression of VEGF, ICAM-1, and other inflammatory cytokines and also with reduced oxidative/nitrative stress parameters.

The proinflammatory effects of MSU have been largely explained by its role as an alarmin (18) through activation of the NLRP3 inflammasome (17,19). Sterile inflammation underlies the development of cardiovascular disease (13) and of diabetic complications (26). Our data show that in STZ-rats, there was an upregulation of constituents of the NLRP3 inflammasome, including NLRP3, TLR4, and IL-1 $\beta$ , which was halted by UA-lowering drugs, thus establishing a cause-and-effect relationship between UA and sterile inflammation in the diabetic rat retina. Most importantly, our data analyzing human postmortem retinas further confirmed the involvement of sterile inflammation in UA/MSU proinflammatory activity by evidencing the upregulation of the NLRP3 inflammasome in postmortem donors with DR and demonstrating the existence of a positive correlation between IL-1 $\beta$  and UA vitreous levels in these samples.

Our studies further show that along with elevation of systemic UA/MSU levels, there is increased retinal production of this alarmin due to upregulation of XOD expression. In line with this evidence, allopurinol, which specifically blocks XOD (32), showed greater ability to prevent retinal inflammation than benzbromarone, which primarily exerts systemic UA levels by increasing its urinary excretion (33). Additional effects of allopurinol may also involve decreased retinal oxidative stress due to XOD blockade (49).

In summary, our study suggests that monitoring of MSU levels may have important predictive and prognostic value for DR and warrant the realization of specific clinical studies to confirm uricemia as new risk factor for DR and validate the use of hypouricemic drugs as an adjunctive therapy for the treatment of this potentially blinding complication of diabetes.

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**Author Contributions.** M.C.T. performed the experiments and developed the project. A.M. organized and performed the experiments with human samples. F.L.P. contributed to the *in vivo* experiments. P.M. participated in the *in vitro* and *in vivo* experiments. D.R.G. contributed to data analysis and manuscript preparation. A.B. analyzed human vitreous samples. G.R. provided the vitreous samples. A.R. provided the vitreous samples and clinical expertise for data analysis. A.T. performed and analyzed the FA data. P.M.M. contributed to data analysis and writing the manuscript. F.F. contributed to the realization of the human studies and data analysis. M.B. developed the idea, designed the studies described here, and provided financial support for their realization. M.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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