Studies of Rat and Human Retinas Predict a Role for the Polyol Pathway in Human Diabetic Retinopathy

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The polyol (sorbitol) pathway of glucose metabolism is activated in many cell types when intracellular glucose concentrations are high, and it can generate cellular stress through several mechanisms. The role of the polyol pathway in the pathogenesis of diabetic retinopathy has remained uncertain, in part because it has been examined preferentially in galactose-induced retinopathy and in part because inhibition studies may not have achieved full blockade of the pathway. Having observed that the streptozotocin-induced diabetic rat accurately models many cellular processes characteristic of human diabetic retinopathy, we tested in the diabetic rat if documented inhibition of the polyol pathway prevents a sequence of retinal vascular abnormalities also present in human diabetes. An inhibitor of aldose reductase, the rate-limiting enzyme in the pathway, prevented the early activation of complement in the wall of retinal vessels and the decreased levels of complement inhibitors in diabetic rats, as well as the later apoptosis of vascular pericytes and endothelial cells and the development of acellular capillaries. Both rat and human retinal endothelial cells showed aldose reductase immunoreactivity, and human retinas exposed to high glucose in organ culture increased the production of sorbitol by a degree similar to that observed in the rat. Excess aldose reductase activity can be a mechanism for human diabetic retinopathy. ***Diabetes*** **53:2404–2411, 2004**

Most cells of the retina are affected by the metabolic abnormalities of diabetes (1), but the sight-threatening manifestations of diabetic retinopathy are ultimately attributable to capillary damage. Abnormal permeability of barrier capillaries can cause macular edema, and capillary closure causes ischemia and unregulated angiogenesis (2). The current means to maintain a normal metabolic status in diabetic patients are imperfect, and they are successful in only a fraction of patients. It would thus be desirable to complement antidiabetic therapy with drugs that target processes specific to the complications of diabetes.

The polyol pathway of glucose metabolism becomes active when intracellular glucose levels are elevated (3,4). Aldose reductase, the first and rate-limiting enzyme in the pathway, reduces glucose to sorbitol using NADPH as a cofactor; sorbitol is then metabolized to fructose by sorbitol dehydrogenase, which uses NAD^+ as a cofactor. The polyol pathway is both a "dream" and a "dread" target when devising strategies to prevent diabetic retinopathy. The pathway is a dream target because its activation is immediately linked to hyperglycemia, generates various types of cellular stress (4–6), and occurs prominently in the tissues that develop complications (3–6), thus promising returns beyond retinopathy. In addition, polymorphisms of the aldose reductase gene may help predict individual susceptibility to retinopathy and other microvascular complications (7), and the enzymatic function of aldose reductase can be specifically inhibited (4) with the expectation of only minor consequences on physiology (8). The polyol pathway has, however, become a dread target because aldose reductase inhibitors have yielded inconsistent results in the diabetic or diabetic-like retinopathy of experimental animals (9,10) and only minor benefits in human diabetic retinopathy (11). It must be stated that the great majority of animal studies addressed the effects of aldose reductase inhibitors on the diabetic-like retinopathy of experimental galactosemia rather than diabetic retinopathy and that the dose of the aldose reductase inhibitor sorbinil given in the clinical Sorbinil Retinopathy Trial (11) was limited by toxicity and may not have achieved complete suppression of the glucose flux through aldose reductase (4).

We returned to the polyol pathway recently (12) because it was a rational candidate mechanism for the ganglion cell apoptosis and Müller glial cell activation that occur early in both human and rat diabetes (1). Ganglion and Müller cells are the retinal cells most consistently found to contain aldose reductase in all species studied, including humans (13–16). In rats with 2.5 months of streptozotocin-induced diabetes, retinal neurons undergoing apoptosis were positive for aldose reductase, and sorbinil prevented both neuronal apoptosis and Müller cell reactivity (12). Since neuroglial changes may cause vascular changes (17), and there is agreement that at least the pericytes of retinal capillaries contain aldose reductase (14,18), the question arose as to whether inhibition of the polyol pathway would also prevent the vascular abnormal-

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DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; MAC, membrane attack complex; NSE, neuron-specific enolase, PECAM-1, platelet endothelial cell adhesion molecule-1; RIPA, radioimmunoprecipitation assay; TUNEL, transferase-mediated dUPT nick-end labeling; vWf, von Willebrand factor.

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ities spanning all the way to the development of acellular capillaries in diabetic rats. We found no studies addressing this question comprehensively in diabetic rats, despite the fact that the diabetic rat is the most widely used animal model for the study of diabetic retinopathy. Moreover, the diabetic rat accurately mimics many of the newly discovered as well as long-known processes that occur in human diabetic retinopathy. We mentioned earlier that neuroglial abnormalities are found in the human and rat diabetic retina (1). In both human and rat diabetes, there is early activation of complement in the wall of retinal vessels associated with abnormalities of complement inhibitors (19), and there is later evidence of apoptosis of vascular pericytes and endothelial cells (20), with development of acellular and nonperfused capillaries (21–23). In this study, we set out to test whether the sequence of vascular abnormalities common to human and rat diabetic retinopathy is susceptible to aldose reductase inhibition in the rat and whether aldose reductase has the potential for being a mechanism of the abnormalities in human retinopathy.

RESEARCH DESIGN AND METHODS

Experiments were performed following institutional guidelines for the use and care of laboratory animals. Sprague-Dawley male rats were randomly assigned to one of the following groups: control, diabetic, and diabetic treated with sorbinil. The experiments carried out for 2.5 months also included a group of control rats treated with sorbinil. Diabetes was induced with streptozotocin (57.5 mg/kg body wt) dissolved in citrate buffer, pH 4.5, and injected via the tail vein. Development of diabetes (blood glucose >250 mg/dl) was verified 3 days after the streptozotocin injection. Body weight was recorded three times a week in the diabetic rats, and 2–4 units of NPH insulin were administered subcutaneously as needed to prevent weight loss without preventing hyperglycemia. Sorbinil (Pfizer, Groton, CT) was given from the onset of diabetes at a dose of 65 mg $\cdot$ kg body wt^{-1} $\cdot$ day^{-1} mixed with the powdered diet. This sorbinil dose prevents the accumulation of both sorbitol and fructose in the retina of rats with 2.5 months of diabetes (12), indicating complete suppression of polyol pathway activity (4). Such preventative effects of sorbinil (given at 50 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}) have also been documented in rats with 10 months of diabetes (J.R. Williamson, personal communication). The rats were killed after 2.5 or 9 months of diabetes together with age-matched controls. At the time of death, blood was obtained by cardiac puncture for the measurement of GHb (Glyc-Affin GHb assay; PerkinElmer, Norton, OH). Whole eyes were embedded in OCT (optimal cutting temperature) compound on dry ice for sectioning or fixed in formalin for the preparation of retinal trypsin digests. The retinas that were immediately dissected were used for protein studies, for preparation of fresh microvessels and dispersed cells, and for organ culture experiments.

Human eyes and specimens. Human postmortem eyes were obtained from certified eye banks through the National Disease Research Interchange. The donors were 63, 66, and 75 years of age, two men and one woman, and all Caucasians. The cause of death was cancer for two donors and cardiac arrest for the third; none had diabetes. The time elapsed from death to enucleation was 4.6 ± 1.5 h and 30 ± 3 h to receipt of the eyes in the laboratory. The retinas were immediately isolated and placed in organ culture or used to prepare dissociated cells and protein lysates.

Retinal trypsin digests. Retinas from rats with 9 months of diabetes and age-matched controls were fixed in 10% buffered formalin for 2–8 days and processed for trypsin digestion to isolate the intact microvascular network (20,24).

Isolation of fresh microvessels. The intact microvascular network was isolated from fresh retinas of normal rats by the hypotonic lysis method as used previously (25).

Preparation of dissociated retinal cells. Fresh rat and human retinas were incubated with 0.1% collagenase type I (Sigma, St. Louis, MO) in serum-free Dulbecco's modified Eagle's medium (DMEM) at 37°C for 50 and 70 min, respectively (26). The reaction was stopped by the addition of DMEM containing 10% FCS. Suspended cells were centrifuged at 200*g* for 5 min at 4°C, resuspended in PBS, and smeared on Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA). The slides were air dried and stored at −20°C.

Organ culture. Retinas dissected sterilely from postmortem human eyes were cut into halves or quarters and placed flat onto the insert of a 6-well Transwell plate (Corning, Corning, NY). The tissue was covered with DMEM containing 10% FCS and antibiotics/antimycotics and placed in a tissue culture incubator at 37°C. The medium contained either 5 or 30 mmol/l glucose. Rat retinas were studied in parallel; each whole rat retina was flattened by two or three peripheral incisions and placed in organ culture onto the insert of a 12-well Transwell plate.

Immunohistochemistry. Retinal cryosections (6 μm) and dissociated cells were fixed in ice-cold acetone for 10 min; microvessels were fixed in formalin for 15 min. Immunohistochemistry was performed as described (12,25). The primary antibodies were mouse monoclonal antibody (mAb) 2A1 anti-rat C5b-9, to detect the membrane attack complex (MAC) of complement in rat retina (1:200; provided by W.G. Couser); mouse mAb anti-rat platelet endothelial cell adhesion molecule-1 (PECAM-1) (1:1,000; BD Pharmingen, San Diego, CA); mouse mAb anti-human von Willebrand factor (vWf) and rabbit anti-human vWf (both used at 1:500; Dako, Carpinteria, CA); and two different rabbit anti-rat aldose reductase (one provided by D. Carper and one by R. Sorenson, both diluted 1:1,000). Negative controls were obtained by substituting the primary antibodies with an equivalent concentration of nonimmune isotypic mouse or rabbit IgG as appropriate.

Semiquantitative scoring of MAC staining in rat retinal sections was performed by masked observers, taking into account the frequency of staining (number of retinal vessels positive for MAC over the total number of vessels detected in each of several fields by the vWf staining) and the area stained in each vessel (varying from the whole vessel wall to speckles). The scoring was from 0 to 4, with 0 indicating no staining and 4 uniform staining of all vessels.

Immunoblotting. Proteins were isolated from retinas and retinal microvessels lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 5 mmol/l EDTA, 1% Triton X-100, 0.5% Na deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (27). Protein concentration was determined with the bicinchoninic acid method, using BSA as standard (Micro BCA Protein Assay Kit; Pierce, Rockford, IL). Retinal proteins were resolved by SDS-PAGE and immunoblotted as previously described (27). Nonreducing conditions were used only for the study of CD59, which was detected with mouse mAb 6D1 (1:1,000; provided by B.P. Morgan [28]). CD55 (H-319), CD46 (H-294), and caveolin-1 (N-20) were detected with rabbit antibodies (all at 1:1,000; Santa Cruz, Santa Cruz, CA), occludin with mouse mAb anti-human occludin (1:1,000; Zymed, South San Francisco, CA), and neuron-specific enolase (NSE) with rabbit anti-cow NSE (1:20,000; Chemicon International, Temecula, CA). The blots were subsequently reacted with mouse mAb AC-15 anti–β-actin (1:200,000; Sigma) to verify even loading. Protein lysates from human retinas were tested for Hb to determine erythrocyte content using goat antibodies to human Hb (1:1,000; Bethyl Laboratories, Montgomery, TX). The human Hb used as standard was purchased from Sigma.

Transferase-mediated dUPT nick-end labeling reaction. Rat retinal trypsin digests were studied with the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Mannheim, Germany). The count of transferase-mediated dUPT nick-end labeling (TUNEL)-positive nuclei and their attribution to pericytes or endothelial cells were performed by masked observers as described previously (20). TUNEL-positive chromatin with uninformative shape or topography was counted as "undetermined." Counts of TUNEL-positive cells are presented per whole retina.

Counts of pericytes, endothelial cells, and acellular capillaries. Cell nuclei and acellular capillaries were counted in rat retinal trypsin digests stained with periodic acid Schiff hematoxylin and viewed at 20× magnification. Counts were performed in six to eight fields in mid-retina using the AxioVision4 program (Carl Zeiss, Göttingen, Germany). Nuclei were attributed to pericytes or endothelial cells on the basis of shape, location along the vessel, and intensity of staining. Pericyte nuclei are round or slightly oval and appear to protrude from the vessel wall, staining heavily with hematoxylin. Endothelial cell nuclei are ellipsoid in shape, larger, and lie in the axis of the capillary, taking a lighter stain. Capillary length was determined in each field, and counts are expressed as the number of cells per millimeter of capillary length. Acellular capillaries were those that had *1*) no visible nuclei along the vessel between bifurcations, *2*) length of at least 40 μm, and *3*) width equal to at least 20% of an average capillary. Acellular capillaries counts are reported as number of acellular capillaries per mm^2 retinal trypsin digest.

Retinal sorbitol levels. The concentration of retinal sorbitol was determined by a modification (29) of the enzymatic method of Malone et al. (30). In the modified assay, after sorbitol is oxidized to fructose by sorbitol dehydrogenase and NAD$^+$ concomitantly reduced to NADH, NADH is oxidized back to NAD$^+$ by diaphorase, with the redox dye resazurin being reduced to the highly fluorescent derivative resorufin. The fluorescence of resorufin is stoichiometric with the amount of sorbitol. Retinas (a minimum of one-eighth of a human

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TABLE 1
Weight, insulin treatment, and GHb in rats with different duration of diabetes

Duration of diabetes	*n*	Body weight (g)	Insulin (units · rat^{-1} · week^{-1})*	GHb (%)
2.5 months				
Control rats	32	532 ± 67	—	4.4 ± 0.6
Control rats treated with sorbinil	6	482 ± 25	—	4.9 ± 0.2
Diabetic rats	33	298 ± 64†	8.9 ± 2.0	15.8 ± 2.8†
Diabetic rats treated with sorbinil	37	293 ± 59†	9.5 ± 2.4	14.9 ± 2.5†
9 months				
Control rats	10	694 ± 63	—	4.5 ± 0.2
Diabetic rats	10	336 ± 64†	12.6 ± 2.5‡	16.6 ± 1.6†
Diabetic rats treated with sorbinil	9	306 ± 80†	11.6 ± 2.9‡	17.3 ± 1.7†

Data are means ± SD. Before streptozotocin injection, the rats had a body weight of 246 ± 30 g. *NPH insulin was injected subcutaneously three times a week as needed to prevent weight loss but not hyperglycemia. †$P < 0.0001$ vs. control rats; ‡$P < 0.03$ vs. 2.5 months with diabetes.

retina or one whole rat retina) collected in 6% (wt/vol) ice-cold perchloric acid were homogenized by a tissue homogenizer and centrifuged at 20,000*g* for 15 min. The supernatant was removed and neutralized with 3 mol/l sodium carbonate. The sample was vortexed and centrifuged again as before. An aliquot of the supernatant was added to the assay solution (0.2 mol/l triethanolamine buffer, pH 8.5, 1.2 μmol/l resazurin, 1 mmol/l NAD^+, and 0.3 units/ml diaphorase with and without 1.0 units/ml sheep sorbitol dehydrogenase). The samples were incubated at room temperature for 1 h, and fluorescence intensity was measured at 580 nm, with the excitation wavelength being 560 nm. Sorbitol concentrations were calculated by comparison with a series of known sorbitol standards. The pellet from the first centrifugation was neutralized with 0.1 N NaOH, briefly sonicated, and rotated overnight at 4°C, and protein was assayed with the BioRad DC protein-assay reagent. Sorbitol levels are expressed as nanomoles per milligram protein.

Statistical analysis. Data are summarized as means ± SD, except scores and counts, which are summarized with the median and range and presented as box plots because they are noncontinuous variables. ANOVA followed by the Fisher's protected least significant difference test was used to compare study groups with regard to metabolic data and levels of CD55 and CD59. The nonparametric Kruskal-Wallis followed by multiple comparisons with the Mann-Whitney *U* test was used to analyze scores and counts. The paired *t* test was used to analyze sorbitol accumulation under normal- or high-glucose conditions in organ culture.

RESULTS

Characteristics of experimental rats. After both 2.5 and 9 months of diabetes, the diabetic rats showed lower body weight and higher GHb levels than the age-matched controls (Table 1). In the diabetic rats treated with sorbinil, weight and GHb levels were not different from those of the untreated diabetic rats. The amount of insulin required to prevent weight loss was greater after the longer duration of diabetes. Sorbinil consistently prevented cataract formation.

Early vascular changes (complement activation and decreased levels of complement inhibitors) are prevented by sorbinil. The retinal vessels of rats with 2.5 months of diabetes showed MAC staining, which was almost completely absent from the vessels of control rats (Fig. 1*A* and *B*), confirming our previous observations in both rat and human diabetes (19). MAC deposition was much reduced in the retinal vessels of diabetic rats treated with sorbinil (Fig. 1*C*). In these rats, the masked assess-

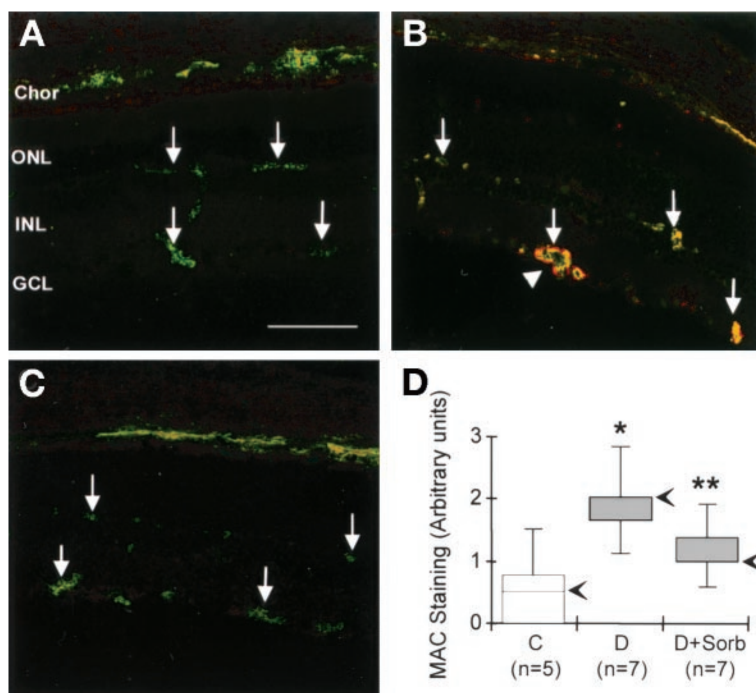

FIG. 1. MAC in the retina of diabetic rats (2.5 months of diabetes) and diabetic rats treated with sorbinil. Retinal sections were doubly immunostained for C5b-9 (MAC, red) and vWf (green, to identify vascular endothelial cells). *A*: Retina of a control rat; arrows point to vessels (choroidal vessels also stain for vWf). *B*: Retina of a diabetic rat showing MAC colocalized with vWf (yellow) in most vessels and red MAC staining in the outer layers of vessels (arrowhead). *C*: Retina of a diabetic rat treated with sorbinil. *D*: Box plot of scores attributed to MAC staining in the retina of control (C) and diabetic (D) rats and diabetic rats treated with sorbinil (D+Sorb). (The bars encompass from the 90th to the 10th percentile of the scores and the box from the 75th to the 25th percentile; arrow points to the median.) Chor, choroid; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Bar = 100 μm. *$P < 0.05$ vs. control rats; **$P < 0.05$ vs. untreated diabetic rats (in all figures).

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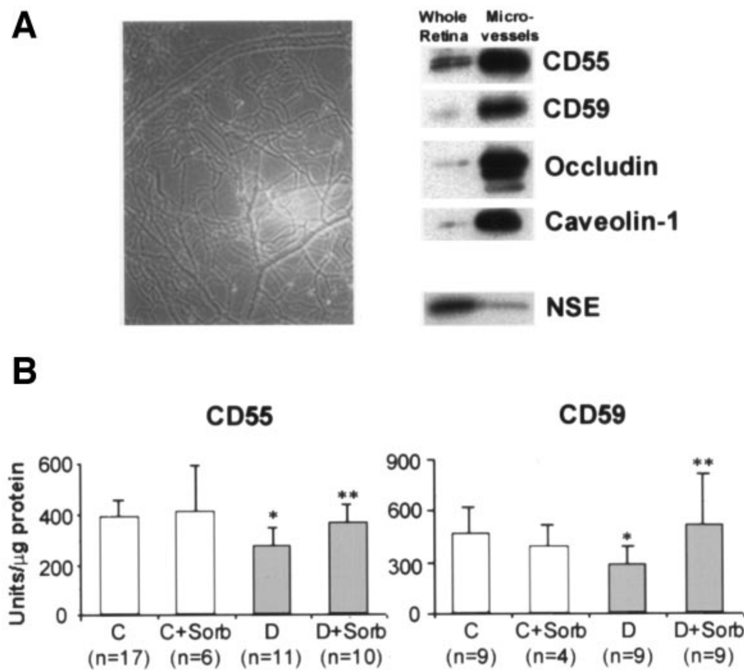

FIG. 2. Complement inhibitors in the retina of diabetic rats (2.5 months of diabetes) and diabetic rats treated with sorbinil. *A*: CD55 and CD59 enrichment in rat retinal microvessels. Microvessels (shown in phase microscopy) were isolated from fresh rat retinas. Protein lysates from microvessel and whole retinas (5 µg per lane) were subjected to SDS-PAGE. CD55 and CD59 were enriched >10-fold in the microvessels, similar to the proteins occludin and caveolin-1, which are known to have an almost exclusive vascular localization. Low levels of NSE in the microvessel preparation indicate minimal contamination by neural tissue. *B*: Quantitation of the signals from retinal CD55 and CD59 immunoblots. The bars represent mean ± SD of the values obtained in the indicated number of animals. C, control rats; C+Sorb, control rats treated with sorbinil; D, diabetic rats; D+Sorb, diabetic rats treated with sorbinil.

ment of frequency and extent of MAC staining yielded a score of 1 (range 0.5–2), which was lower than that in untreated diabetic rats (2 [1–3], $P = 0.02$), but not different from that in control rats (0.5 [0–1.5]) (Fig. 1D). As previously observed in the human retina (19), in the rat retina the two glycosylphosphatidylinositol-anchored complement inhibitors CD55 and CD59 were also mostly present in vessels. This was documented by comparing vascular versus total retinal enrichment in CD55 and CD59 (Fig. 2*A*) because we did not find antibodies that stained these proteins satisfactorily in situ in the rat retina. Diabetic rats showed decreased levels of CD55 and CD59 in comparison to controls, and sorbinil prevented the changes (Fig. 2*B*).

Late and irreversible vascular changes (apoptosis, loss of pericytes, and acellular capillaries) are prevented by sorbinil. Rats with 9 months of diabetes showed an increased number of apoptotic retinal pericytes, endothelial cells, and total capillary cells when compared with nondiabetic controls, and the increase was prevented by sorbinil (Fig. 3*A* and *B*). Apoptosis is expected to decrease the number of pericytes irreversibly because pericytes do not replicate, but it may not affect the number of endothelial cells because the loss is compensated, at least for some time, by increased replication (31). Consistent with this paradigm, there were fewer pericyte nuclei in the diabetic vessels (9 per mm capillary length [range 4–11]) compared with control vessels (12 [9–13]; $P = 0.03$), while the number of endothelial cell nuclei was similar (12 [4–14] in diabetic retinas and 13 [10–15] in control retinas). In accordance with its prevention of microvascular apoptosis, sorbinil treatment prevented the loss of pericytes (13 [11–14]; $P = 0.01$ vs. untreated diabetic rats).

Eventually, retinal capillaries become tubes of basement membrane devoid of both pericytes and endothelial cells (Fig. 4*A*). After 9 months of diabetes, acellular capillaries were already more numerous than in control rats (15 per mm^2 retina [range 7–22] vs. 6 [2–8]; $P = 0.008$), and the increase was prevented by sorbinil treatment (5 [3–13]) (Fig. 4*B*).

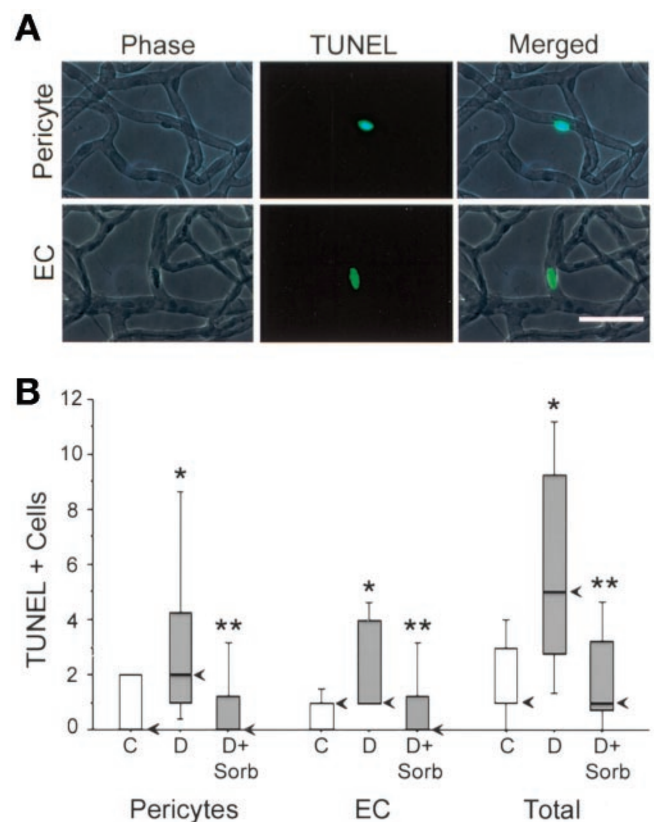

FIG. 3. Vascular cell apoptosis in retinal trypsin digests of diabetic rats (9 months of diabetes) and diabetic rats treated with sorbinil. *A*: Image of a TUNEL-positive pericyte nucleus and endothelial cell nucleus. Bar = 25 µm. *B*: Box plot of the TUNEL-positive nuclei per retina; the total includes TUNEL-positive nuclei of undetermined cellular attribution ($n = 10$ in control [C], 9 in diabetes [D], and 9 in diabetic rats treated with sorbinil [D+Sorb]).

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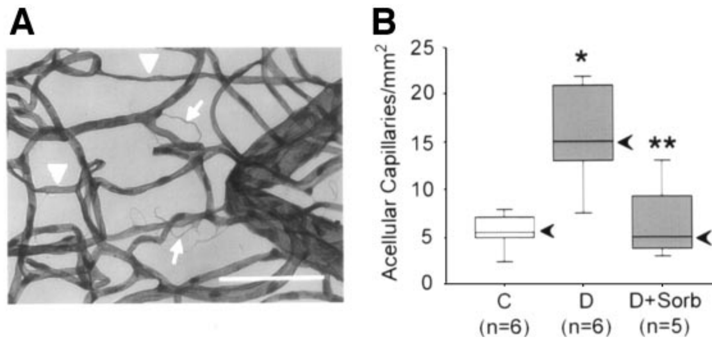

FIG. 4. Acellular capillaries in retinal trypsin digests of diabetic rats (9 months of diabetes) and diabetic rats treated with sorbinil. *A*: Morphology of acellular capillaries (arrowheads) contrasted with the string-like strands (arrows) that do not represent pathology. Bar = 100 μm. *B*: Box plot of the number of acellular capillaries per mm^2 retinal trypsin digest. C, control rats; D, diabetic rats; D+Sorb, diabetic rats treated with sorbinil.

Aldose reductase is present in rat retinal pericytes and endothelial cells. The prevention of microvascular cell apoptosis and acellular capillaries by sorbinil generated the question of whether sorbinil could act directly on vascular cells. Aldose reductase immunoreactivity has been reported in retinal pericytes and endothelial cells of the BB rat (14) but not detected in these cell types in other rat studies (13). The methods used for tissue preparation appear to affect aldose reductase immunoreactivity (18). We could not detect signal in the capillary network prepared by trypsin digestion of retinas fixed in 10% formalin for 24–96 h, but we did in capillaries isolated from fresh retinas and fixed in formalin for 15 min (Fig. 5*A*). Pericytes were positive for aldose reductase, showing rims of cytoplasmic staining around the characteristic round nuclei protruding from the capillary wall. In some areas, the capillary wall stained for both aldose reductase and PECAM-1, an endothelial cell marker (32). Since the dual staining could result from the presence of aldose reductase–positive pericyte processes enveloping PECAM-1–positive endothelial cells, we examined endothelial cells independent of pericytes in dissociated cell preparations (Fig. 5*B* and *C*). Cells positive for PECAM-1 were also positive for aldose reductase, indicating that rat retinal endothelial cells contain aldose reductase. No cells in the preparations examined stained for myeloperoxidase (stain performed in the Clinical Hematology Laboratory of the Massachusetts General Hospital), making it unlikely that PECAM-1–positive cells were granulocytes (32).

Aldose reductase is present in human retinal endothelial cells. Human retinal pericytes are known to contain aldose reductase (18), but endothelial cells have been reported not to show immunoreactivity (16,18). The retinal tissue examined in the above studies was fixed in 4% paraformaldehyde for at least 8 h (16,18). Using preparations of dissociated human retinal cells fixed briefly with acetone, we observed (Fig. 6) aldose reductase immunoreactivity in cells showing the granular perinuclear fluorescence of vWf characteristically seen in retinal endothelial cells in situ (24). Hence, both pericytes and endothelial cells of human retinal vessels contain aldose reductase.

Aldose reductase in the human retina responds to high glucose. Aldose reductase survives for many hours postmortem in several human tissues (33). To establish if aldose reductase in the human retina is enzymatically active, we measured sorbitol in retinas isolated from nondiabetic eye donors and exposed to normal or elevated glucose concentrations in organ culture for 6, 12, 24, and 48 h and compared the response with that exhibited by rat retinas. The peak sorbitol levels were observed after 24 h of incubation in high glucose, at which point the human retinas showed a greater than fourfold increase in sorbitol accumulation (Fig. 7*A*). The magnitude of the change, as well as the absolute sorbitol levels, were very similar to those observed in rat retinas cultured in parallel. Erythrocytes trapped in retinal vessels were not a major contributor to sorbitol levels. Basal sorbitol levels and the increment in response to high glucose in the human retinas were of the magnitude measured per gram Hb in human erythrocytes (30), whereas the Hb levels in the whole human retina (~0.1 μg/20 μg retinal lysate) did not

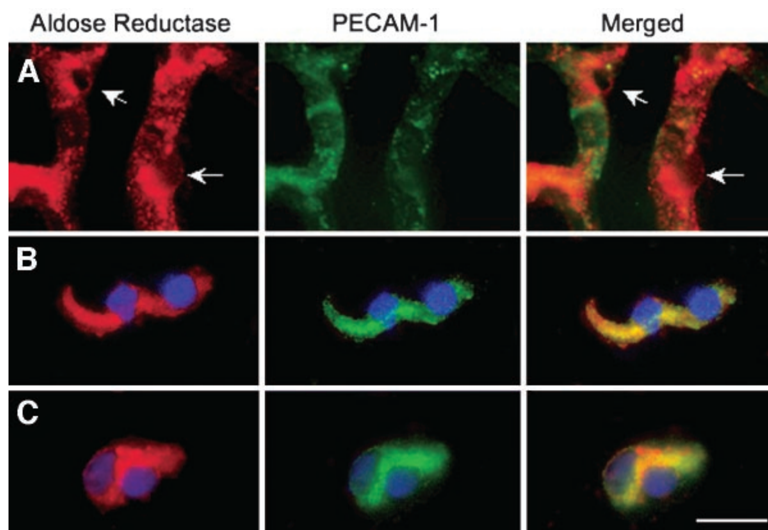

FIG. 5. Aldose reductase in retinal pericytes and endothelial cells of normal rat. *A*: In microvessels immunostained for aldose reductase (red) and PECAM-1 (green, to identify endothelial cells), aldose reductase contours the protruding nucleus of pericytes (arrows) and is present throughout the vessel, often colocalizing (yellow) with PECAM-1. *B* and *C*: In dissociated retinal cell preparations, endothelial cells observed independent of pericytes show aldose reductase immunoreactivity (D. Carper's antibodies in *B*; R. Sorenson's in *C*) overlapping with PECAM-1. Nuclei are stained with DAPI (blue). Bar = 20 μm.

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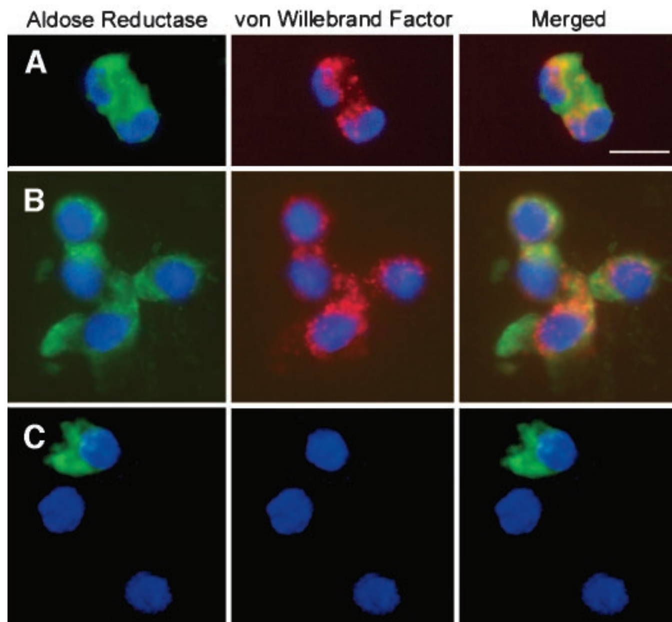

FIG. 6. Aldose reductase in human retinal endothelial cells. *A* and *B*: In dissociated cell preparations from human retinas immunostained for aldose reductase (green) and vWf (red), endothelial cells show aldose reductase immunoreactivity (D. Carper's antibodies in *A*; R. Sorenson's in *B*) overlapping the characteristic granular pattern of vWf in Weibel-Palade bodies. *C*: In the same preparations, some retinal cells stain only for aldose reductase and others for neither protein. Bar = 20 μm.

exceed 40 μg (Fig. 7*B*). Thus, cells of the human retina metabolize glucose to sorbitol when exposed to high glucose levels, and the magnitude of sorbitol accumulation is similar to that observed in the rat retina. In both human and rat retinas, there was sorbitol dehydrogenase activity, as indicated by increased levels of fructose after a 24- to 48-h incubation in high glucose (Y.S.P., unpublished observations).

DISCUSSION

This study supports the concept that, in the rat, activity of aldose reductase is a mechanism for cardinal manifestations of diabetic retinal microangiopathy common to rat and human. We document here that an aldose reductase inhibitor prevented vascular processes culminating in the development of acellular capillaries. Other investigators have shown beneficial effects of different classes of aldose reductase inhibitors on increased vascular permeability (34,35), another clinically important manifestation of diabetic retinal microangiopathy. We reported a preventative effect also on the neuronal apoptosis and glial reactivity (12) found in the diabetic rat retina and occurring in the human retina. These combined observations make the polyol pathway the only mechanism of glucose toxicity currently proven to be responsible for the spectrum of neural, glial, and vascular abnormalities detectable during the development of diabetic retinopathy.

The preventative effect of sorbinil in our studies was not only comprehensive but also noteworthy for three additional reasons. First, the degree of prevention of acellular capillaries was almost complete, while in the same diabetic rat model antioxidant therapy, as tested to date, has shown only partial (36) or absent (37) benefit. Second, prevention was afforded in the presence of severe hyperglycemia, which would be expected to activate several proposed pathways of tissue damage, including excess superoxide production (38), formation of advanced glycation end products (39), and activation of protein kinase C isoforms (40) along with the polyol pathway. Third, prevention of retinopathy occurred despite the fact that aldose reductase, like other members of the aldo-keto reductase superfamily, functions in some circumstances as a detoxification system for lipid peroxidation products (41) that are increased in the retina of diabetic rats (42). Inhibition of the enzyme could have increased toxic products and thus tissue damage, whereas the retinal abnormalities of diabetic rats addressed in our studies were corrected by sorbinil. A likely explanation is that accumulation of lipid peroxidation products in the diabetic rat retina appears to be consequent to polyol pathway activity (42). One should ask if the beneficial effects of sorbinil are solely attributable to inhibition of aldose reductase. The hydantoin class of aldose reductase inhibitors, to which sorbinil belongs, inhibits aldehyde reductase almost as much as aldose reductase (43). However, any contribution of aldehyde reductase to glucose toxicity has yet to be demonstrated and will arguably be low given that the enzyme has a K_m for glucose in the molar range (43). There is no credible evidence that sorbinil and other aldose reductase inhibitors are active in vivo as direct antioxidants (44).

Another novel finding of the present study was the detection of aldose reductase in retinal endothelial cells, both in the rat and in humans. The lack of success of previous attempts may be attributable to the different antibodies used and/or high sensitivity of endothelial (and pericyte) aldose reductase to tissue preparatory steps (fixation in particular). Perhaps the amount of enzyme is

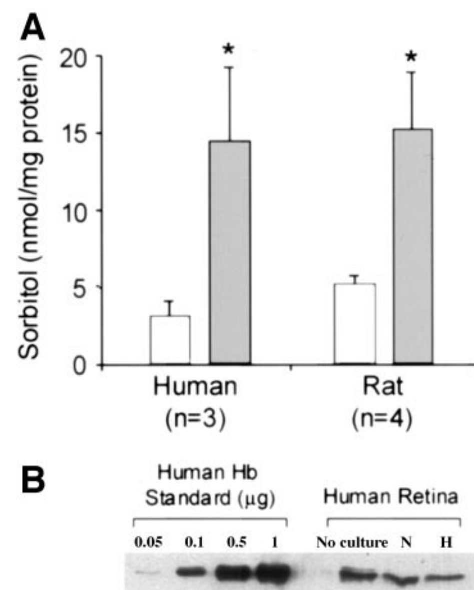

FIG. 7. Aldose reductase activity in nondiabetic human retina. *A*: Retinas were exposed for 24 h in organ culture to normal (5 mmol/l, white) or high (30 mmol/l, gray) glucose, and sorbitol levels were measured. The bars represent the means ± SD of the measurements performed in the indicated number of subjects. *B*: Hb immunoblot in human postmortem retina. Protein lysate (20 μg per lane) from fresh human retina or retina incubated in normal (N) or high (H) glucose was subjected to SDS-PAGE together with human Hb standards and probed with antibodies to Hb.

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smaller in vascular cells than in glial cells or neurons, and any disturbance of the antigenic determinants brings the protein below the threshold of detection by immunohistochemistry. Endothelial cells, which are arbiters of the most consequential lesions of retinopathy, such as capillary permeability and occlusion/obliteration, have been the most reticent among retinal cells to make known how they are affected by diabetes (45). Knowing that they are endowed with aldose reductase offers new ways of probing the effects of diabetes. We can look for a “signature” of polyol pathway activity on the endothelial cells of retinal vessels and eventually reconstruct if endothelial dysfunction and death in diabetic retinopathy is a direct consequence of polyol pathway activation in the endothelium or is attributable to other mechanisms, perhaps extrinsic to the endothelium, such as the death of pericytes or the reactivity of glial cells.

The accumulation of sorbitol by human retinas exposed to high glucose in organ culture is perhaps the first evidence that human retinal aldose reductase is activated under conditions mimicking hyperglycemia. When considering that several factors can influence sorbitol levels, and more may be operative in organ culture, it was noteworthy that the magnitude of changes induced by high glucose in the rat retina was exactly the same as that induced by diabetes (12,42) and that the human retina behaved like the rat retina.

Our data, and the mounting evidence that an allelic variant in the promoter region of the human aldose reductase gene is associated with susceptibility to diabetic retinopathy in both type 1 and type 2 diabetes and in different races (7), renew the rationale for pursuing the role of aldose reductase activation in human diabetic retinopathy. The clinical trial with sorbinil was inconclusive (11), but there was no information that the dose of drug given (20-fold lower than that used in our rat experiments, limited in humans by hypersensitivity reactions) was sufficient to affect polyol pathway activity in situ in the retina. There are precedents for clinical trials failing to prove a plausible hypothesis in diabetes and additional trials proving the hypothesis resoundingly. The University Group Diabetes Program set out to test the “glucose hypothesis” of the cardiovascular complications of diabetes but failed to prove it because the trial was flawed in multiple ways (46). Well-designed and powerful later trials such as the U.K. Prospective Diabetes Study (47) and the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (48) generated clear evidence in support of the role of hyperglycemia. Also with regard to the “polyol pathway hypothesis” of diabetic retinopathy, the definitive studies will be new trials of aldose reductase inhibitors. It is encouraging that new inhibitors are becoming available (35,42); some with novel structures free from the hydantoin nucleus found in inhibitors such as sorbinil, better tissue penetration and activity than the carboxylic acid inhibitors, and higher selectivity against aldose (versus aldehyde) reductase (49). Thus, there are both new reasons and new probes that encourage testing if the polyol pathway is, after all, a “dream” target in the prevention of diabetic retinopathy and other complications.

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REFERENCES

1. Lorenzi M, Gerhardinger C: Early cellular and molecular changes induced by diabetes in the retina. *Diabetologia* 44:791–804, 2001
2. Kohner EM: Diabetic retinopathy. *Br Med Bull* 45:148–173, 1989
3. Gabbay KH: The sorbitol pathway and the complications of diabetes. *N Engl J Med* 288:831–836, 1973
4. Oates PJ: The polyol pathway and diabetic peripheral neuropathy. In *International Review of Neurobiology*: *The Neurobiology of Diabetic Neuropathy*. Vol. 50, Tomlinson DR, Ed. London, Academic Press, 2002, p. 325–392
5. Obrosova IG, Fathallah L, Lang HJ: Interaction between osmotic and oxidative stress in diabetic precataractous lens: studies with a sorbitol dehydrogenase inhibitor. *Biochem Pharmacol* 58:1945–1954, 1999
6. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, Van Den Enden M, Kilo C, Tilton RG: Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42:801–813, 1993
7. Demaine AG, Cross DF: Genetics of aldose reductase and susceptibility to diabetic retinopathy. *Appl Genomics Proteomics* 1:1–13, 2002
8. Ho HTB, Chung SK, Law JWS, Ko BCB, Tam SCF, Brooks HL, Knepper MA, Chung SSM: Aldose reductase-deficient mice develop nephrogenic diabetes insipidus. *Mol Cell Biol* 20:5840–5846, 2000
9. Kador PF, Akagi Y, Takahashi Y, Ikebe H, Wyman M, Kinoshita JH: Prevention of retinal vessel changes associated with diabetic retinopathy in galactose-fed dogs by aldose reductase inhibitors. *Arch Ophthalmol* 108:1301–1309, 1990
10. Engerman RL, Kern TS: Aldose reductase inhibition fails to prevent retinopathy in diabetic and galactosemic dogs. *Diabetes* 42:820–825, 1993
11. Sorbinil Retinopathy Trial Research Group: A randomized trial of sorbinil, an aldose reductase inhibitor, in diabetic retinopathy. *Arch Ophthalmol* 108:1234–1244, 1990
12. Asnaghi V, Gerhardinger C, Hoehn T, Adeboje A, Lorenzi M: A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. *Diabetes* 52:506–511, 2003
13. Ludvigson MA, Sorenson RI: Immunohistochemical localization of aldose reductase. II. Rat eye and kidney. *Diabetes* 29:450–459, 1980
14. Chakrabarti S, Sima AAF, Nakajima T, Yagihashi S, Greene DA: Aldose reductase in the BB rat: isolation, immunological identification and localization in the retina and peripheral nerve. *Diabetologia* 30:244–251, 1987
15. Kern TS, Engerman RL: Distribution of aldose reductase in ocular tissues. *Exp Eye Res* 33:175–182, 1981
16. Akagi Y, Yajima Y, Kador PF, Kuwabara T, Kinoshita JH: Localization of aldose reductase in the human eye. *Diabetes* 33:562–566, 1984
17. Dyer MA, Cepko CL: Control of Müller glial cell proliferation and activation following retinal injury. *Nat Neurosci* 3:873–880, 2000
18. Akagi Y, Kador PF, Kuwabara T, Kinoshita JH: Aldose reductase localization in human retinal mural cells. *Invest Ophthalmol Vis Sci* 24:1516–1519, 1983
19. Zhang J, Gerhardinger C, Lorenzi M: Early complement activation and decreased levels of glycosylphosphatidylinositol-anchored complement inhibitors in human and experimental diabetic retinopathy. *Diabetes* 51:3499–3504, 2002
20. Mizutani M, Kern TS, Lorenzi M: Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest* 97:2883–2890, 1996
21. Cogan DG, Toussaint D, Kuwabara T: Retinal vascular patterns. IV. Diabetic retinopathy. *Arch Ophthalmol* 66:366–378, 1961

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22. Kohner EM, Henkind P: Correlation of fluorescein angiogram and retinal digest in diabetic retinopathy. *Am J Ophthalmol* 69:403–414, 1970
23. Kern TS, Engerman RL: Comparison of retinal lesions in alloxan-diabetic rats and galactose-fed rats. *Curr Eye Res* 13:863–867, 1994
24. Boeri B, Cagliero E, Podestá F, Lorenzi M: Vascular wall von Willebrand factor in human diabetic retinopathy. *Invest Ophthalmol Vis Sci* 35:600–607, 1994
25. Podestá F, Romeo G, Liu W-H, Krajewski S, Reed JC, Gerhardinger C, Lorenzi M: Bax is increased in the retina of diabetic subjects and is associated with pericyte apoptosis in vivo and in vitro. *Am J Pathol* 156:1025–1032, 2000
26. Su X, Sorenson CM, Sheibani N: Isolation and characterization of murine retinal endothelial cells. *Molec Vision* 9:171–178, 2003
27. Gerhardinger C, McClure KD, Romeo G, Podestá F, Lorenzi M: IGF-I mRNA and signaling in the diabetic retina. *Diabetes* 50:175–183, 2001
28. Spiller OB, Hanna SM, Morgan BP: Tissue distribution of the rat analogue of decay-accelerating factor. *Immunology* 97:374–384, 1999
29. Gupta S, Chough E, Daley J, Oates P, Tornheim K, Ruderman NB, Keaney JF Jr: Hyperglycemia increases endothelial superoxide that impairs smooth muscle cell Na^+ -K^+ -ATPase activity. *Am J Physiol Cell Physiol* 282:C560–C566, 2002
30. Malone JI, Knox G, Benford S, Tedesco TA: Red cell sorbitol: an indicator of diabetic control. *Diabetes* 29:861–864, 1980
31. Sharma NK, Gardiner TA, Archer DB: A morphologic and autoradiographic study of cell death and regeneration in the retinal microvasculature of normal and diabetic rats. *Am J Ophthalmol* 100:51–60, 1985
32. Newman PJ: The biology of PECAM-1. *J Clin Invest* 99:3–8, 1997
33. Das B, Srivastava SK: Activation of aldose reductase from human tissues. *Diabetes* 34:1145–1151, 1985
34. Tilton RG, Chang K, Pugliese G, Eades DM, Province MA, Sherman WR, Kilo C, Williamson JR: Prevention of hemodynamic and vascular albumin filtration changes in diabetic rats by aldose reductase inhibitors. *Diabetes* 37:1258–1270, 1989
35. Amano S, Yamagishi S, Kato N, Inagaki Y, Okamoto T, Makino M, Taniko K, Hirooka H, Jomori T, Takeuchi M: Sorbitol dehydrogenase overexpression potentiates glucose toxicity to cultured retinal pericytes. *Biochem Biophys Res Commun* 299:183–188, 2002
36. Kowluru RA, Tang J, Kern TS: Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. *Diabetes* 50:1938–1942, 2001
37. Hammes HP, Bartmann A, Engel L, Wülfroth P: Antioxidant treatment of experimental diabetic retinopathy in rats with nicanartine. *Diabetologia* 40:629–634, 1997
38. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes H-P, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
39. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 48:1–9, 1999
40. Koya D, King GL: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866, 1998
41. Rittner HL, Hafner V, Klimiuk PA, Szweda LI, Goronzy JJ, Weyand CM: Aldose reductase functions as a detoxification system for lipid peroxidation products in vasculitis. *J Clin Invest* 103:1007–1013, 1999
42. Obrosova IG, Minchenko AG, Vasupuram R, White L, Abatan OI, Kumagai AK, Frank RN, Stevens MJ: Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor overexpression in streptozotocin-diabetic rats. *Diabetes* 52:864–871, 2003
43. Sato S, Kador PF: Human kidney aldose and aldehyde reductases. *J Diabetes Complications* 7:179–187, 1993
44. Ramasamy R, Oates PJ: Aldose reductase and vascular stress. In *Diabetes and Cardiovascular Disease.* Marso SP, Stern DM, Eds. Philadelphia, Lippincott, Williams & Wilkins, 2004, p. 55–74
45. Romeo G, Liu W-H, Asnaghi V, Kern TS, Lorenzi M: Activation of nuclear factor-κB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. *Diabetes* 51:2241–2248, 2002
46. Genuth S: Exogenous insulin administration and cardiovascular risk in non-insulin-dependent and insulin-dependent diabetes mellitus. *Ann Intern Med* 124:104–109, 1996
47. Stratton IM, Adler AI, Neil HAW, Matthews DR, Manley SE, Cull CA, Hadden D, Turner RC, Holman RR, UK Prospective Diabetes Study Group: Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *Br Med J* 321:405–412, 2000
48. The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group: Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus *N Engl J Med* 348:2294–2303, 2000
49. Mylari BL, Armento SJ, Beebe DA, Conn EL, Coutcher JB, Dina MS, O'Gorman MT, Linhares MC, Martin WH, Oates PJ, Tess DA, Withbroe GJ, Zembrowski WJ: A highly selective, non-hydantoin, non-carboxylic acid inhibitor of aldose reductase with potent oral activity in diabetic rat models: 6-(5-chloro-3-methylbenzofuran-2-sulfonyl)-2-*H*-pyridazin-3-one. *J Med Chem* 46:2283–2286, 2003

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