Original Article

A Selective Aldose Reductase Inhibitor of a New Structural Class Prevents or Reverses Early Retinal Abnormalities in Experimental Diabetic Retinopathy

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Previously studied inhibitors of aldose reductase were largely from two chemical classes, spirosuccinamide/hydantoins and carboxylic acids. Each class has its own drawbacks regarding selectivity, in vivo potency, and human safety; as a result, the pathogenic role of aldose reductase in diabetic retinopathy remains controversial. ARI-809 is a recently discovered aldose reductase inhibitor (ARI) of a new structural class, pyridazinones, and has high selectivity for aldose versus aldehyde reductase. To further test the possible pathogenic role of aldose reductase in the development of diabetic retinopathy, we examined the retinal effects of this structurally novel and highly selective ARI in insulinized streptozotocin-induced diabetic rats. ARI-809 treatment was initiated 1 month after diabetes induction and continued for 3 months at a dose that inhibited the polyol pathway in the retina of diabetic rats to a similar extent as sorbinil, a poorly selective hydantoin ARI previously shown to prevent retinopathy in this model. ARI-809 improved survival, inhibited cataract development, normalized retinal sorbitol and fructose, and protected the retina from abnormalities that also occur in human diabetes: neuronal apoptosis, glial reactivity, and complement deposition. Because ARI-809 is a novel chemotype highly selective for aldose reductase, these results support the notion that aldose reductase is the key relay that converts hyperglycemia into glucose toxicity in neural and glial cell types in the retina. *Diabetes* 55:2757–2762, 2006

We recently defined the polyol pathway as both a "dream" and a "dread" target in the quest for strategies to prevent diabetes complications, retinopathy in particular (1). New findings in experimental animal models have since reinforced its credentials as a dream target. For example, genetic deletion of aldose reductase, the first and rate-limiting enzyme in the pathway, prevents all early effects of diabetes on neural, glial, and vascular cells of the retina (2); an aldose reductase inhibitor (ARI) prevents a spectrum of retinal abnormalities more comprehensively than other types of drugs (3); and aldose reductase contributes to myocardial ischemic injury (4) and diabetic atherosclerosis (5).

On the other hand, the polyol pathway remains a dread target (1) because ARIs have yielded at best only minor benefits in clinical studies (rev. in 6), and this could indicate that the polyol pathway is not a major pathogenic pathway in human diabetes. Alternatively, the past failures may simply reflect insufficient inhibition of the pathway in target tissues by the doses of drugs used in humans (6). Of the two chemical classes of ARIs tested to date in phase III trials, the carboxylic acid inhibitors (e.g., zopolrestat) penetrate tissues poorly and are not very potent in vivo, whereas the spiroimide (spirohydantoin) inhibitors penetrate tissues more efficiently, but many have caused skin reactions or liver toxicity that limited the doses usable in humans to doses that were subtherapeutic in animal models (7,8). For example, the dose of sorbinil that was used in the essentially negative Sorbinil Retinopathy Trial (8) is ~20-fold lower than the dose shown to prevent retinal polyol pathway activation and the development of retinopathy in diabetic rats (1,9). An aspect of the toleration problem with drugs such as sorbinil may be the relatively poor selectivity for aldose reductase versus aldehyde reductase, a closely related enzyme that plays a role in the detoxification of reactive aldehydes (10,11).

Therefore, testing the hypothesis that the polyol pathway contributes to the complications of human diabetes will require new ARIs combining higher levels of efficacy, selectivity, and safety in humans. Currently, there are new ARIs of the spiroimide/hydantoin class that show greater potency than sorbinil and have achieved in diabetic patients a robust inhibition of the polyol pathway in sural nerves (12), as well as improvement in signs and symptoms of sensorimotor polyneuropathy at well-tolerated doses (13,14). Recently, an ARI from an entirely new structural class was described (Fig. 1) (7) and reported to prevent elevated urinary albumin excretion in diabetic rats (15,16). CP-744809, or compound 19m (7) (henceforth referred to as ARI-809), is a sulfonylpyridazone that in initial characterization studies was profiled as one of the most potent and selective ARIs yet described (7). ARI-809 is a highly selective (1:930) (7) inhibitor of aldose reductase relative to aldehyde reductase, and such selectivity distinguishes it from sorbinil, which inhibits aldose and aldehyde reductase to a comparable extent (11). Sorbinil

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ARI, aldose reductase inhibitor; GFAP, glial fibrillary acidic protein; ICAM-1, intercellular adhesion molecule-1; MAC, membrane attack complex; TUNEL, transferase-mediated dUTP nick-end labeling.

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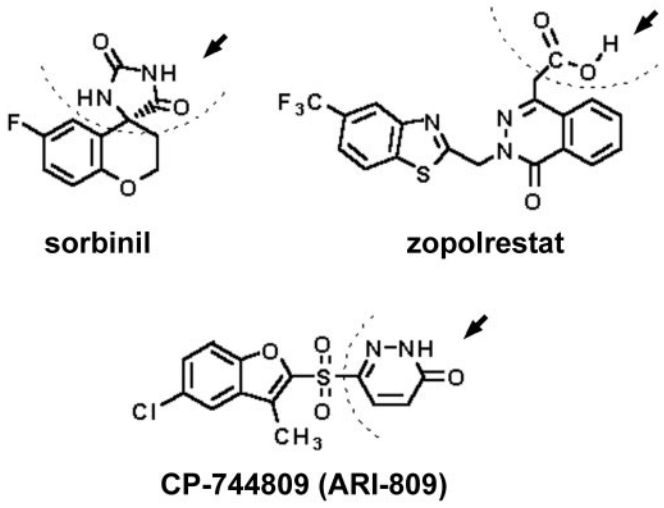

FIG. 1. Structure of ARI-809 compared with other classes of ARIs. Sorbinil is a representative of the spirohydantoin (broadly referred to as spiroimide) ARI class. The eponymous spirohydantoin substituent is the portion of the sorbinil molecule above the dotted line (arrow). Zopolrestat is a representative of the carboxylic acid ARI class. The carboxylic acid moiety is indicated by the atoms above the dotted line (arrow). ARI-809 contains neither a spirohydantoin nor a carboxylic acid substituent but is characterized by a pyridazinone group, denoted by those atoms to the right of the dotted line (arrow).

was previously shown to prevent the development of experimental diabetic retinopathy (1,3,9). The structural uniqueness and high selectivity of ARI-809 now presented an important opportunity to critically target the role of the polyol pathway in the early stages of the development of experimental diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Procedures involving animals were approved by the animal care and use committee of the Schepens Eye Research Institute or of Pfizer Global Research and Development, as appropriate. All rats had free access to food and water. Pilot experiments were performed to identify the dose of ARI-809 to be used for chronic inhibition of sorbitol and fructose accumulation in the retina of diabetic rats. Male Sprague-Dawley rats (175–225 g) made diabetic by tail vein injection of streptozotocin (85 mg/kg body wt) (7), were treated with ARI-809 (Pfizer) at doses of 10, 40, 65, and 100 mg · kg^{-1} · day^{-1} mixed with powdered diet. A group of diabetic rats was treated with positive control sorbinil (Pfizer) at 65 mg · kg^{-1} · day^{-1} (1,9) to obtain comparative biochemical data; treatments were started on day 8 after streptozotocin injection and continued until day 12. Diabetic and control rats were killed by cervical dislocation, and the two retinas from each rat were removed and placed in 1 ml of ice-cold 6% (wt/vol) perchloric acid and frozen for later analysis. On the day of analysis, samples were thawed, homogenized, and neutralized with potassium carbonate. Aliquots of supernatants were used to determine sorbitol and fructose concentrations by fluorometric enzyme assays (17,18).

To investigate the effects of ARI-809 on retinal abnormalities induced by diabetes, male Sprague-Dawley rats were randomly assigned to one of the following groups: control, diabetic, and diabetic treated with ARI-809 at 50 mg · kg^{-1} · day^{-1}. The dose of streptozotocin used to induce diabetes for these longer-term experiments was 57.5 mg/kg body wt (1). Body weight was recorded three times per week in the diabetic rats, and 2–4 units of NPH insulin were administered subcutaneously as needed to prevent weight loss without preventing hyperglycemia. Treatment with ARI-809 was initiated 1 month after induction of diabetes and continued for 3 months. At death, blood was obtained by cardiac puncture for the assay of HbA_{1c} (A1C; Helena GLYCO-Tek affinity column method; Helena Laboratories, Beaumont, TX) and serum ARI-809 levels (measured by Carolyn Soglia at Pfizer Global Research and Development, Groton, CT, using high-pressure liquid chromatography–tandem mass spectrometry). One retina from three rats in each group was immediately placed in 0.5 ml ice-cold 6% perchloric acid for measurement of sorbitol and fructose content. After homogenization and centrifugation, the precipitate was used to determine protein concentration, using a BioRad DC protein-assay reagent (1). The sorbitol and fructose levels measured in the supernatant (17,18) are expressed as nanomoles per milligram retinal protein.

Apoptosis. Whole-mounted retinas prepared and processed as previously described (9) were tested with a transferase-mediated dUTP nick-end labeling (TUNEL) reaction (in situ cell death detection kit; Roche, Mannheim, Germany) to detect apoptosis. The retinas were mounted vitread side up, and observation at different planes of focus permitted localization of the TUNEL-positive nuclei to the planes bracketed by retinal capillaries. Nuclei were classified as apoptotic when showing TUNEL staining and chromatin condensation. The observers were blinded to the identity of the specimens.

Immunohistochemistry. Retinal cryosections (6 μm) were fixed in buffered formalin or ice-cold acetone for 10 min. Immunohistochemistry was performed as previously described (9). The primary antibodies were rabbit anti-cow glial fibrillary acidic protein (GFAP; 1:4,000; Dako, Carpinteria, CA), goat anti-rat intercellular adhesion molecule-1 (ICAM-1; 1:100; R&D Systems, Minneapolis, MN), mouse monoclonal antibody 2A1 anti-rat C5b-9 to detect the membrane attack complex (MAC) of complement (1:50; provided by W.G. Couser), and rabbit anti-human von Willebrand factor (1:500; Dako). Negative controls were obtained by substituting the primary antibodies with an equivalent concentration of nonimmune IgG of the appropriate species.

Immunoblotting. Proteins were isolated from retinas lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (1). Measurement of protein concentration, SDS-PAGE, and immunoblotting was performed as previously described (1). GFAP and ICAM-1 were detected with the antibodies described above; the blots were subsequently reacted with mouse monoclonal antibody AC-15 anti–β-actin (Sigma, St. Louis, MO) to verify even loading.

Statistical analysis. Data are the means ± SD or the median and range for the counts of TUNEL-positive nuclei (noncontinuous variable). The multiple treatment groups were compared with *1*) ANOVA followed by Fisher's projected least significant difference test, *2*) the nonparametric Kruskal-Wallis test followed by multiple comparisons with the Mann-Whitney *U* test for the TUNEL data, and *3*) Fisher's exact test for the survival and cataract data.

RESULTS

Dose of ARI-809 that inhibits polyol pathway activity in the retina of diabetic rats. Polyol pathway inhibition is reflected by the reduction of the elevated tissue pools of both sorbitol and fructose (7). We first sought to identify a dose of ARI-809 that matched, on retinal fructose accumulation, the effects of sorbinil at 65 mg · kg^{-1} · day^{-1}, which is a dose that prevented all neural, glial, and vascular abnormalities evaluated in the retina of diabetic rats (1,9). We found that sorbinil at 65 mg · kg^{-1} · day^{-1} overnormal-

TABLE 1
Characteristics and retinal polyol pathway activity of rats with 4 months of diabetes receiving or not receiving ARI-809 (50 mg · kg^{-1} · day^{-1}) for the final 3 months

	n	Serum ARI-809 level (μg/ml)	Survival (% of rats)*	Cataract (% of eyes)*	Body weight (g)	A1C (%)	Retinal sorbitol (nmol/mg protein)†	Retinal fructose (nmol/mg protein)†
Control	9	NA	100	0	623 ± 63	5.4 ± 1.4	0.697 ± 0.214	1.807 ± 0.696
Diabetes	8	NA	77‡	95‡	319 ± 30‡	13.6 ± 1.1‡	7.729 ± 4.798‡	31.215 ± 6.759‡
Diabetes with ARI-809	9	226 ± 34	90	0	334 ± 41‡	13.3 ± 1.2‡	0.273 ± 0.050	4.507 ± 0.636

Data are means ± SD. *Computed based on a total of 22 control rats, 26 diabetic rats, and 10 diabetic rats treated with ARI-809. The rats in excess of those included in this study were killed at a later date as part of an unrelated experiment. The prevalence of cataracts was computed in surviving rats. †Measurements were performed in three rats from each group; ‡$P < 0.02$ compared with control rats. NA, not applicable.

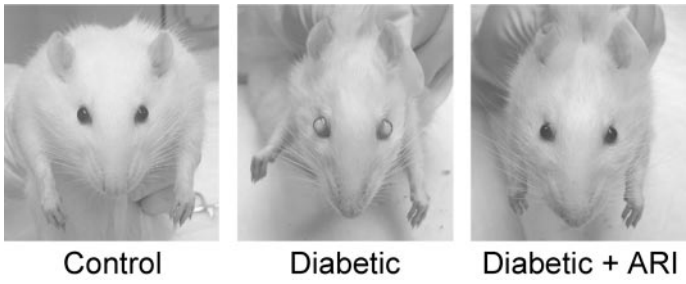

FIG. 2. Effect of ARI-809 on cataracts in rats with 4-months' duration of diabetes. Drug treatment was initiated after 1 month of diabetes and continued for 3 months. No cataracts were detected in treated diabetic rats.

ized retinal sorbitol and reduced fructose accumulation in the retina of diabetic rats by 91%. Results from the multiple doses of ARI-809 tested in the same experiments showed that equivalent retinal polyol reduction was achieved by ~49 mg · kg^{-1} · day^{-1} (data not shown). We therefore used ARI-809 at 50 mg · kg^{-1} · day^{-1} in the experiments targeting retinal abnormalities.

Gross observations, drug serum levels, and retinal polyol pathway activity in ARI-809–treated diabetic rats. Survival was lower in the diabetic than in control rats, but it was no longer different from control in the diabetic rats treated with ARI-809 (Table 1). The development of cataract was powerfully inhibited by ARI-809, even though administration of the drug was initiated after 1 full month of hyperglycemia (Table 1 and Fig. 2). We did not detect signs or symptoms of untoward drug effects in the daily inspections of the rats, nor did we find an effect of ARI-809 on the lower body weight and increased A1C levels characteristic of diabetic rats. Serum levels of ARI-809 were highly consistent among the treated diabetic rats. The relationship between dose and serum levels in our experiments was identical to that observed for a smaller single dose of the drug given by oral gavage (7), indicating linearity of drug dose versus blood levels, despite different experimental conditions. ARI-809 inhibited polyol pathway activity in the retina of diabetic rats to the extent expected from the pilot data, resulting in overnormalization of sorbitol and normalization of fructose levels (Table 1).

ARI-809 prevents or reverses retinal neuroglial abnormalities induced by diabetes. The inner retina of diabetic rats showed multiple nuclei with apoptotic features (TUNEL reaction and chromatin condensation) that by location and size are attributable to ganglion cells (Fig. 3*A*). Treatment with ARI-809 prevented the excess apoptosis caused by diabetes (Fig. 3*B*). Müller glial cells were activated in the diabetic rats and expressed GFAP along the full length of their processes throughout the thickness of the retina. Treatment with ARI-809 returned GFAP to its normal topography of expression in astrocytes and in Müller cell endfeet in the innermost retina (Fig. 4*A*), and it returned GFAP to normal levels (Fig. 4*B* and *C*). Another early abnormality in the retina of diabetic rats is increased expression of ICAM-1, localized mostly at the inner limiting membrane at structures also staining for GFAP. Treatment with ARI-809 normalized ICAM-1 levels (Fig. 5). Finally, an early event in the diabetic retina that also involves the vessels is the deposition of MAC, indicative of complement activation. Absent from the retina of control rats, MAC staining was present in the retina of diabetic rats both in patches along the inner limiting membrane and in the vessel walls, often colocalized with the endothelial marker von Willebrand factor (Fig. 6). MAC staining was virtually undetectable in the retina of diabetic rats treated with ARI-809.

DISCUSSION

We report that a highly selective ARI with novel structure, given at a dose that inhibited retinal polyol accumulation in diabetes, was well tolerated in diabetic rats and prevented or reversed multiple early effects of diabetes on neural, glial, and vascular cells of the retina. These results confirm and extend our previous findings with sorbinil (1,9), an ARI of a different structural class and a less selective inhibitor of aldose reductase. The data are also consistent with recent evidence that deletion of the gene for aldose reductase prevents retinal abnormalities triggered by diabetes in *db/db* mice (2), and they are supportive of the genetic data linking aldose reductase alleles associated with elevated expression of the protein to higher risk for development of diabetic retinopathy in humans (19). Although each of these different types of data has potential limitations when taken individually

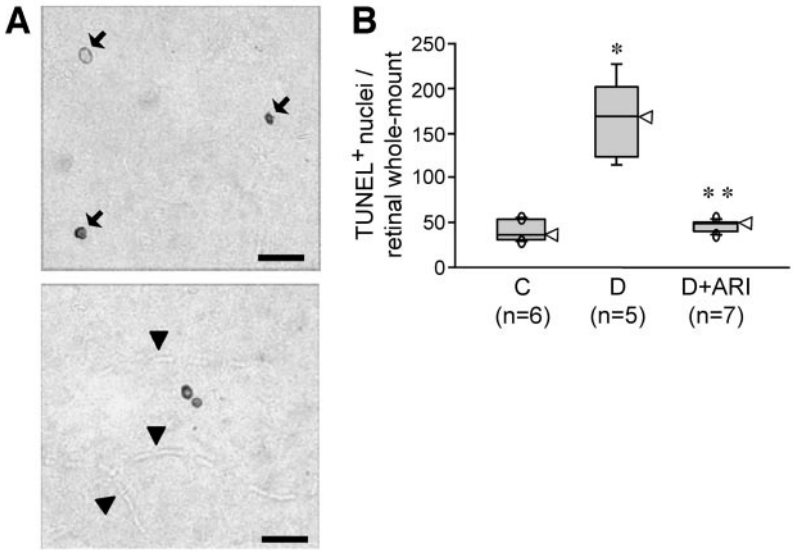

FIG. 3. Effect of ARI-809 on neuronal apoptosis in the retina of diabetic rats. *A*: In the whole-mounted retina of a diabetic rat, TUNEL-positive nuclei are detected at various stages of chromatin condensation (arrows) and are located in the inner retina but are topographically independent of blood vessels (arrowheads). Bar = 20 μm. *B*: Counts of TUNEL-positive nonvascular cell nuclei in retinal whole mounts. In boxplots, the bars encompass from the 90th to the 10th percentile of the scores, and the boxes encompass from the 75th to the 25th percentile; arrowheads point to the median. **P* = 0.006 vs. control rats; ***P* = 0.004 vs. untreated diabetic rats. The number of TUNEL-positive nuclei was no longer increased in treated diabetic rats. C, control rats; D, diabetic rats; D+ARI, diabetic rats treated with ARI-809; n, number of rats tested.

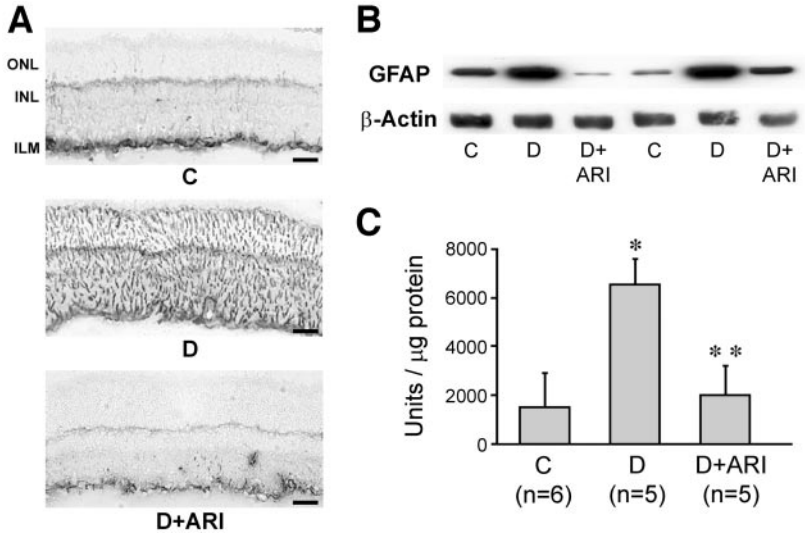

FIG. 4. Effect of ARI-809 on GFAP upregulation in the retina of diabetic rats. *A*: GFAP immunostaining in retinal sections shows in a diabetic rat the presence of GFAP throughout the length of the Müller cell process that span the retina but only in the glia of the inner retina in a diabetic rat treated with ARI-809. Bar = 50 μm. *B*: Representative immunoblot of retinal GFAP and β-actin. *C*: Quantitation of the signals from immunoblots of retinal GFAP. Bars represent the means ± SD. GFAP expression was completely corrected in the treated diabetic rats. **P* = 0.0001 vs. control rats; ***P* = 0.0001 vs. untreated diabetic rats. C, control rats; D, diabetic rats; D+ARI, diabetic rats treated with ARI-809; ILM, inner limiting membrane; INL, inner nuclear layer; ONL, outer nuclear layer.

(e.g., unsuspected off-target effects in the drug studies, unknown but functionally meaningful consequences of gene deletion, and incomplete definition of the functional effects of gene polymorphisms), when taken together they make a compelling case that aldose reductase plays a critical role in the development of diabetic retinopathy.

It appears from our current observations that processes that are eventually irreversible (e.g., cataract formation and apoptosis of retinal neurons) are either not yet initiated or are still at reversible stages after 1 month of sustained hyperglycemia in the rat, and that intervening with effective polyol pathway inhibition can abort these processes. This early tolerance of the metabolic insult is consistent with clinical data. The Diabetes Control and Complications Trial showed that diabetic retinopathy can be largely prevented when near normoglycemia is restored within a few years of diabetes but much less effectively thereafter (20). It is conceivable that the transition to irreversibility and autonomous progression of the process is a function of the response mounted by the tissue (e.g., apoptosis, remodeling).

The aldose reductase present in neural, glial, and vascular cells of the retina (1,9) can be viewed as a relay that converts hyperglycemia into intracellular glucose toxicity. This concept applies now firmly to the rat retina, where *1*) diabetes activates the polyol pathway (1,9,21,22), *2*) activation of the pathway accounts for oxidative and nitrosative stress (21,22), and *3*) multiple manifestations of tissue damage are prevented by structurally distinct ARIs (1,9,23,24), including one highly selective for aldose versus aldehyde reductase (this work). Of note, aldose reductase activation was found to also be the mechanism for the oxidative and nitrosative stress imposed by high glucose on retinal endothelial cells (from bovine retina) (25). Aldose reductase may also be an important relay to glucose toxicity in the human retina, which *1*) shows the

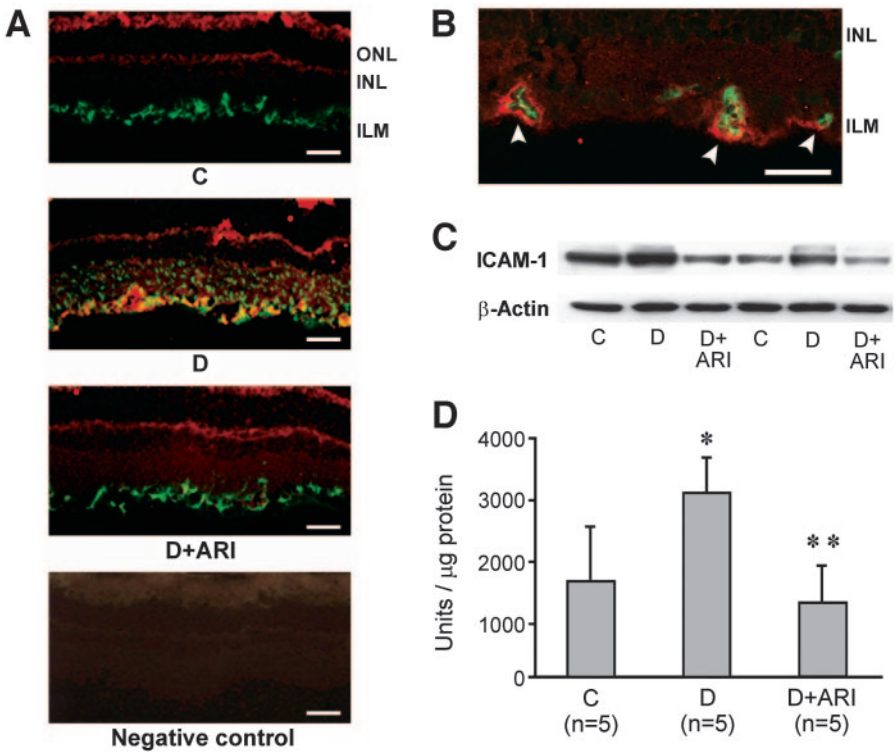

FIG. 5. Effect of ARI-809 on ICAM-1 upregulation in the retina of diabetic rats. *A*: Immunostaining for ICAM-1 (red) and GFAP (green) in retinal sections shows in a control rat GFAP staining only. In a diabetic rat, ICAM-1 is visible along the inner limiting membrane and in the wall of a large vessel; ICAM-1 staining is absent in a diabetic rat treated with ARI-809. Bar = 50 μm. *B*: Immunostaining for ICAM-1 (red) and von Willebrand factor (green, to identify vascular endothelial cells) in the retinal section of a diabetic rat shows that ICAM-1 is peripheral to the endothelium. Bar = 50 μm. *C*: Representative immunoblot of retinal ICAM-1 and β-actin. *D*: Quantitation of the signals from immunoblots of retinal ICAM-1. Bars represent the means ± SD. **P* = 0.004 vs. control rats; ***P* = 0.0005 vs. untreated diabetic rats. ICAM-1 expression was completely corrected in the treated diabetic rats. C, control rats; D, diabetic rats; D+ARI, diabetic rats treated with ARI-809; ILM, inner limiting membrane; INL, inner nuclear layer; ONL, outer nuclear layer.

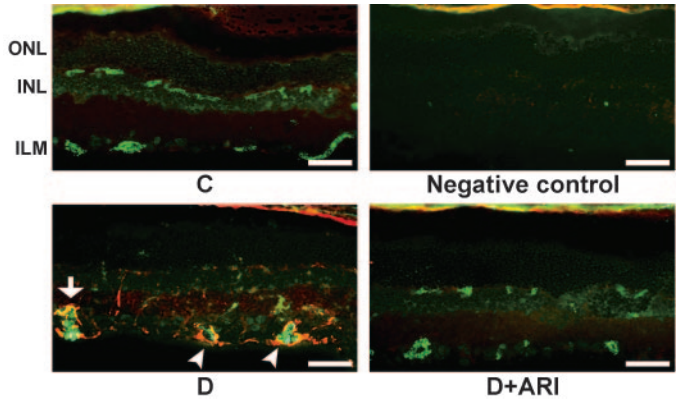

FIG. 6. Effect of ARI-809 on MAC deposition in the retina of diabetic rats. Retinal sections were immunostained for C5b-9 (MAC, red) and von Willebrand factor (green). In the retina of a diabetic rat, MAC is present along the inner limiting membrane and in the wall of vessels, at times peripheral to the endothelium (arrowheads) and at times colocalized with von Willebrand factor (yellow color, arrow). MAC is not detected in the retina of a diabetic rat treated with ARI-809. Bar = 50 μm. C, control rats; D, diabetic rats; D+ARI, diabetic rats treated with ARI-809; ILM, inner limiting membrane; INL, inner nuclear layer; ONL, outer nuclear layer.

presence of aldose reductase in neural, glial, and vascular cells (1,26,27); *2*) activates the polyol pathway in response to high glucose levels in vitro (1); and *3*) manifests in diabetes the very same abnormalities—neuronal apoptosis (28), glial reactivity (29), and complement activation (30)—that in the retina of diabetic rats are prevented by ARIs. However, the causal link between polyol pathway activation and tissue pathology has not yet been demonstrated in humans, and only potent and safe ARIs will permit us to eventually affirm or reject the link all the way to microangiopathy.

A question still to be addressed in preclinical studies is whether ARI-809 is effective in preventing longer-term retinal pathology, including the acellular capillaries, the ultimate index of vascular failure leading in humans to proliferative retinopathy. The prevention of pericyte loss (1,24) and acellular capillaries (1) exerted by sorbinil, as well as fidarestat, in rats with 9–15 months of diabetes is encouraging, and the safety of ARI-809 during this 4-month study makes longer-term administration possible.

ARIs are only one of several types of drugs that have shown prevention of late, irreversible vascular damage in experimental diabetic retinopathy (3,31–33), and they are candidate adjunct treatments for retinopathy and other complications of diabetes in humans. It is valuable and desirable that more than one candidate drug be developed because prevention of the complications of diabetes will require drugs that can be used effectively and safely over many years in the context of the unique requirements and sensitivities of individual patients.

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