SPANDIDOS PUBLICATIONS

Evaluation of the aldose reductase inhibitor fidarestat on ischemia-reperfusion injury in rat retina

IRINA G. OBROSOVA[1], YURY MAKSIMCHYK[1], PAL PACHER[2], ELISABET AGARDH[4], MAJ-LIS SMITH[4], AZZA B. EL-REMESSY[3] and CARL-DAVID AGARDH[4]

[1]Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA; [2]Section on Oxidative Stress Tissue Injury, Laboratory of Physiological Studies, NIH/NIAAA, Bethesda, MD; [3]Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA, USA; [4]The Department of Clinical Sciences, University Hospital MAS, Lund University, Malmö, Sweden

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Abstract. This study evaluated the effects of retinal ischemia-reperfusion (IR) injury and pre-treatment with the potent and specific aldose reductase inhibitor fidarestat on apoptosis, aldose reductase and sorbitol dehydrogenase expression, sorbitol pathway intermediate concentrations, and oxidative-nitrosative stress. Female Wistar rats were pre-treated with either vehicle (N-methyl-D-glucamine) or fidarestat, 32 $mgkg^{-1}d^{-1}$ for both, in the right jugular vein, for 3 consecutive days. A group of vehicle- and fidarestat-treated rats were subjected to 45-min retinal ischemia followed by 24-h reperfusion. Ischemia was induced 30 min after the last vehicle or fidarestat administration. Retinal IR resulted in a remarkable increase in retinal cell death. The number of TUNEL-positive nuclei increased 48-fold in the IR group compared with non-ischemic controls ($p<0.01$), and this increase was partially prevented by fidarestat. AR expression (Western blot analysis) increased by 19% in the IR group ($p<0.05$), and this increase was prevented by fidarestat. Sorbitol dehydrogenase and nitrated protein expressions were similar among all experimental groups. Retinal sorbitol concentrations tended to increase in the IR group but the difference with non-ischemic controls did not achieve statistical significance ($p=0.08$). Retinal fructose concentrations were 2.2-fold greater in the IR group than in the non-ischemic controls ($p<0.05$). Fidarestat pre-treatment of rats subjected to IR reduced retinal sorbitol concentration to the levels in non-ischemic controls. Retinal fructose concentrations were reduced by 41% in fidarestat-pre-treated IR group vs. untreated ischemic controls ($p=0.0517$), but remained 30% higher than in the non-ischemic control group. In conclusion, IR injury to rat retina is associated with a dramatic increase in cell death, elevated AR expression and sorbitol pathway intermediate accumulation. These changes were prevented or alleviated by the AR inhibitor fidarestat. The results identify AR as an important therapeutic target for diseases involving IR injury, and provide the rationale for development of fidarestat and other AR inhibitors.

Correspondence to: Dr Irina G. Obrosova, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808, USA
E-mail: obrosoig@pbrc.edu

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Introduction

Sorbitol pathway of glucose metabolism consists of two reactions, aldose reductase (AR)-catalyzed NADPH-dependent reduction of glucose to its sugar alcohol, sorbitol, followed by sorbitol dehydrogenase(SDH)-catalyzed NAD-dependent oxidation of sorbitol to fructose. For almost 50 years after its discovery (1), it has been considered that activation of the sorbitol pathway of glucose metabolism is a direct consequence of hyperglycemia or hypergalactosemia. This notion has been supported by numerous studies with AR inhibitors in animal models of diabetes and galactose feeding which provided evidence for the important role of increased sorbitol pathway activity in diabetic or diabetes-like complications including cataract (2-4), retinopathy (2,5,6), peripheral and autonomic neuropathy (7-11), and nephropathy (12,13). The important role for the first enzyme of the sorbitol pathway, AR, in several diabetic complications has further been confirmed in studies with AR-overexpressing and AR-knockout mice (14-17).

Recent reports suggest that increased sorbitol pathway activity is also involved in the pathogenesis of diseases that are not accompanied by hyperglycemia including atherosclerosis (18), cancer (19), and a number of inflammatory conditions and disorders such polymicrobial sepsis (20), asthma (21) and uveitis (22). Evidence for the important role of this mechanism in ischemia-reperfusion (IR) injury, and, in particular, in cardiac (23-25) and, recently, retinal (26,27) IR injury is emerging.

In human disease, retinal IR injury takes place when retinal blood flow is interrupted for a long period of time and then restarted. It occurs in transient ischemia-related diseases, such as central retinal artery occlusion, angle-closure glaucoma, amaurosis fugax, as well as in diabetes, atherosclerosis, and hypertension (26-29). Retinal ischemia and diabetic retinopathy share many pathophysiological and pathological features

including but not limited to b-wave abnormalities in the electroretinogram (30,31), glial activation (32,33), retinal neuronal and ganglion cell degeneration (26,34), increased vascular permeability and breakdown of blood-retinal barrier (35,36), edema (26,37), capillary cell loss (38,39), and accelerated angiogenesis (40,41). Also note, that whereas retinal ischemia plays an important role in the progression of diabetic retinopathy towards its advanced, or proliferative stage (41), its treatment with scatter laser photocoagulation restoring oxygen supply is associated with the development of sight-threatening macular edema (42). Sixty percent of eyes treated with scatter photocoagulation showed an increase in foveal thickness, detectable by a scanning retinal thickness analyzer, after photocoagulation (42).

A recent study in AR-deficient mouse model implicated increased AR activity in neuronal loss and edema associated with retinal IR injury (26). In the same report, IR-associated increase in TUNEL positivity in retinal ganglion cell and inner neuronal layers, retinal thickening, as well as cytoarchitectural disorganization were prevented or markedly reduced by the ARI fidarestat. In our recent experiments in the rat model of retinal IR injury, pre-treatment with fidarestat alleviated inflammatory response (27). The present study conducted in the same animal model evaluated the effects of IR injury and fidarestat pre-treatment on retinal apoptosis, AR and sorbitol dehydrogenase (SDH) protein expressions, sorbitol pathway intermediate concentrations, and oxidative-nitrosative stress.

Materials and methods

Reagents. Unless otherwise stated, all chemicals were of reagent-grade quality, and were purchased from Sigma Chemical Co., St. Louis, MO. Rabbit polyclonal anti-nitrotyrosine antibody was purchased from Upstate, Lake Placid, NY, USA. Mouse anti-AR monoclonal antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Mouse anti-SDH monoclonal antibody was obtained from Abcam, Cambridge, MA, USA. ApopTag® Peroxidase In Situ Apoptosis Detection kit was purchased from Chemicon International, Inc., Temecula, CA. Other reagents for immunohistochemistry were purchased from Dako Laboratories, Inc., Santa Barbara, CA.

Animals. The experiments were performed in accordance with regulations specified by The Guide for the Care and Handling of Laboratory Animals (NIH Publication no. 85-23) and The Animal Ethics Committee of Malmö/Lund. Female Wistar rats (Taconic Europe, Ry, Denmark), body weight 200-250 g, were housed in individually ventilated cages in a temperature-controlled environment with free access to food and water and a 12-h light-dark cycle. The rats have randomly been divided into groups pre-treated with the ARI fidarestat (Sanwa Kagaku Kenkyusho Co., Nagoya, Japan), at 32 $mgkg^{-1}d^{-1}$ i.v. or the vehicle N-methyl-D-glucamine (NMDG). A group of vehicle- and fidarestat-pre-treated rats were then subjected to 45-min retinal ischemia followed by 24 h of reperfusion.

Administration of fidarestat and NMDG. On day 1, the animals in the fidarestat and NMDG groups were anesthetized with 3% isoflurane (IsoFlo Vet, Orion Pharma Animal Health, Sollentuna, Sweden). A neck incision was made and the right jugular vein was exposed and catheterized to allow repeated i.v. injections. The catheter was tunneled under the skin to appear through a small cut in the neck scruff. After injection, the catheter was closed and replaced under the skin and wounds were sutured. Injections were repeated on day 2 under 2% isoflurane anesthesia. On day 3, the animals were anesthetized with fentanyl (300 μg/kg i.p., B. Braun, Melsungen, Germany) followed by medetomidin (300 μg/kg i.p., Domitor Vet, Orion Pharma Animal Health, Sollentuna, Sweden), and a third dose of fidarestat or NMDG was given 30 min before induction of ischemia.

Induction of ischemia and reperfusion. The pupils were dilated with cyclopentolate 1% (Cyclogyl®, Alcon, Stockholm, Sweden) followed by the application of a local anesthetic, tetracaine 1% (Tetrakain Chauvin, Novartis Ophthalmic, Copenhagen, Denmark) which allowed the direct observation of retinal blood flow under a stereomicroscope. Retinal ischemia was induced 30 min after the vehicle or fidarestat injection, by careful application of a 5-0 silk suture (Ethicon, Sollentuna, Sweden) around the vessels and the accompanying optic nerve of the left eye as described (43). The ligature was tightened until complete cessation of the retinal blood flow occurred. The right eye served as a non-ischemic control. During induction of ischemia, rats were placed in cages supplied with a heating pad, to avoid a decrease in body temperature. After 45 min, the ligature was carefully removed. After confirmation of restoration of retinal blood flow, anesthesia was reversed by bupenorfin (30 μg/kg s.c., Temgesic®, Schering-Plough, Kenilworth, NJ, USA) and atipamezol (1 mg/kg s.c., Antisedan Vet, Orion Pharma Animal Health, Sollentuna, Sweden), and reperfusion continued for 24 h, after which all animals were sacrificed.

Euthanasia and retinal dissection. The rats were sedated by CO_2, and were immediately sacrificed by cervical dislocation. In approximately half of animals in each experimental group, both eyes were rapidly enucleated, the lenses were removed, and the retinas gently peeled off from the pigment epithelium, snap-frozen in liquid nitrogen, and stored at -80˚C prior to analysis of sorbitol pathway intermediate concentrations and AR, SDH, and nitrated protein expression. In the other half, the eyes were fixed in 4% paraformaldehyde in PBS and later used for preparation of flat mounted retinas and quantitation of apoptosis.

Specific methods

Retinal glucose and sorbitol pathway intermediate concentrations. Retinal glucose, sorbitol and fructose concentrations were assessed by spectrofluorometric enzymatic methods with hexokinase/glucose 6-phosphate dehydrogenase, sorbitol dehydrogenase, and fructose dehydrogenase as we described in detail (44,45).

Western blot analysis of AR, SDH, and nitrated proteins. Western blot analyses of AR, SDH, and nitrated proteins in individual retinas (one retina from each rat) were performed as described previously (13,33). Protein bands were visualized with the BM Chemiluminescence Blotting Substrate (POD) (Roche, Indianapolis, IN). Membranes were then stripped and reprobed with ß-actin antibody to confirm equal protein

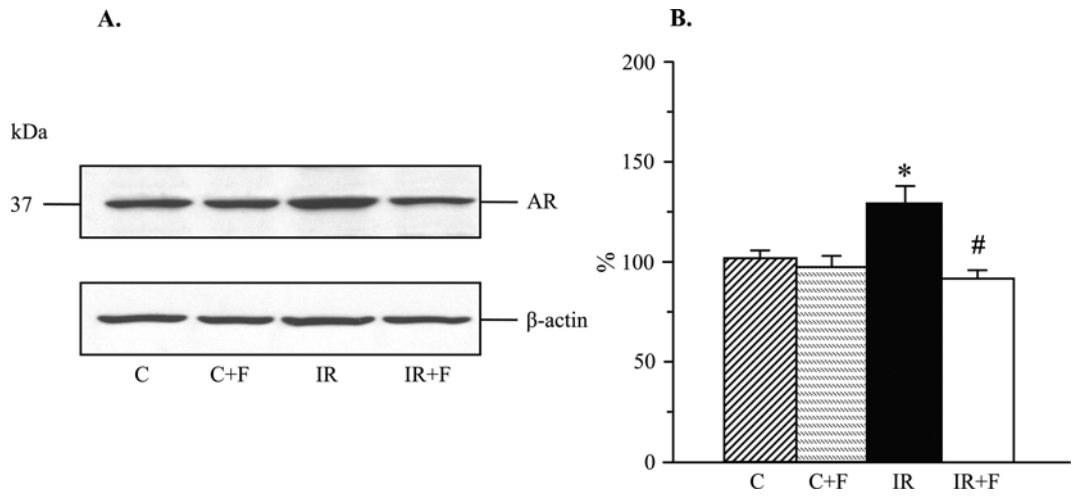

Figure 1. Representative Western blot analysis of retinal aldose reductase expression (A) and aldose reductase protein content (densitometry, B) in non-ischemic and ischemia-reperfusion-subjected retinas after vehicle or fidarestat pre-treatment. C, control; IR, ischemia-reperfusion; F, fidarestat. Mean ± SEM, n=4-5 per group. *p<0.05 vs. the non-ischemic control group, #p<0.05 vs. the vehicle-pre-treated ischemia-reperfusion group.

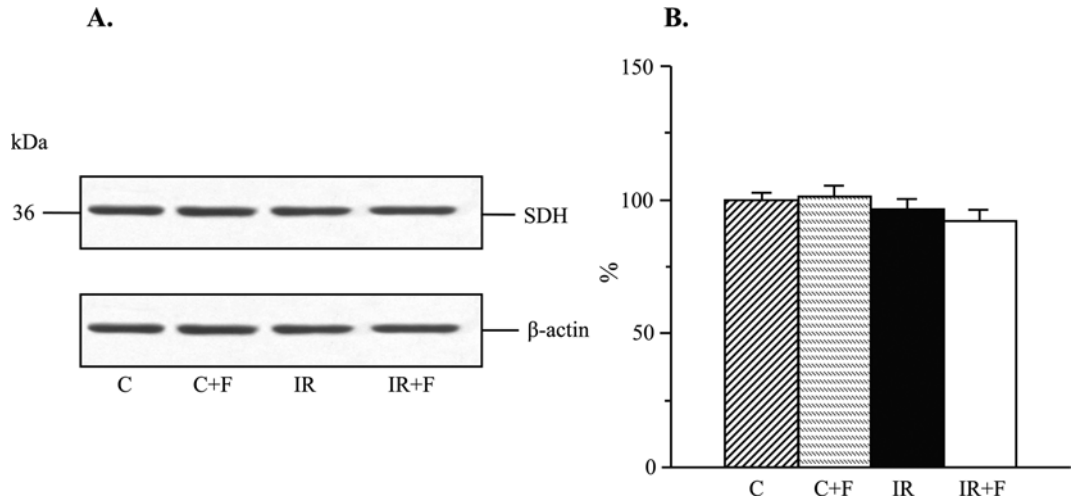

Figure 2. Representative Western blot analysis of retinal sorbitol dehydrogenase expression (A) and sorbitol dehydrogenase protein content (densitometry, B) in non-ischemic and ischemia-reperfusion-subjected retinas after vehicle or fidarestat pre-treatment. C, control; IR, ischemia-reperfusion and F, fidarestat. Mean ± SEM, n=5 per group.

loading. The data were quantified by densitometry (Quantity One 4.5.0 software, Bio-Rad Laboratories, Richmond, CA).

Immunohistochemical assessment of TUNEL positivity. All flat mounted retinas were processed by a single investigator and evaluated blindly. TUNEL positivity was quantified with the ApopTag® Peroxidase In Situ Apoptosis Detection kit as described (34,46).

Statistical analysis. The results are expressed as mean ± SEM. Data were subjected to equality of variance F test, and then to log transformation, if necessary, before one-way analysis of variance. Where overall significance ($p<0.05$) was attained, individual between group comparisons for multiple groups were made using the Student-Newman-Keuls multiple range test. When between-group variance differences could not be normalized by log transformation (data sets for body weights and blood glucose), the data were analyzed by the non-parametric Kruskal-Wallis one-way analysis of variance, followed by the Bonferroni/Dunn test for multiple comparisons. Significance was defined at $p\leq0.05$.

Results

Both AR and SDH expressions in the retina were clearly identifiable in all experimental conditions. Retinal IR injury was associated with a 19% increase in AR protein expression ($p<0.05$ vs. the non-ischemic control group, Fig. 1). Fidarestat pre-treatment prevented IR-associated AR protein expression ($p<0.05$ vs. the vehicle-pretreated ischemic group). Vehicle pre-treatment did not affect AR expression in non-ischemic retina.

Retinal SDH expression did not differ among the experimental groups (Fig. 2). Retinal glucose concentration tended

Table I. Retinal glucose and sorbitol pathway intermediate concentrations (nmol/mg protein) in non-ischemic and ischemia-reperfusion-subjected rat retinas after vehicle or fidarestat pretreatment.

Group	Variable		
	Glucose	Sorbitol	Fructose
Control + Vehicle	12.3±2.1	0.9±0.1	7.3±2.0
Control + Fidarestat	11.2±1.5	0.8±0.1	8.9±1.7
IR	16.9±1.0	1.4±0.2[a]	16.1±2.4[a]
IR + Fidarestat	9.8±1.0[c]	0.9±0.1[b]	9.5±2.1

Data are means ± SEM, n=4-9 per group. IR, ischemia-reperfusion. [a]p<0.05 vs controls; [b,c]p<0.05, p<0.01 vs. untreated group of mice.

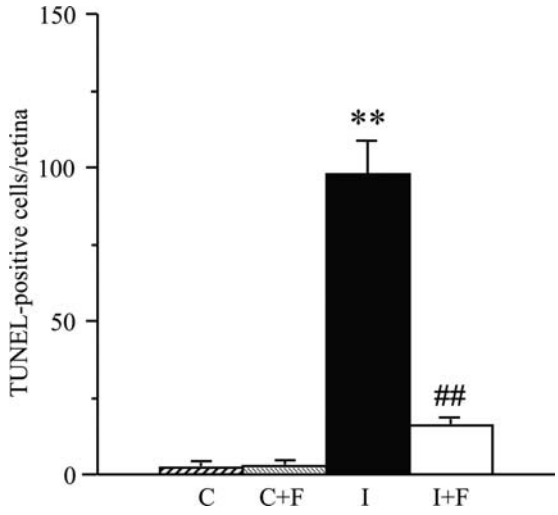

Figure 3. TUNEL-positive cell counts in non-ischemic and ischemia-reperfusion-subjected retinas after vehicle or fidarestat pre-treatment. C, control; IR, ischemia-reperfusion; F, fidarestat. Mean ± SEM, n=5-9 per group, **p<0.01 vs. the non-ischemic control group; ##p<0.01 vs. the vehicle-pre-treated IR group.

to increase in the IR group (probably, due to impaired aerobic glucose metabolism), but the difference with the non-ischemic group did not achieve statistical significance (Table I). Retinal sorbitol and fructose concentrations were increased by 56% and 120% in the IR group (p<0.05 vs. the non-ischemic control group). Pre-treatment with fidarestat completely prevented IR-induced changes in retinal glucose and sorbitol concentrations. Retinal fructose concentrations were 41% lower in fidarestat-pre-treated rats compared with the vehicle-pre-treated group (p=0.052), but still remained 30% higher than in non-ischemic controls (p=0.44). Vehicle pre-treatment did not affect retinal glucose and sorbitol pathway intermediate concentrations in the non-ischemic rats.

Retinal IR injury resulted in a dramatic increase in cell death (Fig. 3). The number of TUNEL-positive cells per retina was 48-fold greater in the IR group than in the non-ischemic controls (p<0.01). Pre-treatment with fidarestat reduced TUNEL positivity 6.1-fold, compared with the vehicle-pre-treated group (p<0.01). However, the number of TUNEL-positive cells was 7.8-fold greater in fidarestat-pre-treated IR-subjected retinas compared with the non-ischemic controls (p<0.01). Retinal nitrated protein levels were similar among non-ischemic and IR-subjected retinas pre-treated with vehicle or fidarestat (Fig. 4).

Discussion

The results discussed herein implicate increased sorbitol pathway activity in accelerated cell death associated with retinal IR injury. Retinal IR induced AR protein over-expression and sorbitol pathway intermediate accumulation that were completely or partially prevented by pre-treatment with the ARI fidarestat. Fidarestat markedly reduced IR-related retinal cell death. Neither IR injury nor fidarestat-pre-treatment were associated with any changes in the whole retina nitrated protein expression.

In the present study, IR injury tended to increase retinal glucose concentrations, probably due to decreased glucose metabolism under ischemic conditions. Fidarestat pre-treatment prevented IR-induced increase in retinal glucose. Whereas the mechanism underlying this phenomenon in IR-subjected retina has not been clarified, an ARI treatment is known to be associated with an increase in retinal cytosolic and mitochondrial NAD^+/NADH ratios in the diabetic rat model (45). Such changes in free cytosolic and mitochondrial NAD^+/NADH redox states accelerate glycolysis and tricarboxylic acid cycle thus promoting intracellular glucose utilization.

The findings of increased sorbitol pathway activity manifest by sorbitol and fructose accumulation in retinal IR model in the present study are in line with previous reports demonstrating beneficial effects of ARIs on cardiac IR injury (23-25,47). The mechanisms underlying IR-associated sorbitol pathway activation are not understood; furthermore, it is unclear whether it occurs because of ischemia, reperfusion, or both phases of IR injury. A recent demonstration of increased sorbitol and fructose concentrations in the muscle and kidney of mice subjected to hindlimb ischemia (48) suggests that ischemia rather than reperfusion leads to sorbitol pathway activation. In another study (26), IR, but not ischemia alone, was associated with increased cardiac fructose concentration, whereas sorbitol concentrations were indistinguishable in non-ischemic hearts and those subjected to ischemia alone or IR. Pre-treatment with fidarestat counteracted IR-induced retinal sorbitol pathway intermediate accumulation in the present study, consistent with the effects of fidarestat (48) and another ARI (25) on ischemia- or IR-associated increase in sorbitol and/or fructose concentrations in the muscle, kidney, and heart in the two above-mentioned reports.

Ischemia- or IR-related sorbitol pathway activation is not necessarily associated with increased AR expression. AR expression in IR-subjected retinas was increased in the present study, consistent with induction of AR immunoreactivity in the retinal inner neuronal layer by transient ischemia in another report (26). In contrast, no induction in AR expression was found in the mouse ischemic kidney or muscle (48).

The role for the second enzyme of sorbitol pathway, SDH, in diabetic complications has been an area of intense controversy for more than ten years, and the findings suggesting

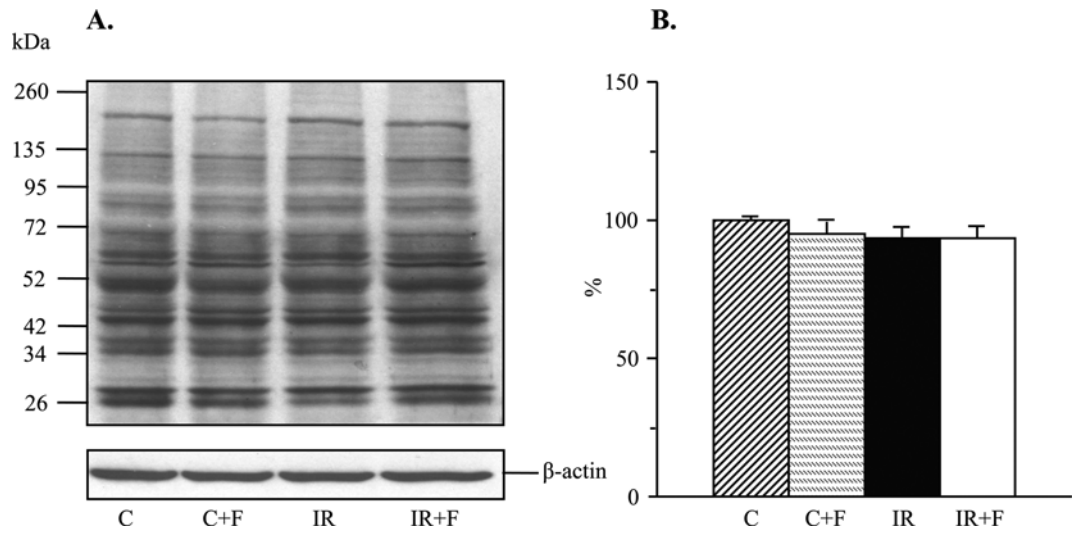

Figure 4. Representative Western blot analysis of nitrated protein expression (A) and nitrated protein content (densitometry, B) in non-ischemic or ischemia-reperfusion-subjected retinas after vehicle or fidarestat pre-treatment. C, control; I, ischemia-reperfusion and F, fidarestat. Mean ± SEM, n=5 per group.

protective (49-53) or detrimental (54,55) functions of this enzyme activity or its minor importance (56,57) in the pathogenetic process have been produced by different laboratories. Two groups (25,58) obtained evidence of the important role for this enzyme in cardiac IR injury. In contrast, SDH does not seem to play an important role in retinal IR injury as, in contrast to AR-deficient or fidarestat-pre-treated mice, SDH-deficient or the SDH inhibitor CP-470,711-pre-treated mice were not protected from apoptotic neuronal death or edema caused by transient retinal ischemia (26). In the present study, SDH expression in the whole retina was unchanged by IR, whereas SDH immunoreactivity in inner neuronal layer was found decreased by transient retinal ischemia in the aforementioned report (26).

Studies in the field of diabetic complications suggest that increased AR activity contributes to multiple biochemical changes in tissue-sites for diabetic complications including retina (reviewed in ref. 59). In particular, increased AR activity leads to vascular endothelial growth factor formation (6,17), mitochondrial and cytosolic NAD^+/NADH imbalances (10,45) and energy deficiency (10,60), Ca^{2+} overload (61), increased formation of precursors of advanced glycation endproducts (AGE), i.e., fructose, fructose 3-phosphate (62), methylglyoxal (63), and 3-deoxyglucosone (64), and AGE *per se* (65,66). Increased AR activity also contributes to diabetes-associated diversion of the glycolytic flux from glyceraldehyde 3-phosphate dehydrogenase towards the formation of α-glycerophosphate (51), the protein kinase C activator diacylglycerol (67), and protein kinase C activation (68). In recent years, AR has been implicated in activation of mitogen-activated protein kinases (69), poly(ADP-ribose) polymerase [PARP (13,70)], and cyclooxygenase-2 (71), as well as nuclear factor-κB (NF-κB) and activator protein-1 (72). Many of these mechanisms, and, in particular, NAD^+/NADH redox imbalances, accumulation of intracellular Ca^{2+}, activation of PARP and cyclooxygenase-2 (COX-2), and activation of NF-κB and resultant increase in cytokine production and pro-inflammatory response, have also been implicated in IR injury (25,58,73-75). Cytokines (75), PARP and COX-2 activations (73,74), and overexpression of c-Jun N-terminal kinase (76) have also been implicated in IR-induced retinal apoptosis. Whereas relations among increased AR and these factors in IR still remain to be explored, it is not excluded that retinoprotective effect of fidarestat is mediated through one of the afore-mentioned mechanisms. Note, that PARP activation manifest by increased poly(ADP-ribose) immunoreactivity, was detected in retinal ganglion cell layer and inner neuronal layer of wild-type but not AR-deficient mice (26). A complete or partial prevention of retinal apoptosis has been reported in ARI-treated diabetic animals (3,4,17), ARI-treated high glucose exposed retinal pericytes (77,78), as well as ARI-treated and AR-deficient mice subjected to IR injury (26).

In the present study, IR-related increase in retinal cell death and its partial prevention by fidarestat pre-treatment were not related to the corresponding changes in nitrated protein expression, a stable footprint of peroxynitrite damage (79), in the whole retina. The latter is quite surprising considering that peroxynitrite plays a role in IR injury in general (79), and protein nitration has been identified as a contributing factor to retinal IR injury (80) as well as to apoptosis associated with diabetic retinopathy (81). Also, increased AR activity, a major factor responsible for impaired antioxidative defense (reviewed in ref. 59), has been found to contribute to nitrosative stress in diabetic (4,13,17,70) and IR (25,26) models and high glucose-exposed cultured endothelial cells (82). Probably, Western blot analysis of nitrated proteins in the whole retinal samples is not the optimal method for evaluation of focal nitrosative stress induced by IR injury (26), and immunohistochemical assessment of nitrotyrosine immunoreactivity remains the only option for retinal IR-related studies, at least, in rodent models.

In conclusion, IR injury to rat retina is associated with a dramatic increase in cell death, elevated AR expression and sorbitol pathway intermediate accumulation. These changes were prevented or alleviated by the aldose reductase inhibitor fidarestat. The results identify AR as an important therapeutic target for diseases involving IR injury, and provide the rationale for development of fidarestat and other ARIs.

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