Aldose Reductase Inhibitor Fidarestat Prevents Retinal Oxidative Stress and Vascular Endothelial Growth Factor Overexpression in Streptozotocin-Diabetic Rats

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The study addressed the role for aldose reductase (AR) in 1) retinal oxidative stress and vascular endothelial growth factor (VEGF) overexpression in early diabetes, and 2) high glucose-induced oxidative stress in retinal endothelial cells. In vivo experiments were performed on control rats and diabetic rats treated with or without low or high dose of the AR inhibitor (ARI) fidarestat (2 or 16 mg·kg⁻¹·day⁻¹). In vitro studies were performed on bovine retinal endothelial cells (BREC) cultured in either 5 or 30 mmol/l glucose with or without 1 μmol/l fidarestat. Intracellular reactive oxygen species were assessed using the 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) probe and flow cytometry. Both low and high doses of fidarestat (i.e., the doses that partially and completely inhibited sorbitol pathway hyperactivity) arrested diabetes-induced retinal lipid peroxidation. This was achieved due to upregulation of the key antioxidative defense enzyme activities rather than changes in reduced glutathione, oxidized glutathione, ascorbate and dehydroascorbate concentrations, and the glutathione and ascorbate redox states. Diabetes-associated 2.1-fold VEGF protein overexpression (enzyme-linked immunosorbent assay; ELISA) was dose-dependently prevented by fidarestat, whereas total VEGF mRNA and VEGF-164 mRNA (RT-PCR) abundance were not affected by either diabetes or the ARI. In BREC, fidarestat corrected hyperglycemia-induced increase in H₂DCFDA fluorescence but not oxidative stress caused by three different pro-oxidants in normoglycemic conditions. In conclusion, increased AR activity contributes to retinal oxidative stress and VEGF protein overexpression in early diabetes. The findings justify the rationale for evaluation of fidarestat on diabetic retinopathy. Diabetes 52:864–871, 2003

Both increased aldose reductase (AR) activity and oxidative stress have been implicated in the pathogenesis of diabetic complications (1–4). The important role of the two mechanisms in diabetic retinopathy (DR) is supported by findings in animal models of diabetes and galactose feeding that manifest virtually identical alterations in gene expression (R.N.F., A. Kennedy, T.A. Twomey, S.J. Land, M.R. Hughes, D. Guberski, unpublished observations) and similar biochemical (5,6), functional (7), and histological (8–10) abnormalities in the retina. Both AR inhibitors (ARIs) and antioxidants prevent formation of retinal pericyte ghosts and acellular capillaries (1,6,10), increased vascular permeability (11–13), decreased blood flow and oxygenation (7,14), and impaired leukocyte-endothelial cell interactions resulting in retinal inflammation (15,16). Furthermore, our earlier ARI study in long-term galactose-fed rats (2) and a more recent antioxidant study in short-term diabetic rats (17) revealed the capacity of both classes of agents to prevent increased expression of retinal vascular endothelial growth factor (VEGF). VEGF has been implicated in the increased vascular permeability, breakdown of the blood-retinal barrier, and vascular cell proliferation characteristic of diabetes (18).

The relation between diabetes-associated increase in retinal AR activity and oxidative injury remains unexplored, and hyperglycemia-induced oxidative stress is present in cells with both relatively high (retinal pigment epithelial and Muller cells, pericytes) and low (endothelial cells) AR activity [(19), R.N. Frank and A. Kennedy, unpublished]. According to some investigators (20), AR activity in vascular cells is far too low to be responsible for diabetes-induced vascular complications. However, this view contradicts the experimental findings of, at least, ten groups demonstrating prevention of 1) diabetes-induced neurovascular dysfunction (4,21–23) and 2) vascular abnormalities of diabetic or diabetes-like galactosemic retinopathy (1,2,7,9–11,13,16) with an ARI treatment. Furthermore, the AR activity in high glucose–exposed aortic endothelium was recently found to be sufficient to almost completely account for hyperglycemia-associated superoxide generation (24).

The present study was designed to assess the role for AR in 1) retinal oxidative stress and VEGF overexpression in early diabetes and 2) high glucose–induced oxidative

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AA, ascorbate; AGE, glycation end products; AR, aldose reductase; ARI, AR inhibitor; BSO, L-buthionine(S,R)-sulfoximine; BREC, bovine retinal endothelial cells; DHAA, dehydroascorbate; DMEM, Dulbecco’s modified Eagle’s medium; DR, diabetic retinopathy; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, reduced glutathione; GSH Px, glutathione peroxidase; GSSG, oxidized glutathione; GSSG Red, glutathione reductase; GSSG Trans, glutathione transreductase; 4-HA, 4-hydroxyalkenals; MDA, malondialdehyde; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor.
stress in retinal endothelial cells. These aims were achieved by evaluating the potent (13,25,26) and well-tolerated (27) ARI fidarestat (SNK-860, Sanwa Kagaku Kenkyusho) on I) retinal lipid peroxidation, antioxidative defense, and VEGF protein and mRNA abundance in streptozotocin-diabetic rats with 6-week duration of diabetes; and (2) high-glucose-induced generation of reactive oxygen species (ROS) in bovine retinal endothelial cells (BREC).

**RESEARCH DESIGN AND METHODS**

The experiments were performed in accordance with regulations specified by The Guiding Principles in the Care and Use of Animals (DHHEW Publication, NIH 80-23) and the University of Michigan Protocol for Animal Studies. Animals. Male Wistar rats (Charles River, Wilmington, MA), initial body weight 227-267 g, were fed a standard rat chow diet (ICN Biomedicals, Cleveland, OH) and had ad libitum access to water. Diabetes was induced by a single injection of streptozotocin (55 mg/kg body wt i.p.). Blood samples for measurements of glucose were taken from the tail vein ~48 h after streptozotocin injection and the day before the rats were killed. Rats with blood glucose of 13.9 mmol/l or more were considered diabetic. The experimental groups included control rats and 6-week diabetic rats treated with or without low (2 mg/kg per day) or high (16 mg/kg per day) doses of fidarestat administered in the diet. The treatments were started after induction of diabetes.

Reagents. Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma Chemical, St. Louis, MO. Methanol (high-performance liquid chromatography grade), perchloric acid, hydrochloric acid, and sodium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA). Ethyl alcohol (200 proof dehydrated alcohol, U.S.P. punctilious) was purchased from McCormick Distilling (Weston, MO). β-D-Glucose, sorbitol, N.F., and α-fructose, U.S.P., were purchased from Pfanstiehl Laboratories (Waukegan, IL). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from GIBCO (Grand Island, NY). 5-(and-6)-chloromethyl-2

**FIG. 1.** Representative calibration curves for human and rat VEGF mRNAs obtained with the Quantikine mRNA kit for human VEGF mRNA.

![Graph showing calibration curves for human and rat VEGF mRNAs](image)

1) VEGF ELISA. The retinas were homogenized, and the homogenates centrifuged as we described previously (17). Fifty microliter aliquots of the supernatant were used for VEGF measurements. VEGF was assayed by a sandwich ELISA with an affinity-purified polyclonal antibody specific for mouse VEGF and mouse VEGF standard according to the manufacturer’s instructions. Good linearities (r_{human} = 0.996 and r_{rat} = 0.986) were obtained with this antibody using both mouse and rat VEGF standards in the range of 5–500 pg/ml as well as rat VEGF standards in the range of 15–500 pg/ml added to rat retinal homogenates (r = 0.964). VEGF concentrations were normalized to total protein quantified according to the manufacturer’s instructions. 2) RNA preparation. The isolation of total RNAs was performed in accordance with the manufacturer’s instructions. The absorbance of isolated RNAs was assessed spectrophotometrically at λ = 260 nm and λ = 280 nm. The absorbance-absorbance ratio was used as a criterion of the RNA purity. The RNA yield from individual samples was calculated by multiplying the absorbance at 260 nm by 4, i.e., the absorbance of 1 μg/ml RNA solution in the 1-cm path quartz cuvette. The integrity of isolated RNAs was assessed by resolving four randomly selected RNA samples on a formaldehyde-denaturing agarose gel and comparing the quality and distribution of the ribosomal RNA 28S and 18S fragments. 3) Quantitation of total VEGF mRNA. The measurements were performed in 3.5-μg RNA samples in duplicate in accordance with the manufacturer’s instructions. Taking into consideration that the assay has been developed for human VEGF mRNA, we have obtained calibration curves with human and rat VEGF mRNA preliminary synthesized from the plasmids for rat and human VEGF probes. Representative calibration curves are presented in Fig. 1. The results of this comparison indicate that the kit can be used for measurements of rat VEGF mRNA. 4) Quantitation of VEGF-164 mRNA. VEGF-164 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were analyzed by RT-PCR. First, the mRNA template was copied into the complementary single-stranded cDNA (first-strand cDNA synthesis). This was performed using 50 ng retinal RNA, oligo(dT), and Sensiscript Reverse Transcriptase Kit according to the manufacturer’s protocol. Second, VEGF cDNA fragment was amplified by PCR, using forward primer (5’-CCGAAACCATGAACTTTCTGC-3’) and reverse primer (3’-GTGACAAAGCCAAGGCGGTGAG-5’). The reverse primer oligonucleotide and the forward primer oligonucleotide AAAACATGAAGTTTCCAGC-3’ and reverse primer (3’-ATGATTTTCTCGGTCGGTGC-5’) correspond to the nucleotide sequences 588–578 and 1–17 of rat VEGF mRNA (GenBank accession no. MS21467). The GAPDH cDNA fragment was amplified using the forward primer (5’-CACACACATGGAAGAAGGTG-3’) and the reverse primer (5’-ATGATTTTCTCGGTCGGTGC-3’) that correspond to 330–349 and 648–625 nucleotide sequences of the rat GAPDH gene, respectively (GenBank accession no. NM017008). The amplification was performed using HotStarTaq DNA Polymerase Kit and 0.25 μl of deoxyctydine 5’-[α-32P]triphosphate according to the manufacturer’s protocol. Amplified PCR products were analyzed by electrophoresis in 2% agarose gel. Gels were photographed using the Quantity One BioRad System (BioRad Laboratories,
were mixed with 0.2 ml of ice-cold 10% metaphosphoric acid and centrifuged
were added for 24 h at the end of the experiment. Parallel pro-oxidant
ethyl maleate (DEM, 60
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ANOVA, followed by the Bonferroni-Dunn test for multiple comparisons.

5) Sorbitol pathway intermediates. Individual retinæ were homogenized in 1 ml of 0.1 mol/l sodium-phosphate buffer, pH 6.5. Deproteinization and spectrofluorometric measurements of glucose, sorbitol, and fructose by enzymatic procedures were performed as we described previously (28).

6) MDA plus 4-HA, GSH, and GSSG. 0.2 ml of the retinal homogenates obtained in section five were used for measurements of MDA plus 4-HA performed in accordance with the manufacturer’s instructions. Another 0.2 ml were used for GSH and GSSG measurements (29).

7) DHAA and AA. 0.2 ml of the homogenates described in the section five were mixed with 0.2 ml of ice-cold 10% metaphosphoric acid and centrifuged at 4,000g for 10 min. Total AA and DHAA concentrations were measured as we have described previously (17). AA concentrations were calculated as the difference between total AA and DHAA.

8) Antioxidative defense enzyme activities. Individual retinæ were homogenized in 1 ml of 0.1 mol/l sodium-phosphate buffer, pH 6.5, and centrifuged at 20,000g for 20 min. The supernatants were used for spectrophotometric measurements of SOD, catalase, GSSGRed, GSHPx, and GSH-Trans activities (29,30).

BREC cultures. BREC cultures were established from fresh calf eyes as we described previously (31). Passages 2–10 were used for all experiments. Purity of cultures was confirmed by characteristic endothelial cell morphology and by either >90% uptake of acetylated LDL or >90% immunopositivity for factor VIII, blood endothelial cell specific marker (31).

Assessment of ROS generation by BREC. Experiment 1. The BREC were cultured (eight plates per condition) for 7 days in DMEM containing either 5 or 30 mmol/l glucose with or without 1 μmol/l fludarate added for 4 h at the end of the experiment. 10 μl of 10 μmol/l CM-H$_2$DCFDA, the dichloro-fluorometric derivative with the best retention properties among all the studied analogs, was added for 30 min at the end of the experiment. The cells were then washed and trypsinized, and the CM-H$_2$DCFDA fluorescence, an index of ROS generation, was measured by flow cytometry (λ excitation 480 nm; λ emission 520 nm). After flow cytometry, the BREC were counted, and CM-H$_2$DCFDA fluorescence was expressed per 10$^5$ cells. Parallel cultures (eight plates per condition) were grown without CM-H$_2$DCFDA and used for measurements of the sorbitol pathway intermediates.

Experiment 2. The BREC (eight plates per condition) were cultured for 2 days in DMEM containing 5 mmol/l glucose with or without 1 μmol/l CM-H$_2$DCFDA and the dichloro-fluorometric derivative with the best retention properties among all the studied analogs, was added for 30 min at the end of the experiment. The cells were then washed and trypsinized, and the CM-H$_2$DCFDA fluorescence, an index of ROS generation, was measured by flow cytometry (λ excitation 480 nm; λ emission 520 nm). After flow cytometry, the BREC were counted, and CM-H$_2$DCFDA fluorescence was expressed per 10$^5$ cells. Parallel cultures (eight plates per condition) were grown without CM-H$_2$DCFDA and used for measurements of the sorbitol pathway intermediates.

Statistical analysis. The results are expressed as mean ± SE. Data were subjected to equality of variance F test and then to log transformation if necessary, before one-way ANOVA. When overall significance (P < 0.05) was attained, individual between-group comparisons were made using the Student-Newman-Keuls multiple range test. Significance was defined at P = 0.05. When between-group variance differences could not be normalized by log transformation, the data were analyzed by the nonparametric Kruskal-Wallis one-way ANOVA, followed by the Bonferroni-Dunn test for multiple comparisons.

RESULTS

The final body weights were lower in diabetic rats than in the control group (Table 1). The initial body weights were similar in control and diabetic groups. No statistically significant difference was found between final body weights in diabetic rats treated with either low or high doses of fludarate and the corresponding untreated group.

Blood glucose concentration was increased 4.5-fold in diabetic rats compared with controls. Blood glucose concentrations in diabetic rats were not affected by the low- or high-dose fludarate treatment.

Retinal glucose, sorbitol, and fructose concentrations were 8.3-fold, 5.1-fold, and 4.3-fold higher in untreated diabetic rats than in the control rats (Table 2). Diabetes-induced increase in retinal glucose concentrations was not

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + LF</th>
<th>Diabetic + HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>423 ± 6</td>
<td>310 ± 8†</td>
<td>323 ± 8†</td>
<td>314 ± 12†</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>4.44 ± 0.11</td>
<td>20.1 ± 0.33†</td>
<td>19.3 ± 0.39†</td>
<td>18.9 ± 0.39†</td>
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Data are means ± SE. *n = 38 in the control group and n = 39 in three other groups. †Significantly different from controls (P < 0.01). LF, low dose fludarate; HF, high dose fludarate. **Sorbitol pathway intermediate concentrations in control and diabetic rats treated with or without fludarate (n = 8)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + LF</th>
<th>Diabetic + HF</th>
</tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>10.4 ± 0.63</td>
<td>86 ± 6.3*</td>
<td>92 ± 9.2*</td>
<td>84 ± 6.2*</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>2.00 ± 0.12</td>
<td>10.2 ± 0.77*</td>
<td>4.1 ± 0.56†</td>
<td>1.70 ± 0.33‡</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.18 ± 0.10</td>
<td>5.08 ± 0.52*</td>
<td>3.63 ± 0.58*</td>
<td>1.85 ± 0.37‡</td>
</tr>
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</table>

Data are means ± SE. *Significantly different from controls (P < 0.01); †significantly different from controls (P < 0.05); ‡significantly different from untreated diabetic group (P < 0.01). LF, low dose fludarate; HF, high dose fludarate.
treated with or without low or high doses of the ARI. GSSGRed and GSHPx activities tended to increase with the low-dose fi darestat treatment but the difference with the untreated diabetic group did not achieve statistical significance. GSSGRed activity was essentially normalized by the high dose of fi darestat, whereas GSHPx responded similarly to the low-dose and high-dose fi darestat treatments. In contrast, GSHTrans was normalized by low dose, but not high dose, of fi darestat. It tended to increase with the high-dose fi darestat treatment, but the difference with the untreated diabetic group did not achieve statistical significance.

Glucose, sorbitol, and fructose concentrations were 24.5-fold, 11.7-fold, and 6.7-fold higher in BREC cultured in 30 mmol/l glucose than in those cultured in 5 mmol/l glucose (Table 4). High glucose–induced increase in BREC glucose concentrations was not affected by fi darestat treatment, whereas sorbitol and fructose concentrations were reduced 2.3-fold and 2-fold, respectively.

Intracellular ROS abundance was 2.4-fold higher in BREC cultured in 30 mmol/l glucose compared with those cultured in 5 mmol/l glucose (Fig. 4A). Hyperglycemia-induced increase in ROS generation was essentially corrected by 1 μmol/l fi darestat.

Intracellular ROS abundance was 3.2-fold, 5.4-fold, and 16.7-fold higher in BREC cultured in 5 mmol/l glucose supplemented with BSO, DEM, or primaquine, respectively, than in 5 mmol/l glucose without pro-oxidants (Fig. 4B). 1 μmol/l fi darestat did not affect oxidative stress induced by any of the three pro-oxidants.

FIG. 2. Retinal VEGF protein (A) and mRNA (B) concentrations in control and diabetic rats treated with or without fi darestat (mean ± SE, n = 6–12). **Significantly different versus controls (P < 0.01); #significantly different versus untreated diabetic group (P < 0.05); ##significantly different versus untreated diabetic group (P < 0.01).

DISCUSSION

The present study provides the first experimental evidence of the major contribution of increased AR activity to enhanced oxidative stress and VEGF protein expression in the retina in short-term diabetes. The relation between increased AR activity and oxidative stress deserves the most thorough consideration because, in the last several years, the continuing debate about a “primary mechanism” of diabetic complications has centered on oxidative stress and its relation with other hyperglycemia-initiated factors. Several groups (32,33) have suggested that the key physiological role of AR is the detoxification of lipid peroxidation products, and thus, AR inhibition in the diabetic conditions could be detrimental rather than beneficial. However, this view is not supported by the studies in ARI-treated nondiabetic animals that did not reveal any appearance of oxidative stress or diabetes-like complications (21,22), as observed with modulation of antioxidant deficiency (34) or pro-oxidants (3,4). Furthermore, it is well known that the effects of ARIs and antioxidants in diabetic animal models are unidirectional, i.e., both classes of agents delay, prevent, or correct the development of diabetic complications including DR (1–4,7,9–11, 13,16,19,21–23). One should keep in mind that although AR has been found to metabolize 4-hydroxynonenal, in in situ perfused organs (33,35), the aforementioned studies employed additions of relatively high concentrations of 4-hydroxynonenal, 100–200 μmol/l. Until now, no evidence of
elevated concentrations of the products of 4-hydroxynonenal metabolism by AR, i.e., glutathione-4-hydroxynonenal conjugate, 1,4-dihydroxy-2-nonenone, and 4-hydroxy-2-nonen-enoic acid, in tissue-sites for diabetic complications, and no evidence of the presence of those products in vivo, has been generated. Recently, one group hypothesized that three pathways leading to diabetic complications, i.e., increased sorbitol pathway activity, nonenzymatic glycation/glycoxidation, and protein kinase C (PKC) activation, originate from oxidative stress and, in particular, production of superoxide anion radicals in mitochondria (20). However, this concept, at least the part related to the sorbitol pathway, is not supported by experimental studies demonstrating the absence of any suppression of tissue sorbitol pathway activity by antioxidants, including those neutralizing superoxide anion radicals (DL-α-lipoic acid and taurine) in diabetic animal models (28,36). Based on evidence of contribution of increased AR activity (4,23,37,38), the Maillard reaction (39), the interaction of advanced glycation end products (AGE) with their receptors (40), and recently, PKC activation (41) to hyperglycemia-induced oxidative injury, it would be more logical to assume that the common component for the pathways leading to diabetic complications, i.e., oxidative stress, is localized downstream from primary hyperglycemia-initiated mechanism(s). The importance of extramitochondrial versus intramitochondrial mechanisms of high glucose–induced ROS generation remains to be clarified. A recent study indicates the predominantly extramitochondrial origin (xanthine oxidase) of oxidative stress in patients with type 1 diabetes (42). At least, two extramitochondrial mechanisms of free radical production, i.e., xanthine oxidase and semicarbazide-sensitive amine oxidase, are of key importance in vasa nervorum (4). Our findings suggest that the extramitochondrial enzyme AR has an important role in diabetes-associated retinal oxidative stress.

The demonstration of the importance of AR in diabetes-induced retinal oxidative stress complement previous findings in the lens and peripheral nerve obtained with a number of ARIs (4,19,23,37,43). These reports complemented by studies in AR-overexpressing (38) mice implicating AR in increased lipid peroxidation (19,23,37,38), depletion of major nonenzymatic antioxidants, i.e., GSH (4,19,23,37,38,43), ascorbate (19,23), and taurine (19), and downregulation of superoxide dismutase activity (19) in the two tissue sites for diabetic complications. Fidarestat appeared remarkably effective in arresting diabetes-induced retinal lipid peroxidation. Preservation of normal MDA plus 4-HA concentration was achieved with 2 mg/kg per day, i.e., the dose that was at least 50-fold lower than the effective doses of direct antioxidants previously tested in a rat model of diabetes (17,28,29,35). Note, that unlike in lens or peripheral nerve, the arrest of retinal lipid peroxidation with AR inhibition was not due to a “sparing/regeneration” of the key nonenzymatic antioxidants, GSH, and AA. In contrast to other tissues of rats with short-term diabetes (4,19,23,28,37,43), retinal GSH and AA concentrations are not depleted, and the glutathione and ascorbate redox states are not compromised. It is quite plausible that the remarkable efficacy of the low dose of fidarestat on retinal lipid peroxidation is due to an upregulation of GSHTransferase, which neutralizes 4-HA by their conjugation with GSH, combined with a faster turnover of the glutathione redox cycle due to increase in both GSSGRed and GSHPx activities. Note that retinal SOD activity was significantly different from untreated diabetic group (P < 0.01); †significantly different from controls (P < 0.05); ‡significantly different from untreated diabetic group (P < 0.01). F, fidarestat.
superoxide-generating enzyme, by an excessive sorbitol accumulation induced by a sorbitol dehydrogenase inhibitor in the lens of diabetic rats, as described in our previous studies (19,30).

Although the presence of AR in endothelial cells remained a subject of debate for a long time, a rigorous study (44) has generated a positive answer. Our findings indicate a major contribution of AR to high glucose–induced oxidative stress in retinal endothelial cells. The arrest of hyperglycemia-induced ROS generation by fidareset is not due to its direct antioxidant properties, which are not related to an ability to inhibit the sorbitol pathway of glucose metabolism. Fidareset did not affect oxidative stress induced by three unrelated pro-oxidants with different mechanisms of action that included hydrogen peroxide generation and initiation of hydrogen peroxide–stimulated lipid peroxidation (primaquine) and GSH depletion due to inhibition of glutathione biosynthesis (BSO) or monovalent thiol oxidation (DEM). Therefore, fidareset does not interfere with increased production or compromised neutralization of ROS caused by mechanisms other than hyperglycemia. The present study, together with the findings obtained with another structurally diverse ARI zopolrestat (24), support an important role for AR activity in diabetes-induced ROS generation in vascular endothelium. Furthermore, the major contribution of AR to hyperglycemia-induced endothelial superoxide production (24) strongly implies that AR is also involved in generation of the highly reactive hydroxyl radicals produced from superoxide and hydrogen peroxide in the Fenton and Haber-Weiss reactions.

Increasing evidence indicates that such diabetes-associated phenomena as increased retinal AR activity, oxidative stress, VEGF overexpression, and increased vascular permeability are interrelated. The present study, together with the previous observations by our group and others, suggest that AR triggers the whole cascade by causing oxidative stress, which, in turn, leads to overexpression of VEGF (17,45) responsible for increased vascular permeability (13,18). Indeed, all three components of this cascade, i.e., enhanced oxidative stress, VEGF protein overexpression, and increased vascular permeability, have been found preventable by an ARI treatment (2,11,13, and in the present study). Several studies (12,17,45), including one from our group (17), also relate VEGF upregulation and increased vascular permeability to oxidative stress.

Our findings revealed that retinal VEGF protein, but not VEGF mRNA expression, is increased in early diabetes. The absence of diabetes-induced retinal VEGF mRNA overexpression in the present study using the commercial kit with a sensitivity of a Northern blotting as well as RT-PCR was also found in another study from our group using a ribonuclease protection assay (17). The present findings, together with our previous observations with two other classes of agents, i.e., the antioxidants DL-alpha-lipoic acid and taurine (17) and the poly-(ADP-ribose) synthetase inhibitors, 3-aminobenzamide and 1,5-isoquinolinediol (46), suggest that early diabetes-induced upregulation of retinal VEGF protein occurs at a post-transcriptional level. Based on current knowledge, one can speculate that three factors, i.e., increased AR activity, oxidative stress, and poly(ADP-ribosyl)ation, interfere with PKC-dependent phosphorylation of the initiation factor (eIF) 4E, known to play an important role in the translational regulation of VEGF (47). The role for AR (48), oxidative stress (6), and recently, poly(ADP-ribosyl)ation (49) in PKC activation has already been demonstrated. Both low and high doses of fidareset decreased diabetes-induced retinal VEGF overexpression, which supports the findings of the favorable effects of long-term treatment with 2 mg/kg of fidareset on retinal pathological changes and increased vascular permeability in streptozotocin-diabetic rats (13). It remains to be established whether this dose of fidareset will also be effective in preventing VEGF-unrelated phenomena in the pathogenesis of DR, i.e., pericyte loss and formation of acellular capillaries, which ultimately leads to retinal capillary closure. The initial event, pericyte dropout, is likely to occur due to high glucose–induced apoptosis, which is known to be preventable by fidareset (50).

In conclusion, increased AR activity has a key role in diabetes-induced oxidative stress in the retina and high glucose–induced oxidative stress in retinal endothelial cells. AR is also responsible for retinal VEGF protein expression in early diabetes. Both oxidative stress and VEGF overexpression are prevented by the potent and well-tolerated ARI fidareset. The findings support the “AR concept” of DR and provide the rationale for further evaluation of fidareset to prevent the onset and progression of diabetes-induced retinal vascular changes. A recent 52-week multicenter placebo-controlled double-blind parallel group trial (27) demonstrated clinical efficacy of the very low dose of fidareset (1 mg/day) in improving nerve
conduction deficits and symptomatic indexes of diabetic neuropathy.

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