

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

Irina G. Obrosova,¹ Alexander G. Minchenko,² Rukmini Vasupuram,¹ Lauren White,¹ Omorodola I. Abatan,¹ Arno K. Kumagai,¹ Robert N. Frank,³ and Martin J. Stevens¹

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

From the¹Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan; the²Department of Anesthesiology, Thomas Jefferson University, Philadelphia, Pennsylvania; and the³Department of Ophthalmology, Kresge Eye Institute, Wayne State University School of Medicine, Detroit, Michigan.

Address correspondence and reprint requests to Dr. Irina G. Obrosova, Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Michigan Medical Center 1150 West Medical Center Drive, MSRB II, Rm 5570, Ann Arbor, MI 48109-0678. E-mail: iobrosso@umich.edu.

Received for publication 6 August 2001 and accepted in revised form 12 December 2002.

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; GSHIPx, glutathione peroxidase; GSSG, oxidized glutathione; GSSGRed, glutathione reductase; GSSGTrans, glutathione transferase; 4-HA, 4-hydroxyalkenals; MDA, malondialdehyde; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor.

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 (1,8–10) 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 DR (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

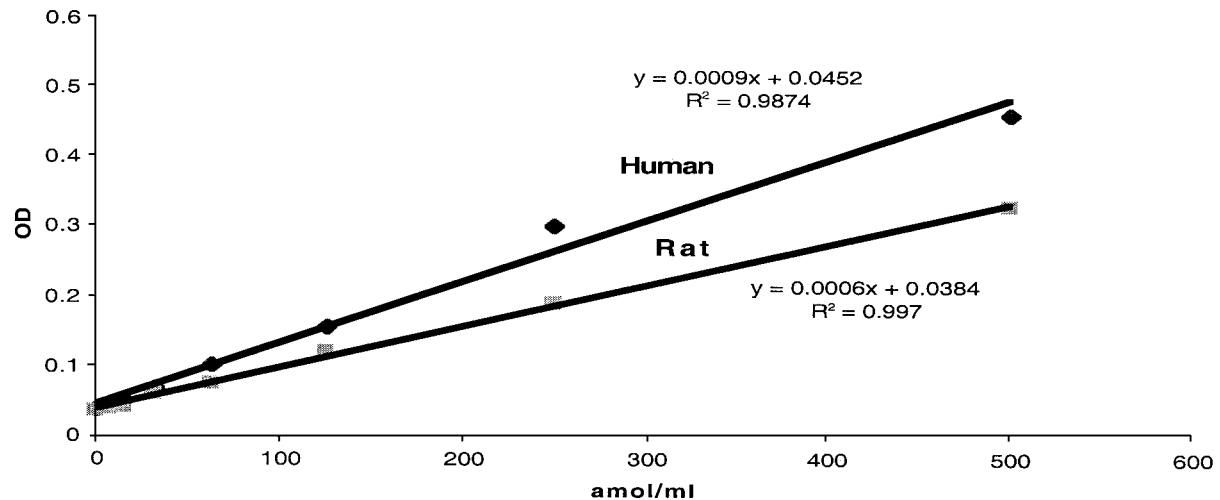


FIG. 1. Representative calibration curves for human and rat VEGF mRNAs obtained with the Quantikine mRNA kit for human VEGF mRNA.

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 (1) 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 (DHEW 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 D-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',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H₂DCFDA) was obtained from Molecular Probes (Eugene, OR). Kits for measurements of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HA) and glutathione peroxidase activity were purchased from Oxis International (Portland, OR); Rneasy kits for RNA isolation, Sensiscript Reverse Transcriptase kits, and HotStarTaq DNA Polymerase kits were from Qiagen (Chatsworth, CA); kits for VEGF protein and mRNA measurements and VEGF protein standard were from R&D Systems (Minneapolis, MN); and bicinchoninic acid protein assay kits were from Pierce (Rockford, IL). Deoxycytidine 5'-[α -³²P]-triphosphate was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Experimental procedure. Rats were sedated with carbon dioxide and immediately killed by cervical dislocation. Both retinas were rapidly dissected and frozen in liquid nitrogen. Three retinas from three animals were pooled for each VEGF protein measurement, and two retinas from one animal for RNA isolation. Other variables (i.e., glucose, sorbitol, fructose, MDA plus 4-HA, reduced glutathione [GSH], oxidized glutathione [GSSG], total ascorbate [AA],

and dehydroascorbate [DHAA] concentrations and superoxide dismutase [SOD], catalase, glutathione peroxidase [GSHPx], glutathione reductase [GSSGRed], and glutathione transferase [GSHTrans] activities) were measured in individual retinas.

Individual measurements.

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 ($r_{\text{mouse}} = 0.996$ and $r_{\text{rat}} = 0.986$) 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 $\lambda = 260$ nm and $\lambda = 280$ nm. The absorbance₂₆₀/absorbance₂₈₀ 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 μ g/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- μ g 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'-CCGAAACCATGAACCTTTCTGC-3') and reverse primer (3'-GTGACAAGCCAAGGCGGTGAG-5'). The reverse primer oligonucleotide and the forward primer oligonucleotide AAACCATGAACCTTTCTGC correspond to the nucleotide sequences 558–578 and 1–17 of rat VEGF mRNA (GenBank accession no. M32167). The GAPDH cDNA fragment was amplified using the forward primer (5'-CACCACCATGGAGAAGGCTG-3') and the reverse primer (5'-ATGATGTTCTGGGCTGCCCC-3') that correspond to 330–349 and 644–625 nucleotide sequences of the rat GAPDH gene, respectively (GenBank accession no. NM017008). The amplification was performed using HotStarTaq DNA Polymerase Kit and 0.25 μ Ci of deoxycytidine 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,

Hercules, CA). The bands corresponding to VEGF-164 and GAPDH mRNAs were cut and the DNAs extracted using Ultrafree-DA column (Millipore Corporation, Bedford, MA). The VEGF-164 cDNA radioactivities were counted using Beckman LS (6000SC) System (Beckman, Fullerton, CA), and normalized to those of the corresponding GAPDH cDNAs.

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, both endothelial cell-specific markers (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 μ mol/l fidarestat added for 4 h at the end of the experiment. 10 μ l of 10 μ mol/l CM-H₂DCFDA, the dichlorofluorescein 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-H₂DCFDA 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-H₂DCFDA fluorescence was expressed per 10⁶ cells. Parallel cultures (eight plates per condition) were grown without CM-H₂DCFDA 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 one of the following pro-oxidants: L-buthionine(S,R)-sulfoximine (BSO, 100 μ mol/l), diethyl maleate (DEM, 60 μ mol/l), or primaquine (50 μ mol/l). The pro-oxidants were added for 24 h at the end of the experiment. Parallel pro-oxidant-treated BREC cultures contained 1 μ mol/l fidarestat added for 4 h at the end of the experiment. ROS generation was assessed by the CM-H₂DCFDA fluorescence assay as described above.

Statistical analysis. The results are expressed as mean \pm 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 $P \leq 0.05$. 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 fidarestat 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 fidarestat treatments.

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 1

Final body weight and blood glucose concentrations in control and diabetic rats treated with or without fidarestat ($n = 38$ –39*)

Variable	Control	Diabetic	Diabetic + LF	Diabetic + HF
Body weight (g)	423 \pm 6	310 \pm 8†	323 \pm 8†	314 \pm 12†
Blood glucose (mmol/l)	4.44 \pm 0.11	20.1 \pm 0.33†	19.3 \pm 0.39†	18.9 \pm 0.39†

Data are means \pm SE. * $n = 38$ in the control group and $n = 39$ in three other groups. †Significantly different from controls ($P < 0.01$). LF, low dose fidarestat; HF, high dose fidarestat.

affected by treatments with either low or high doses of fidarestat. Retinal sorbitol concentrations were reduced 2.5-fold but not completely normalized by the low-dose fidarestat treatment. Fructose concentrations tended to decrease, but the difference with the untreated diabetic group did not achieve statistical significance. High dose of fidarestat completely normalized retinal sorbitol concentration, whereas retinal fructose concentration remained slightly elevated, but the difference with the control group did not achieve statistical significance.

Retinal VEGF protein concentration was increased two-fold in diabetic rats compared with the control group, and this increase was dose-dependently prevented by fidarestat (Fig. 2A). High dose fidarestat completely arrested diabetes-induced retinal VEGF protein overexpression. Total VEGF mRNA (Fig. 2B) and VEGF-164 mRNA abundance (Fig. 3A and B) were similar among the experimental groups.

Retinal lipid peroxidation product concentration was increased 1.6-fold in the diabetic rats compared with the control group, and this increase was totally arrested by both low and high doses of fidarestat (Table 3). Retinal GSH, GSSG, total AA, and AA and DHAA concentrations were similar among control rats and diabetic rats treated with or without fidarestat. Neither glutathione redox state nor ascorbate redox state (expressed as GSSG/GSH and DHAA/AA ratios, respectively) were affected by diabetes or fidarestat treatment. SOD, GSSG Red, and GSHPx activities were decreased in the retina of diabetic rats compared with controls. Catalase activity remained unchanged. GSHPx tended to decrease, but the difference between diabetic and control groups did not achieve statistical significance. Low dose fidarestat did not affect SOD activity, whereas high dose supranormalized it. Catalase activities were indistinguishable among diabetic rats

TABLE 2

Retinal sorbitol pathway intermediate concentrations in control and diabetic rats treated with or without fidarestat (nmol/mg protein, $n = 8$)

Variable	Control	Diabetic	Diabetic + LF	Diabetic + HF
Glucose	10.4 \pm 0.63	86 \pm 6.3*	92 \pm 9.2*	84 \pm 6.2*
Sorbitol	2.00 \pm 0.12	10.2 \pm 0.77*	4.1 \pm 0.56†‡	1.70 \pm 0.33‡
Fructose	1.18 \pm 0.10	5.08 \pm 0.52*	3.63 \pm 0.58*	1.85 \pm 0.37‡

Data are means \pm 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 fidarestat; HF, high dose fidarestat.

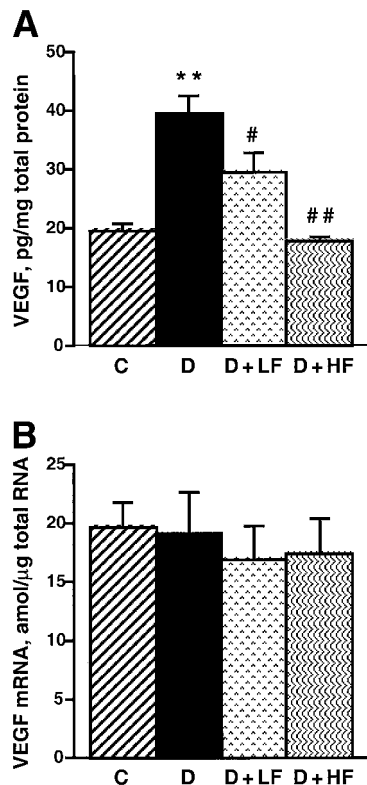


FIG. 2. Retinal VEGF protein (A) and mRNA (B) concentrations in control and diabetic rats treated with or without fidarestat (mean \pm SE, $n = 6-12^a$). ^aFor VEGF protein, $n = 7$, except the control group where $n = 6$. For VEGF mRNA, $n = 12$ in the control group and $n = 6$ in three other groups. **Significantly different versus controls ($P < 0.01$); #significantly different versus untreated diabetic group ($P < 0.05$); ##significantly different versus untreated diabetic group ($P < 0.01$).

treated with or without low or high doses of the ARI. GSSGRed and GSHPx activities tended to increase with the low-dose fidarestat treatment but the difference with the untreated diabetic group did not achieve statistical significance. GSSGRed activity was essentially normalized by the high dose of fidarestat, whereas GSHPx responded similarly to the low-dose and high-dose fidarestat treatments. In contrast, GSHTrans was normalized by low dose, but not high dose, of fidarestat. It tended to increase with the high-dose fidarestat 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 BRECs cultured in 30 mmol/l glucose than in those cultured in 5 mmol/l glucose (Table 4). High glucose-induced increase in BRECs glucose concentrations was not affected by fidarestat treatment, whereas sorbitol and fructose concentrations were reduced 2.3-fold and 2-fold, respectively.

Intracellular ROS abundance was 2.4-fold higher in BRECs 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 μ mol/l fidarestat.

Intracellular ROS abundance was 3.2-fold, 5.4-fold, and 16.7-fold higher in BRECs cultured in 5 mmol/l glucose supplemented with BSO, DEM, or primaquine, respectively, than in 5 mmol/l glucose without pro-oxidants (Fig.

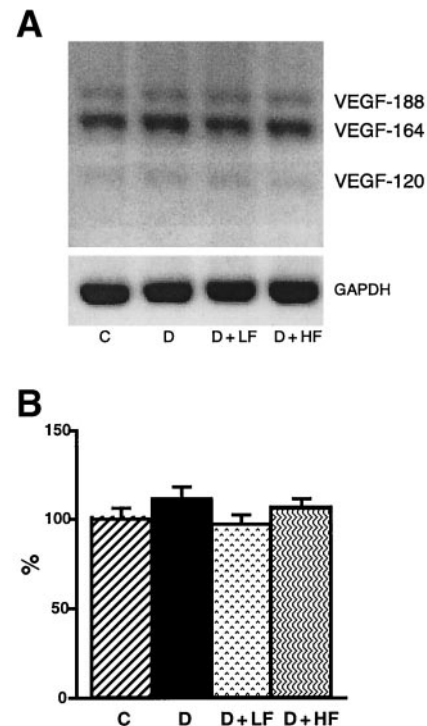


FIG. 3. A: Representative agarose gel analysis of VEGF-164 mRNA and GAPDH mRNA in the retinae of control rats and diabetic rats treated with or without low or high doses of fidarestat using RT-PCR assay. B: Retinal VEGF-164 mRNA abundance (mean \pm SE, $n = 7$) in the retinae of control and diabetic rats treated with or without low or high doses of fidarestat. VEGF-164 mRNA abundance in controls is taken as 100%.

4B). 1 μ mol/l fidarestat did not affect oxidative stress induced by any of the three pro-oxidants.

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 situ perfused organs (33,35), the aforementioned studies employed additions of relatively high concentrations of 4-hydroxynonenal, 100-200 μ mol/l. Until now, no evidence of

TABLE 3

Retinal lipid peroxidation product (MDA + 4-HA) concentrations ($n = 8$), the state of nonenzymatic antioxidants ($n = 8$) and antioxidative enzyme activities ($n = 8^*$) in control and diabetic rats treated with or without fidarestat

Variable	Control	Diabetic	Diabetic + LF	Diabetic + HF
MDA + 4-HA	0.874 ± 0.084	1.416 ± 0.091†	0.821 ± 0.054‡	0.799 ± 0.053‡
GSH	17.9 ± 0.6	18.1 ± 1.1	18.1 ± 1.1	19.2 ± 1.0
GSSG	0.429 ± 0.039	0.426 ± 0.049	0.439 ± 0.029	0.415 ± 0.042
GSSG/GSH	0.024 ± 0.002	0.024 ± 0.003	0.024 ± 0.001	0.022 ± 0.003
Total AA	15.7 ± 0.8	15.8 ± 0.8	15.0 ± 1.1	16.3 ± 0.8
Free AA	13.1 ± 0.8	12.9 ± 0.8	12.3 ± 1.1	13.6 ± 0.8
DHAA	2.60 ± 0.11	2.83 ± 0.20	2.70 ± 0.11	2.76 ± 0.15
DHAA/free AA	0.206 ± 0.019	0.227 ± 0.024	0.233 ± 0.024	0.210 ± 0.018
SOD	936 ± 67	556 ± 65†	540 ± 80§	1229 ± 119‡
Catalase	340 ± 26	307 ± 35	291 ± 33	349 ± 45
GSSGRed	105 ± 3	60 ± 5†	84 ± 13	96 ± 7¶
GSHPx	28 ± 4	19 ± 2	26 ± 2	24 ± 3
GSHTrans	546 ± 88	337 ± 53†	583 ± 39‡	401 ± 3

Data are means ± SE. *Except SOD in the diabetic group treated with high dose fidarestat ($n = 7$) and GSHPx in the control group ($n = 6$). MDA + 4-HA, GSH, GSSG, total AA, AA and DHAA concentrations are expressed in nmol/mg protein. Antioxidative defense enzyme activities are expressed in nmol substrate or NADPH per min per mg protein. LF, low dose fidarestat; HF, high dose fidarestat.

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-nonenic 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 implicate 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 GSHTrans, 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 supranormalized by the complete inhibition of the sorbitol pathway hyperactivity. These results are consistent with 1) the attenuation of diabetes-induced downregulation of SOD activity in the peripheral nerve of diabetic rats by the ARI sorbinil and 2) the upregulation of NADH oxidase, the

TABLE 4

Sorbitol pathway intermediate concentrations in BREC cultured in 5 and 30 mmol/l glucose with or without fidarestat (nmol/mg protein, $n = 8$)

Variable	5 mmol/l glucose	30 mmol/l glucose	30 mmol/l glucose + F
Glucose	5.08 ± 0.59	124.8 ± 11.0*	142.9 ± 19.1*
Sorbitol	0.590 ± 0.067	6.92 ± 0.839*	3.03 ± 0.431†‡
Fructose	3.11 ± 0.224	20.8 ± 2.75*	10.2 ± 1.0†‡

Data are means ± SE. *Significantly different from controls ($P < 0.01$); †significantly different from controls ($P < 0.05$); ‡significantly different from untreated diabetic group ($P < 0.01$). F, fidarestat.

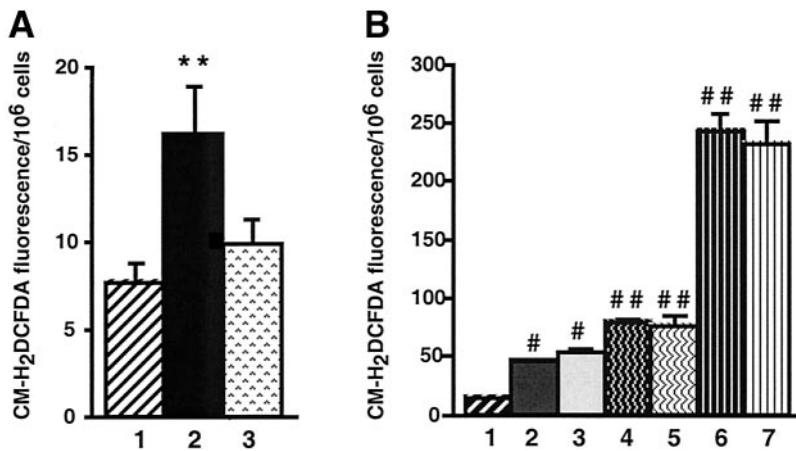


FIG. 4. A: Intracellular ROS abundance in BREC cultured in 5 and 30 mmol/l glucose with or without fidarestat (mean \pm SE, $n = 8$). Lane 1: 5 mmol/l glucose; lane 2: 30 mmol/l glucose; lane 3: 30 mmol/l glucose + fidarestat. **Significantly different versus controls ($P < 0.01$); #Significantly different versus BREC cultured in 30 mmol/l glucose without fidarestat ($P < 0.05$). **B:** Intracellular ROS abundance in BREC cultured in 5 mmol/l glucose and 5 mmol/l glucose plus one of the following pro-oxidants: BSO, DEM, and primaquine and with or without fidarestat (mean \pm SE, $n = 8-9$). Lane 1: 5 mmol/l glucose; lane 2: 5 mmol/l glucose + BSO; lane 3: 5 mmol/l glucose + BSO + fidarestat; lane 4: 5 mmol/l glucose + DEM; lane 5: 5 mmol/l glucose + DEM + fidarestat; lane 6: 5 mmol/l glucose + primaquine; lane 7: 5 mmol/l glucose + primaquine + fidarestat. *Significantly different versus BREC cultured in 5 mmol/l glucose without pro-oxidants ($P < 0.05$); **significantly different versus BREC cultured in 5 mmol/l glucose without pro-oxidants ($P < 0.01$).

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 fidarestat is not due to its direct antioxidant properties, which are not related to an ability to inhibit the sorbitol pathway of glucose metabolism. Fidarestat 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, fidarestat 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- α -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 fidarestat decreased diabetes-induced retinal VEGF overexpression, which supports the findings of the favorable effects of long-term treatment with 2 mg/kg of fidarestat on retinal pathological changes and increased vascular permeability in streptozotocin-diabetic rats (13). It remains to be established whether this dose of fidarestat 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 fidarestat (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 fidarestat. The findings support the "AR concept" of DR and provide the rationale for further evaluation of fidarestat 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 fidarestat (1 mg/day) in improving nerve

conduction deficits and symptomatic indexes of diabetic neuropathy.

ACKNOWLEDGMENTS

This study was supported by a grant from Sanwa Kagaku Kenkyusho (I.G.O.) and the Juvenile Diabetes Research Foundation Center for the Study of Complications of Diabetes Grant 4-200-421 (I.G.O., A.K.K., and M.J.S.).

REFERENCES

- Neuenschwander H, Takahashi Y, Kador PF: Dose-dependent reduction of retinal vessel changes associated with diabetic retinopathy in galactose-fed dogs by the aldose reductase inhibitor M79175. *J Ocul Pharm Ther* 13:517-528, 1997
- Frank RN, Amin R, Kennedy A, Hohman TC: An aldose reductase inhibitor and: aminoguanidine prevent vascular endothelial growth factor expression in rats with long-term galactosemia. *Arch Ophthalmol* 115:1036-1047, 1997
- Purves T, Middlemas A, Agthong S, Jude EB, Boulton AJ, Fernyhough P, Tomlinson DR: A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy. *FASEB J* 15:2508-2514, 2001
- Cameron NE, Eaton SE, Cotter MA, Tesfaye S: Vascular factors and metabolic: interactions in the pathogenesis of diabetic neuropathy. *Diabetologia* 44:1973-1988, 2001
- Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL: Characterization: of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 43:1122-1129, 1994
- 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
- Berkowitz BA, Ito Y, Kern TS, McDonald C, Hawkins R: Correction of early subnormal superior hemiretinal ΔPO_2 predicts therapeutic efficacy in experimental diabetic retinopathy. *Invest Ophthalmol Vis Sci* 42:2964-2969, 2001
- Kern TS, Engerman RL: Comparison of retinal lesions in alloxan-diabetic rats and galactose-fed rats: *Current Eye Res.* 13:863-867, 1994
- Kador PF, Takahashi Y, Wyman M, Ferris F, III: Diabetes-like proliferative retinal: changes in galactose-fed dogs. *Arch Ophthalmol* 113:352-354, 1995
- Robison WG Jr., Jacot JL, Glover JP, Basso MD, Hohman TC: Diabetic-like retinopathy: early and late intervention therapies in galactose-fed rats. *Invest Ophthalmol Vis Sci* 39:1933-1941, 1998
- Vinoves SA, Van Niel E, Swerdloff JL, Campochiaro PA: Electron microscopic immunocytochemical evidence for the mechanism of blood-retinal barrier breakdown in galactosemic rats and its association with aldose reductase expression and inhibition. *Exp Eye Res* 57:723-735, 1993
- Berther P, Farine JC, Barras JP: Calcium dobesilate: pharmacological profile related to its use in diabetic retinopathy. *Int J Clin Pract* 53:631-636, 1999
- Makino M, Taniko K, Ichikawa K, Okuyama M, Furuta Y, Kato N, Nakayama Y, Jomori T: Effects of aldose reductase inhibition on the retinal vascular permeability of streptozotocin-diabetic rats and VEGF production in cultured human retinal cells (Abstract). *Diabetes* 50 (Suppl. 2):A193, 2001
- Bursell SE, Clermont AC, Aiello LP, Aiello LM, Schlossman DK, Feener EP, Laffel L, King GL: High-dose vitamin E supplementation normalizes retinal blood flow and creatinine clearance in patients with type 1 diabetes. *Diabetes Care* 22:1245-1251, 1999
- Abiko T, Abiko A, Martin L, Clermont AC, Tsujikawa A, Shoelson B, King GL, Bursell SE: Regulation of oxidant formation by PKC- β inhibition, a potential mechanism for normalizing retinal leukostasis in diabetic rats (Abstract). *Invest Ophthalmol Vis Sci* 42:S207, 2001
- Cruz JW, Oliveira MA, Hohman TC, Fortes ZB: Influence of tolrestat on the defective leukocyte-endothelial interaction in experimental diabetes. *Eur J Pharmacol* 391:163-174, 2000
- Obrosova IG, Minchenko AG, Marinescu V, Fathallah L, Kennedy A, Stockert CM, Frank RN, Stevens MJ: Antioxidants attenuate early upregulation of retinal vascular endothelial growth factor in streptozotocin-diabetic rats. *Diabetologia* 44:1102-1110, 2001
- Aiello LP: Vascular endothelial growth factor and the eye: biochemical mechanisms of action and implications for novel therapies. *Ophthalm Res* 29:354-362, 1997
- Obrosova IG: L How does glucose generate oxidative stress in peripheral nerve? In *International Review of Neurobiology. Vol. 50. Neurobiology of Diabetic Neuropathy.* Tomlinson DR, Ed. San Diego, Academic Press, 2002, p. 3-35
- Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001
- Cameron NE, Cotter MA, Basso M, Hohman TC: Comparison of the effects of inhibitors of aldose reductase and sorbitol dehydrogenase on neurovascular function, nerve conduction and tissue polyol pathway metabolites in streptozotocin-diabetic rats. *Diabetologia* 40:271-281, 1997
- Nakamura J, Kato K, Hamada Y, Nakayama M, Chaya S, Nakashima E, Naruse K, Kasuya Y, Mizubayashi R, Miwa K, Yasuda Y, Kamiya H, Ienaga K, Sakakibara F, Koh N, Hotta N: A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. *Diabetes* 48:2090-2095, 1999
- Obrosova IG, Van Huysen C, Fathallah L, Cao X, Greene DA, Stevens MJ: An aldose reductase inhibitor reverses early diabetes-induced changes in peripheral nerve function, metabolism, and antioxidative defense. *FASEB J* 16:123-125, 2002
- Gupta S, Chough E, Daley J, Oates P, Tornheim K, Ruderman NB, Keane JF: Hyperglycemia increases endothelial superoxide that impairs smooth muscle cell Na^+K^+ -ATPase activity. *Am J Physiol* 282:C560-C566, 2002
- Mizuno K, Kato N, Makino M, Suzuki T, Shindo M: Continuous inhibition of excessive polyol pathway flux in peripheral nerves by aldose reductase inhibitor fidarestat leads to improvement of diabetic neuropathy. *J Diabetes Complications* 13:141-150, 1999
- Kato N, Mizuno K, Makino M, Suzuki T, Yagihashi S: Effects of 15-month aldose reductase inhibition with fidarestat on the experimental diabetic neuropathy in rats. *Diabetes Res Clin Pract* 50:77-85, 2000
- Hotta N, Toyota T, Matsuoka K, Shigeta Y, Kikkawa R, Kaneko T, Takahashi A, Sugimura K, Koike Y, Ishii J, Sakamoto N, the SNK-860 Diabetic Neuropathy Study Group: clinical efficacy of fidarestat, a novel aldose reductase inhibitor, for diabetic peripheral neuropathy. *Diabetes Care* 24:1776-1782, 2001
- Obrosova IG, Stevens MJ: Effect of dietary taurine supplementation on GSH and NAD(P)-redox status, lipid peroxidation, and energy metabolism in diabetic precataractous lens. *Invest Ophthalmol Vis Sci* 40:680-688, 1999
- Obrosova IG, Fathallah L, Greene DA: Early changes in lipid peroxidation and antioxidative defense in diabetic rat retina: effect of DL- α -lipoic acid. *Eur J Pharmacol* 398:139-146, 2000
- 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
- Sone H, Deo BK, Kumagai AK: Enhancement of glucose transport by vascular endothelial growth factor in retinal endothelial cells. *Invest Ophthalmol Vis Sci* 41:1876-1884, 2000
- Srivastava SK, Bhatnagar A: Metabolism of the lipid peroxidation product, 4-hydroxy-trans-2-nonenal, in isolated perfused rat heart. *J Biol Chem* 273:10893-10900, 1998
- Rittner HL, Hafner V, Klimiuk PA, Szveda 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
- Robison WG Jr., Jacot JL, Katz ML, Glover JP: Retinal vascular changes induced by the oxidative stress of alpha-tocopherol deficiency contrasted with diabetic microangiopathy. *J Ocul Pharmacol Ther* 16:109-120, 2000
- Grune T, Siems WG, Petras T: Identification of metabolic pathways of the lipid peroxidation product 4-hydroxynonenal in in situ perfused rat kidney. *J Lipid Res* 38:1660-1665, 1997
- Kishi Y, Schmelzer JD, Yao JK, Zollman PJ, Nickander KK, Tritschler HJ, Low PA: Alpha-lipoic acid: effect on glucose uptake, sorbitol pathway, and energy metabolism in experimental diabetic neuropathy. *Diabetes* 48:2045-2051, 1999
- Lowitt S, Malone JJ, Salem AF, Korhals J, Benford S: Acetyl-L-carnitine corrects the altered peripheral nerve function of experimental diabetes. *Metabolism* 44:677-680, 1995
- Lee AYW, Chung SSM: Contribution of polyol pathway to oxidative stress in diabetic cataract. *FASEB J* 13:23-30, 1999
- Yim HS, Kang SO, Hah YC, Chock PB, Yim MB: Free radicals generated during the glycation reaction of amino acids by methylglyoxal: a model study of protein-cross-linked free radicals. *J Biol Chem* 270:28228-28233, 1995
- Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, Pinsky D, Stern D: Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 269:9889-9897, 1994
- Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh

- T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H: High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49:1939–1945, 2000
42. Desco MC, Asensi M, Márquez R, Martínez-Valls J, Vento M, Pallardó FV, Sastre J, Viña J: Xanthine oxidase is involved in free radical production in type 1 diabetes: protection by allopurinol. *Diabetes* 51:1118–1124, 2002
43. Lou MF, Dickerson JE Jr., Garadi R, York BM Jr: Glutathione depletion in the lens of galactosemic and diabetic rats. *Exp Eye Res* 46:517–530, 1988
44. Hohman TC, Carper D, Dasgupta S, Kaneko M: Osmotic stress induces aldose reductase in glomerular endothelial cells. In *Enzymology and Molecular Biology of Carbonyl Metabolism*. Vol. 3. Weiner H, Wermuth B, Crabb DW, Eds. Plenum Press, New York, 1999, p. 139–152
45. Ellis EA, Guberski DL, Somogyi-Mann M, Grant MB: Increased H₂O₂, vascular endothelial growth factor and receptors in the retina of the BBZ/Wor diabetic rat. *Free Radic Biol Med* 28:91–101, 2000
46. Obrosova I, Minchenko A, Kennedy A, Szabó C, Frank R, Stevens M: Poly(ADP-ribose)synthetase inhibitors counteract diabetes- and hypoxia-induced retinal VEGF formation (Abstract). *Ophthalm Res* 34 (Suppl. 1):128, 2002
47. Morley SJ: Signalling through either the p38 or ERK mitogen-activated protein (MAP) kinase pathway is obligatory for phorbol ester and T cell receptor complex (TCR-CD3)-stimulated phosphorylation of initiation factor (eIF)4E in Jurkat T cells. *FEBS Lett* 418:327–332, 1997
48. Ishii H, Tada H, Isogai S: An aldose reductase inhibitor prevents glucose-induced increase in transforming growth factor-beta and protein kinase C activity in cultured mesangial cells. *Diabetologia* 41:362–364, 1998
49. Du X, Matsumura T, Szabó C, Edelstein D, Brownlee M: Hyperglycemia-induced superoxide activates PKC, the hexosamine poathway, AGE formation, and NFκB via poly(ADP-ribose)polymerase inhibition of GAPDH (Abstract). *Diabetes* 51 (Suppl. 2):A175, 2002
50. Naruse K, Nakamura J, Hamada Y, Nakayama M, Chaya S, Komori T, Kato K, Kasuya Y, Miwa K, Hotta N: Aldose reductase inhibition prevents glucose-induced apoptosis in cultured bovine retinal microvascular pericytes. *Exp Eye Res* 71:309–315, 2000