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Diabetes-induced Activation of Caspase-3 in Retina: Effect of Antioxidant Therapy

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Apoptosis of retinal endothelial cells and pericytes is postulated to contribute to the development of retinopathy in diabetes. The goal of this study is to investigate diabetes-induced activation of retinal caspase-3, an apoptosis executer enzyme, in retina, and examine the effects of antioxidants on the activation. Caspase-3 activation was determined in the retina of alloxan diabetic rats (2–14 months duration) and in the isolated retinal capillary cells (endothelial cells and pericytes) by measuring cleavage of caspase-3 specific fluorescent substrate, and cleavage of caspase-3 holoenzyme and poly (ADP ribosyl) polymerase. Effect of antioxidants on the activation of caspase-3 was determined by feeding a group of diabetic rats diet supplemented with a comprehensive mixture of antioxidants, including Trolox, α-tocopherol, *N*-acetyl cysteine, ascorbic acid, β-carotene and selenium for 2–14 months, and also under *in vitro* conditions by incubating isolated retinal capillary cells with antioxidants with wide range of actions. Caspase-3 was activated in the rat retina at 14 months of diabetes ($P < 0.05$ vs. normal), but not at 2 months of diabetes, and administration of antioxidants for the entire duration inhibited this activation. In the isolated retinal capillary cells incubated in 25 mM glucose medium, caspase-3 activity was increased by 50% compared to the cells incubated in 5 mM glucose ($P < 0.02$), and antioxidants or caspase-3 inhibitor inhibited this increase. Our results suggest that increased oxidative stress in diabetes is involved in the activation of retinal caspase-3 and apoptosis of endothelial cells and pericytes. Antioxidants might be inhibiting the development of diabetic retinopathy by inhibiting microvascular apoptosis.

Keywords: Antioxidants; Apoptosis; Caspase; Diabetes; Oxidative stress; Retinopathy

INTRODUCTION

Hyperglycemia-induced abnormalities in retinal metabolism, including increased polyol pathway, nonenzymatic glycation, activity of protein kinase C and oxidative stress, have been postulated to contribute to the development of retinopathy,[1–6] but it has been difficult to recognize which abnormalities are critical. In the pathogenesis of diabetic retinopathy retinal microvascular pericytes and endothelial cells are lost selectively before other histopathology is detectable, or loss of vision is evident.[7,8] One of the possible mechanisms postulated to be involved in the retinal cell death in diabetes is the programmed cell death, apoptosis.[8,9] Recent studies have shown that the frequency of early apoptosis in retinal microvascular cells can predict the development of histological lesions.[7]

In diabetes, oxidative stress is increased in the retina and dietary supplementation with a mixture of antioxidants can inhibit diabetes-induced abnormalities of retinal metabolism.[10–12] Our recent studies have demonstrated that long-term administration of antioxidants to diabetic rats and galactose-fed rats (another animal model of diabetic retinopathy) inhibits the development of retinopathy[6] and this, for the first time, shows a strong association between increased oxidative stress and the development of retinopathy in diabetes.

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Caspases, a family of thiol proteases, are important players in the apoptotic cascade, and high glucose is shown to activate retinal caspases.[13,14] The processing of pro-caspase-3 to its active form is considered to be a point of no return in the death-signaling cascade. Caspase-3 is a predominant target involved in the reactive oxygen species-mediated high glucose induced apoptosis in human endothelial cells.[15] Thus, inhibition of the enzymatic activity of caspases might provide a mechanism to abort the apoptotic program.

In the present study, we have investigated the activation of retinal caspase-3 in diabetes, and the effect of long-term administration of antioxidants on its activation. The retinal cells involved in hyperglycemia-induced activation of caspase-3 are identified using isolated bovine retinal endothelial cells and pericytes in culture.

METHODS

Sprague–Dawley rats (male, 200–220 g) were randomly assigned to normal or diabetic groups. Diabetes was induced with alloxan monohydrate (45 mg/kg body weight, intravenously), and if the rats were losing weight, insulin was given as needed to allow slow weight gain while maintaining hyperglycemia (blood glucose levels of 20–25 mM). A group of diabetic rats were fed diet supplemented with a comprehensive mixture of antioxidant consisting ascorbic acid, 1 g/kg; Trolox 500 mg/kg; *dl* α-tocopherol acetate, 250 mg/kg; *N*-acetyl cysteine, 200 mg/kg; β-carotene, 45 mg/kg; and selenium, 0.1 mg/kg of diet. The antioxidant diet was replaced weekly, and food consumption was measured to calculate the amount of antioxidants consumed. Nonenzymatically glycated hemoglobin (GHb) was measured at 2 months of diabetes, and every 3 months thereafter, using affinity columns (Glyc-Affin; Pierce, Rockford, IL). We have described all of these methods previously. Animals were sacrificed at 2 months or 14 months of diabetes after an overnight fast, and the retina was immediately removed. These experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Retinal capillary cells were prepared from bovine eyes using the method described by Kennedy *et al*.[16] This method yields homogenous preparations of both endothelial cells and pericytes; the cells exhibit their typical morphology (endothelial cells with "cobblestone" morphology and pericytes with characteristic irregular polygonal shape and loose monolayers), and positive and negative staining, respectively, for factor VIII.[16] Endothelial cells were grown to 80% confluence in petridishes coated with 0.1% gelatin in Dulbecco's Modified Eagle Medium (DMEM) containing heparin, 10% fetal calf serum (heat inactivated), 10% Nu-serum, endothelial growth supplement (25 μg/ml) and antibiotic/antimycotic in an environment of 95% air and 5% CO_2. Confluent cells from 5th to 7th passage were split and incubated in DMEM supplemented with 1% fetal calf serum (heat inactivated), 2.5% Nu-serum, 2.5 (g/ml endothelial cell growth supplement and heparin, under normoglycemic (5 mM glucose) or hyperglycemic (25 mM glucose) conditions for up to 10 days. Bovine retinal pericytes were grown in DMEM supplemented with 10% fetal calf serum, antibiotics, and antimycotics. Pericytes (4th–6th passage) were incubated in DMEM containing 2.5% fetal bovine serum in 5 or 25 mM glucose. The cells were incubated in the presence or absence of antioxidants: 0.25 mM *N*-acetyl cysteine, 0.25 mM lipoic acid, 0.2 mM *dl* α-tocopherol acetate, 0.2 mM Trolox, 0.1 mM ascorbic acid, or 1 μM β-carotene. Cultures were routinely found to be free of contaminants. Control incubations containing 25 mM mannitol always were run simultaneously to rule out the effect of increased osmolarity. The media were changed every other day, and fresh antioxidants were added at every change.

To further investigate if caspase inhibition can prevent high glucose-induced oxidative stress, the cells were incubated in 5 or 25 mM glucose medium containing 100 μM of Ac-DEVD-CHO (BIOMOL, Plymouth, PA) for 10 days. Each experiment was repeated with at least three different cell preparations, and measurements were done in duplicate.

Lipid peroxides were estimated in retina by measuring the levels of thiobarbituric acid reactive substances (TBARS). The fluorescence of malonaldialdehyde-thiobarbituric acid adducts formed by acid hydrolysis at 100°C was measured at excitation and emission wavelengths of 535 and 553 nm, respectively. In the isolated retinal microvascular cells, lipid peroxides were measured using an assay kit from Cayman Chemical (Ann Arbor, MI). Hydroperoxides were measured directly by the redox reactions with ferrous ions, and the resulting ferric ions were detected using thiocynate ion as the chomogen.

To measure caspase-3 activity, retina or isolated cells were suspended in 100 mM HEPES buffer (pH 7.5) containing 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT and the protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin and leupeptin). The samples were left on ice for 15 min, sonicated, and centrifuged at 10,000*g* for 5 min. Caspase-3 activity was measured in the supernatant using15–20 μg protein/tube, and 25μM fluoregenic substrate specific for caspase-3, *N*-acetyl-As-Glu-1-Val-asp-7-amino-4 trifluoromethyl coumarin (DEVD-AFC). Fluorescence signal emitted by the cleavage of the substrate was quantitated at

excitation and emission wavelengths of 400 and 505 nm, respectively.[14,17] The enzyme activity was linear for up to 100 μg protein.

PARP is cleaved during the apoptotic process, and its cleavage can be detected by measuring the smaller fragment (about 85 kDa) of PARP by Western blot analysis. Retinal protein or cell lysate (40 μg protein) was separated on a 10% reducing polyacrylamide gel, and then transferred to nitrocellulose membranes. The membranes were blocked in 5% milk, followed by the incubation with a polyclonal antibody against PARP. After washing the blots with phospahte buffer saline (PBS) containing 0.1% Tween 20 the membranes were incubated with anti-rabbit IgG horseradish peroxidase-conjugated antibody in blocking buffer for 1 h. The membranes were developed using ECL-Plus Western blotting detection kit from Amersham Pharmacia Biotech, UK.

Activation of caspase-3 results in the proteolytic cleavage of caspase-3 holoenzyme, and the cleavage of caspase-3 was detected in retinal lysate or cell lysate (40 μg protein) by Western blot analysis. Protein was separated on a 12% SDS polyacrylamide gel, the membranes were incubated overnight with anticaspase-3 antibody (Santa Cruz, CA), washed, incubated with secondary antibody for 1 h, and developed using ECL-Plus kit.

Tissue protein was measured by the Bradford method[18] using bovine serum albumin as standard. Data are reported as mean ± SD and analyzed statistically using the nonparametric Kruskal–Wallis test followed by Mann–Whitney test for multiple group comparison. Similar conclusions were reached also by using ANOVA with Fisher or Tukey.

RESULTS

Diabetic rats (both 2 and 14 months durations) presented elevated levels of TBARS in the retina compared to their respective age-matched normal controls (Fig. 1a). Caspase-3 enzyme activity was not detectable in the retina of rats diabetic for 2 months despite increased oxidative stress, but was increased by 35% in the retina of rats diabetic for 14 months as compared to age matched normal rats (Fig. 1b).

In long-term diabetic rats, increased cleavage of the 116 kDa PARP into its 85 kDa fragment was observed in the retina, where as little cleavage could be seen in the retina from age-matched normal rats (Fig. 2a). In addition, the results presented here show that proteolytic cleavage of caspase-3 could be seen in the retina of the rats diabetic for 14 months compared to the age-matched normal, as represented by the increased expression of 17 kDa subunit (Fig. 2b). Administration of antioxidants for the entire duration of diabetes normalized increases in retinal lipid peroxides in the rats at 2 or 14 months of diabetes. In the same long-term diabetic rats, antioxidant administration inhibited diabetes-induced activation of retinal caspase-3, which was demonstrated by the measure of substrate cleavage, and by PARP and caspase-3 cleavage measured by western blots (Figs. 1 and 2). The beneficial effects of antioxidants on caspase-3 activation were not mediated by the reduction in the severity of hyperglycemia: GHb, blood glucose, urine volume and food intake remained comparable between diabetic rats receiving diets supplemented with and without antioxidants (Table I).

Incubation of isolated retinal endothelial cells with 25 mM glucose for 10 days increased lipid peroxides by about 2 folds ($P < 0.02$, Table II), and caspase-3 activity (measured by the substrate cleavage) by 50% (Fig. 3) as compared to the cells incubated in 5 mM glucose. Similar results were obtained when retinal

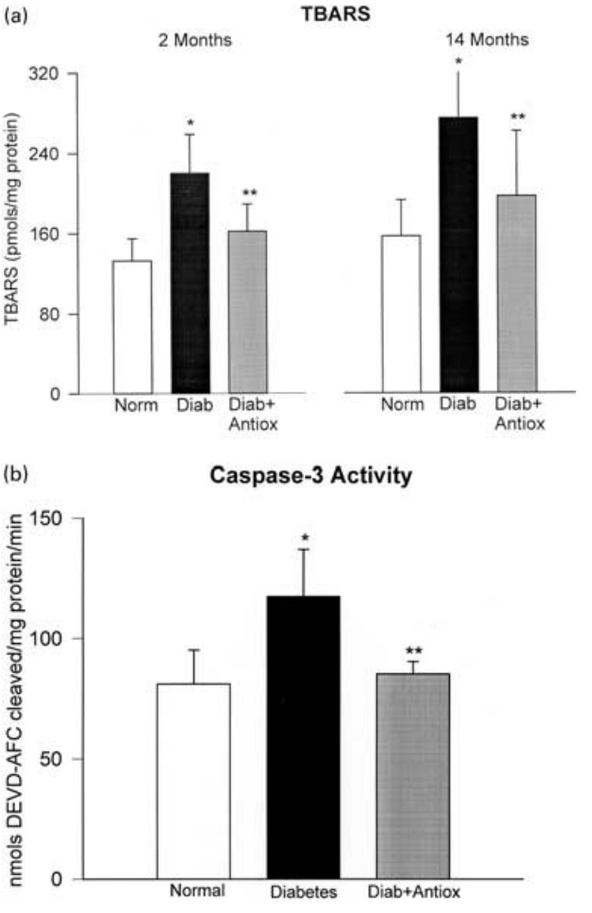

FIGURE 1 Lipid peroxides and caspase-3 activity in the retina of diabetic rats: (a) lipid peroxides and (b) enzyme activity of caspase-3 were determined in the retina of rats diabetic for 2 months and 14 months, and compared with the age-matched normal rats. No measurable caspase-3 activity was detected in the retina at 2 months of diabetes. Six to eight rats were used in each group. *$P < 0.05$ compared to normal, and **$P < 0.05$ compared to diabetes.

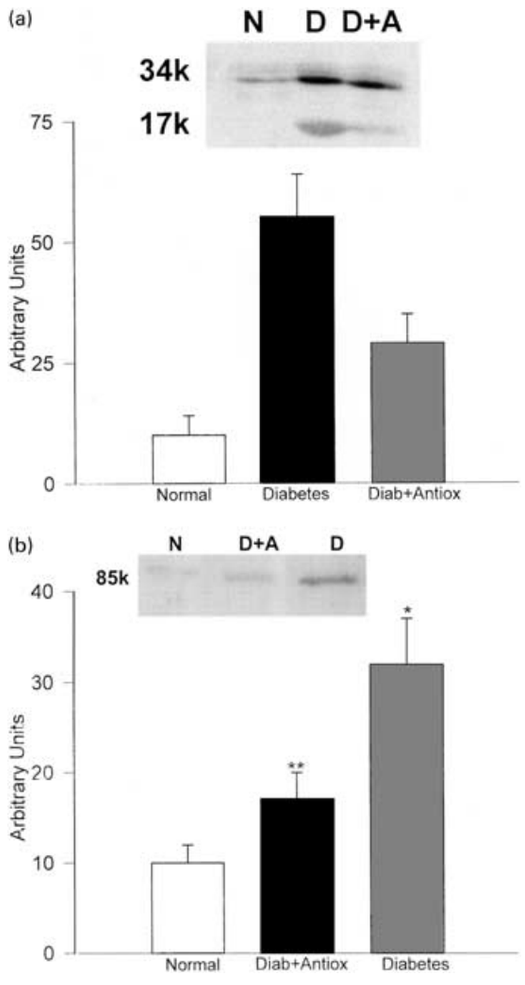

FIGURE 2 Caspase-3 and PARP cleavage in the retina of diabetic rats: retinal lysate (40 μg protein) from normal, diabetic, diabetic + antioxidants rats (14 months duration) were separated on SDS-gel (12% for caspase-3 and 10% for PARP). (a) Cleavage of pro-caspase; (b) cleavage of PARP. N: normal; D: diabetes; D + A: diabetes + antioxidants. The results are representative of at least three different animals in each group.

pericytes were incubated in 25 mM glucose medium (Table II and Fig. 3). Addition of caspase-3 inhibitor at the time of incubation with 25 mM glucose inhibited glucose-induced activation in caspase-3 activity and lipid peroxides in both endothelial cells and pericytes. Moreover, inclusion of individual antioxidants in 25 mM glucose medium inhibited both increases in lipid peroxides and activation of caspase-3 in these capillary cells (Table II and Fig. 3), however, these antioxidants had no effect on the cells incubated in 5 mM glucose medium. PARP and caspase-3 cleavages were also increased in the retinal endothelial cells incubated with high glucose for 10 days, and antioxidants prevented such cleavages. Caspase-3 activity was not measurable in the cells incubated in 5 or 25 mM glucose for only 3 days. Cells incubated in 25 mM mannitol showed similar results as obtained from the cells incubated in 5 mM glucose.

DISCUSSION

The results presented here show that caspase-3, the enzyme responsible for execution of apoptosis, is not active in the retina at 2 months of diabetes in rats (a duration where retinal capillary cell death and histopathology cannot be detected in the retina), but becomes active at 14 months of diabetes, a duration where histopathology is present.[6] In the pathogenesis of diabetic retinopathy, retinal pericytes and endothelial cells are lost selectively by apoptosis,[7,8] retinal cell death enzymes are over expressed,[19] the levels of the proapoptotic protein Bax are increased,[20] and caspases are activated.[13] The present study suggests that increased oxidative stress in diabetes is playing an important role in the activation of caspase-3.

Apoptosis can readily account for the pericyte "drop-out" and formation of "ghosts" in diabetic retinopathy. The cell contains fragmented DNA for only a few hours,[21] defective endothelial cell replication, seen in hyperglycemia,[22] could accelerate the process of retinal ischemia by exhausting the cell's replicative capacity, and poor replicative capabilities of retinal pericytes[23] could add insult to the process. Our results show that caspase-3 activation in diabetes, seen at a duration when histopathology is present,[6] could be playing an active role in the process of cell death.

Increased serum lipid hydroperoxides are associated with increased prevalence of retinopathy in diabetes,[24] and oxidative stress is involved in pericyte dropout seen in diabetic retinopathy.[19] Oxidative stress can result in increased apoptosis by damaging other macromolecules vital to cellular functions, signal transduction and genomic processes,[25–27] and caspase-3 is considered as one of

TABLE I Administration of antioxidants had no effect on the severity of hyperglycemia in rats diabetic for 14 months

	GHb (%)	Blood glucose (mg/dl)	Urine volume (ml/24 h)	Body weight (g)
Normal	4.0 ± 0.6	56 ± 4	5 ± 3	578 ± 76
Diabetes	12.5 ± 0.8*	335 ± 19*	126 ± 21*	304 ± 49*
Diabetes + antioxidant	13.3 ± 2.2*	331 ± 101*	112 ± 20*	286 ± 50*

Blood glucose was measured after an overnight fast, and 24-h urine was measured over 2–3 consecutive days. Values are mean ± SD of 7–9 rats in each group. *$P < 0.02$ compared to normal.

TABLE II Incubation of retinal capillary cells with glucose elevates lipid peroxides, and antioxidants and caspase-3 inhibitor inhibit such activation

	Lipid peroxides (pmols/mg protein)	
	Endothelial cells	Pericytes
5 mM Glucose	25 ± 1.9	21 ± 3.7
25 mM Glucose	60 ± 3.5*	39 ± 6.1*
25 mM Glucose + *N*-acetyl cysteine	26 ± 3.1	25 ± 2.8
25 mM Glucose + lipoic acid	28 ± 1.7	24 ± 4.2
25 mM Glucose + Trolox	26 ± 2.3	22 ± 2.4
25 mM Glucose + α-tocopherol	20 ± 3.2	17 ± 2.6
25 mM Glucose + ascorbic acid	29 ± 6.1	ND
25 mM Glucose + β-carotene	25 ± 1.5	ND
25 mM Glucose + Ac-DEVD-CHO	32 ± 2.9	27 ± 3.4

Values are mean ± SD of 3–4 separate experiments in each group. *$P < 0.02$ compared to 5 mM glucose. ND: Not determined.

the predominant targets involved in the reactive oxygen species-mediated high glucose-induced apoptosis.[15] Our recent studies have shown that long-term administration of a comprehensive mixture of antioxidants inhibits the development of retinopathy in animal models of diabetic retinopathy,[6] and here the data is presented showing that the same antioxidant mixture is capable of inhibiting diabetes-induced activation of caspase-3 in retina. This suggest that in diabetes retinal caspase-3 might be compromised due to increased oxidative stress, and antioxidants, via inhibiting oxidative stress, normalize caspase-3 activation and retinal cell death. However, we cannot rule out the possibility that the antioxidants included in our comprehensive antioxidant mixture might be inhibiting diabetes-induced activation of caspase-3 in the retina by mechanisms other than inhibiting increased oxidative stress, but the results from *in vitro* experiments clearly demonstrate that various individual antioxidants with a broad spectrum of mode of action, are inhibiting hyperglycemia-induced caspase-3 activation.

The data presented here show that in rats diabetic for 2 months we could not detect any activity of caspase-3 in the retina by our method despite increased oxidative stress. This may be due to the possibility that the activation of caspase-3 is a slower process compared to the increase in oxidative stress in diabetes, or caspase-3 activation might be occurring as a consequence of increased oxidative stress. The role of oxidative stress in the activation of caspase-3 in diabetes is strengthened by our recent findings showing that the same antioxidant mixture

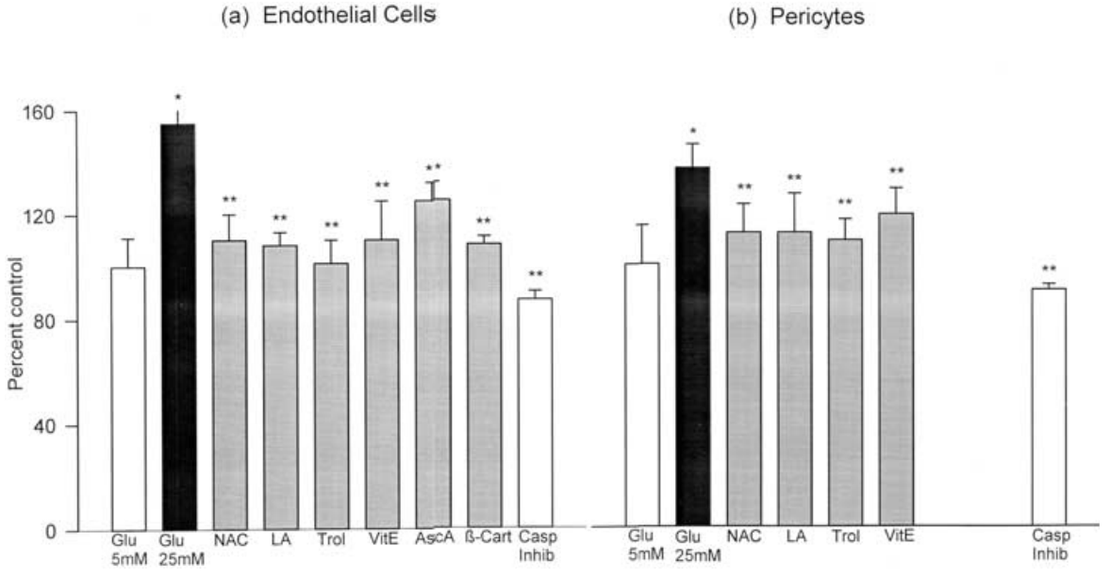

FIGURE 3 Activity of caspase-3 in the isolated endothelial cells and pericytes incubated in high glucose: Bovine retinal endothelial cells or pericytes were incubated in 5 mM glucose (Glu) or 25 mM glucose for 10 days in the presence or absence of 0.25 mM *N*-acetyl cysteine (NAC), 0.25 mM α-lipoic acid (LA), 0.2 mM Trolox (Trol) or *dl* α-tocopherol acetate (VitE), 0.1 mM ascorbic acid AscA), 1 μM β-carotene (β-Cart), or 0.1 mM Ac-DEVD-CHO (Casp Inhib). Each experiment was repeated with at least three different cell preparations, and measurements done in duplicate. Values obtained with 5 mM glucose were considered 100%. *$P < 0.05$ compared to 5 mM glucose, and **$P < 0.05$ compared to 25 mM glucose.

is able to inhibit the development of retinopathy in diabetic rats,[6] and by the *in vitro* results presented here which show that the individual antioxidants, which are known to function through different mechanisms; including metal chelating, scavenging reactive oxygen species, protecting polyunsaturated membrane lipids and DNA, and inhibition of lipid peroxidation,[28–31] inhibit glucose-induced activation of caspase-3.

Caspase-3 activity was measured in the whole retina, the number of vascular cells in retina is small compared to nonvascular neuronal cells, and diabetes is shown to increase transferase-mediated dUTP nick end labeling (TUNEL)-positive staining in the retinal ganglion and Muller cells,[9] and vascular cells.[7] Thus, the contribution of nonvascular cells in diabetes-induced increase in retinal caspase-3 activity, observed in the present study, needs to be recognized. However, the data from the cell culture studies presented here show that incubation of both isolated retinal pericytes or endothelial cells with 25 mM glucose results in the activation of caspase-3, which can be demonstrated by substrate cleavage, PARP and caspase-3 cleavage. In addition, high glucose increases oxidative stress in both of these cells, and antioxidants and caspase inhibitor inhibit oxidative stress, caspase activation, and DNA laddering (data not shown). This suggests that the capillary cells are one of the major contributors to the caspase-3 activation seen in the retina of long-term diabetic rats. The possibility that the cultured cells may not truly be reflecting the nature of stress experienced by the cells *in vivo* cannot be ruled out. However, in our experience,[32] and the experience of others,[27] cultured endothelial cells have predicted the *in vivo* status of retinal endothelial cells.

Our *in vitro* results show that caspase-3 inhibitor (DEVD-CHO) inhibits only the portion of caspase-3 activity induced by high glucose. This may be due to the fact that DEVD-CHO is very specific for caspase-3, but the substrate (*N*-acetyl-As-Glu-1-Val-asp-7-amino-4 trifluoromethyl coumarin) can be cleaved also by other caspases including caspase-7 and caspase-8. Since retina is shown to have multiple caspases,[13] the contribution of other caspases in the measurement of activity by this substrate cleavage cannot be excluded. However, the activation of caspase-3 in retina in hyperglycemia is confirmed by three independent methods including, cleavage of the 116 kDa PARP into its 85 kDa fragment, proteolytic cleavage of caspase-3 holoenzyme resulting in increased expression of 17 kDa subunit, and break down of the fluorogenic substrate. The results from all of these methods show unequivocally that caspase-3 is activated in retina in diabetes.

In summary, our results show that retinal caspase-3 is not active at 2 months of diabetes despite increased oxidative stress, but is active at the duration of diabetes when histological lesions are present. Increased oxidative stress in diabetes could be contributing to the activation of caspase-3 and apoptosis in retinal endothelial cells and pericytes. The mechanism by which antioxidants inhibit the development of retinopathy in diabetes might involve inhibition of apoptosis of retinal capillary cells.

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