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Metabolic memory in diabetes – from in vitro oddity to in vivo problem: Role of Apoptosis

Renu A. Kowluru^{*} and Pooi-See Chan

Kresge Eye Institute, Wayne State University, 4717 St. Antoine, Detroit, MI 48201, United States

Abstract

Retinal capillary cells undergo apoptosis before pathology characteristic of retinopathy can be observed, and the appearance of apoptotic capillary cell can predict the development of pathology. The purpose of this study is to investigate the effect of reversal of hyperglycemia on retinal capillary cell apoptosis, and identify the apoptosis encoding genes. Streptozotocin-diabetic rats were maintained either in poor glycemic control (PC, glycated hemoglobin, GHb >11%) or in good glycemic control (GC, GHb <6%) for 12 months, or allowed to be in PC for 6 months followed by GC for 6 additional months (PC–GC). Capillary cell apoptosis was determined in the trypsin-digested retinal microvasculature by TUNEL staining, and the genes encoding apoptosis were identified by Oligo GEArray rat apoptosis microarray that profiles 113 genes. Six months of good glycemic control that followed 6 months of poor control failed to attenuate the number of TUNEL-positive capillary cells in the retinal microvasculature. Twenty-three retinal genes, mainly from TNF ligand and receptor, caspase, Bcl-2 and death domain subfamilies that were upregulated by least a two-fold in PC rats remain upregulated after reversal of hyperglycemia. Thus, the continued activation of apoptosis plays a major role in the resistance of retinopathy to halt after re-institution of good glycemic control, and the regulation apoptosis machinery could help retard the progression of diabetic retinopathy.

Keywords

Apoptosis; Diabetic retinopathy; Metabolic memory

1. Introduction

In the development of diabetic retinopathy retinal microvascular cells (pericytes and endothelial cells) and other non-capillary cells (e.g. Muller cells and glial cells), are lost selectively via apoptosis [1,17,31]. Although histopathology of diabetic retinopathy takes over decades in humans and about one year in rodents to develop, apoptosis is a rapidly consummated phenomenon, and the cell contains fragmented DNA for only a few hours [34]. Defective endothelial cell replication seen in diabetic conditions accelerates the process of retinal ischemia by exhausting the cell's replicative capacity, thus accelerated apoptosis contributing to the pericyte 'drop-out' and formation of 'ghosts' in diabetic retinopathy. Our previous studies have shown that TdT-mediated dUTP nick end labeling (TUNEL)-positive cells are detectable in rat retinal vasculature at 6–8 months of hyperglycemia [17,31] suggesting that in diabetic retinopathy vascular cells are degenerating

^{*}Corresponding author. Tel.: +1 313 993 6714; fax: +1 313 577 8884., rkowluru@med.wayne.edu (R.A. Kowluru).

Conflict of interest

Drs Renu A. Kowluru and Pooi-see Chan have no financial, personal or other relationship with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) their work.

before other histopathology is detectable, and that the clinically silent initial phase of diabetic retinopathy consists of irreversible cellular events with late structural consequences [17].

Good glycemic control, instituted early in the course of diabetes, is shown to reduce the incidence and progression of retinopathy effectively, and the advantages of intensive glycemic control persist beyond the duration of maintenance of good glycemic control. However, if good glycemic control is established after a profound period of poor glycemic control, it does not immediately benefit the progression of retinopathy [7,8,28,38]. This 'metabolic memory' phenomenon was first reported in isolated human umbilical vein endothelial cells showing that glucose-induced overexpression of fibronectin is not readily reversible after the cells that were pre-exposed to high glucose are exposed to normal glucose [36]. Further studies have duplicated this phenomenon in animal models of diabetic retinopathy, including diabetic or experimentally galactosemic dogs and rats, also show this metabolic memory phenomenon [3,9,11,15,18,21,23,24]. We have identified many metabolic abnormalities associated with the metabolic memory phenomenon; retinal vasculature continues to accumulate elevated levels of nitrotyrosine and its DNA oxidatively modified, inflammatory mediators remain activated, and glyceraldehyde-3-phosphate dehydrogenase compromised at least for 6 months after re-institution of good glycemic control in rats that are allowed to remain in poor glycemic control for 6 months [3,15,18,23].

The process of activation of the enzyme involved in the execution of apoptosis and nuclear transcriptional factor (NF- κ B), which is considered to act as pro-apoptotic in the pathogenesis of diabetic retinopathy, occur early in the development of retinopathy in diabetes, and re-institution of good metabolic control in diabetic rats does not reverse these activations [21,25]. The purpose of this study is to investigate the genes encoding apoptosis by Oligo GEArray rat apoptosis microarray that profiles 113 genes in the retina in diabetes, and determine the effect of good glycemic control after 6 months of poor glycemic control on the apoptosis encoding genes and retinal capillary cell apoptosis.

2. Methods

2.1. Maintenance of rats in glycemic control

Wistar rats (male, 200–220 g body weight) were assigned to normal or diabetes groups. Soon after establishment of diabetes (3–4 days after streptozotocin administration) diabetic rats were divided into two groups; rats in group 1 remained in poor glycemic control (glycated hemoglobin, GHb >11%, PC) for the entire duration of the experiment (12 months), while those in group 2 were in poor glycemic control for 6 months followed by good glycemic control (GHb <6.5%, GC) for 6 additional months (PC–GC). Some of the same rats have been used by us in our previous studies [15,23]. Diabetic rats received insulin (NPH) injections, and the dose and frequency of insulin administration was adjusted to achieve the desired degree of hyperglycemia. The rats in poor glycemic control group received a single injection of insulin (1–2 units) 4–5 times a week to prevent ketosis and weight loss, and the rats in good glycemic control group received insulin twice daily (7–8 units total) to maintain their blood glucose levels below 150 mg/dl and a steady gain in body weight [15,23]. The entire rat colony was housed in metabolism cages, and 24 h urine volumes were measured on a daily basis. Blood glucose was measured once every week using Elite Glucometer (Bayer Corporation), and GHb every 2 months. These experiments conformed to the Association of Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research, as well as to the specific Institutional Guidelines. Twelve months after initiation of diabetes, the animals were sacrificed by an overdose of pentobarbital, and the eyes were enucleated immediately. One eye was fixed in 10%

buffered formalin, and the other eye was used to isolate retina under a dissecting microscope by gently separating sensory retina from choroid using a micro-spatula.

2.2. Preparation of retinal microvasculature and capillary cell apoptosis

Microvessels were prepared from the eyes fixed in formalin by trypsin digestion. The retina was isolated, and incubated with 3% crude trypsin in Tris-HCl buffer (pH 7.8) containing 0.2M sodium fluoride for 90min at 37 °C, and apoptosis was determined by evaluating terminal transferase dUTP nick end labeled cells using a commercially available kit (In Situ Cell Death Kit, Roche Molecular Biochemicals, IN) as routinely used in our laboratory [22,26]. The slides containing trypsin-digested retinal vessels were rehydrated in phosphate buffered saline and permeabilized with 0.25% Triton X-100 for 1 h at room temperature. The slides were incubated with terminal deoxynucleotidyl transferase to add deoxynucleotide to the free 3'-OH end of DNA breaks characteristic of apoptotic cell death, and mounted in VECTASHIELD (Vector Laboratories, CA). Positive control included the retinal vessels exposed to DNase (2000 U/ml in 20mM Tris-HCl, pH 7.5) for 10min at room temperature before the TUNEL reaction. TUNEL-positive cells were identified in a masked; each slide was evaluated systematically under a Zeiss Axiophot photomicroscope by scanning the specimen with downward and upward motion beginning at the upper left margin [17,26].

2.3. Microarray analysis

Total RNA was isolated from the retina by TRIzol reagent (Invitrogen, Carlsbad, CA), and its concentration and integrity were determined spectrophotometrically.

The complementary RNA probe was synthesized (TrueLabeling-Am kit, Super- Array Bioscience, Frederick, MD) together with biotin-16-UTP labeling. The complementary RNA probes were incubated with rat Apoptosis Oligo GEArray (SuperArray Bioscience) membranes that profile 113 genes. The expression levels of each gene were detected with chemiluminescence using a copalyl diphosphate star substrate (SuperArray Bioscience). Membranes were exposed to X-ray film (Kodak, XAR film) and the signals were analyzed by the GEArray Expression Analysis Suite (SuperArray Bioscience) with background subtraction.

2.4. Quantitative real-time polymerase chain reaction (Q-RT-PCR)

To validate the array results representative genes were quantified using the Taq-Man assays on demand for the rat (Applied Biosystems, ABI; Foster City, CA). The Q-RT-PCR assay for Fas, Fas ligand (FasL), CD40, CD40 ligand (CD40L), caspases 3 and 9, Bax and NF- κ B was conducted in 50 ng cDNA template in 96-well plates using the ABI-7500 sequence detection system. The standard PCR conditions included 2 min at 50 °C and 10min at 95°C followed by 40 cycles of extension at 95 °C for 15 s and 1min at 60 °C. Threshold lines were automatically adjusted to intersect amplification lines in the linear portion of the amplification curves. Each sample was analyzed in triplicate. Beta-2-microglobulin (B2 M) was validated as an appropriate housekeeping gene. Data for target genes were normalized with B2M and the fold change in gene expression relative to normal was calculated using the ddCt method [3].

2.5. Statistical analysis

Data are reported as mean $\pm$ SD, and analyzed with nonparametric statistics. Experimental groups were compared using Kruskal-Wallis test followed by Mann-Whitney test for multiple group comparison. Analyses yielded identical results when performed using ANOVA with Fisher or Tukey.

3. Results

As shown in Table 1, the desired glycemic control was achieved by the insulin regimen used. The rats in poor glycemic control group had GHb values around 12% throughout 12 months duration of the experiment, and those in good glycemic control group had GHb around 6%. GHb values in PC–GC group before the initiation of good control were similar to those in poor glycemic control group (GHb >11%) but were reduced to around 6% during good glycemic control phase. Body weights of the rats in the PC–GC group before re-institution of good control were not different from those in poor glycemic control, and these values became comparable to the good glycemic control group. Similarly, 24 h urine volumes of the rats in good glycemic control group were similar to the age-matched normal rats, and were significantly lower than those in poor glycemic control group.

As reported previously [27] trypsin-digested retinal microvasculature from the rats maintained in poor glycemic control for the entire duration of the experiment presented a significantly greater number of TUNEL-positive cells compared to their age-matched normal control rats (Fig. 1). The total nuclei (inclusive of pericytes, endothelial cells, and nuclei with undetermined cellular attribution) positive for TUNEL staining were 2.5 ± 2.0 in the retinal vasculature from normal rats compared with 9.0 ± 5 rats in from poor glycemic control group. However, when good glycemic control was initiated soon after induction of diabetes (GC group), the number of TUNEL-positive cells was significantly lower than that in PC and PC–GC groups (3.1 ± 2.7), and these values were not statistically different from those obtained from normal rats.

In the same diabetic rats, microarray data revealed a total of 23 genes that were significantly ($p < 0.05$) upregulated in the retina, and 92% of these upregulated genes were from tumor necrosis factor (TNF) receptor and ligand, caspase, Bcl2, caspase recruitment domain and the death domain families. TNF receptor family genes Tnfrsf5 (Cd40), Tnfrsf6 (Fas) and Tnfrsf11b and 12a were upregulated by 2.3 to 7.7 fold, and TNF ligand family genes Tnfsf5 (Cd40L), Tnfsf6 (FasL) and Tnfsf9 were upregulated by 2.9–8.4 fold in the retina from poor glycemic control group compared to the retina obtained from age-matched normal rats. Caspase family genes (caspase-3, 8, 9 and 14) were upregulated by 2.5–4.9 fold, and Bcl-2 family genes (Bcl-2-associated death promoter, Bad; Bcl-2-associated $\times$ protein, Bax, and B-cell CLL/lymphoma 10, Bcl10) were upregulated by over 2.5 fold (Table 2). However, due to limited availability of the tissue, some of the parameters were not measured in the retina from GC group.

Twelve months of diabetes in rats also upregulated seven other pro-apoptotic genes in the retina. They included, one gene each from the death domain (Dapk1), CIDE domain (Cidea), TRAF (Traip), p53 families (Trp53bp2), and two from and caspase recruitment domain (Card6 and Ripk3) families (Table 2). In addition, NFkB1, which is shown to act as pro-apoptotic in the pathogenesis of diabetic retinopathy [25] was also significantly unregulated compared to the values obtained from age-matched normal rat retina. However, in the same diabetic rats five genes belonging to various apoptotic families, including TNF receptor (Tnfrsf1b), caspase (Casp4), CIDE domain (Dffa), and p53 families (Trp63 and Trp53inp1) were downregulated (Table 3). As expected, anti-apoptotic gene, Sphk2, was downregulated by 2.1 fold in the retina in diabetes (Table 3), but two other classic anti-apoptotic genes, Akt1 and Atf 5, were upregulated by 2.1 and 4.6 fold, respectively (Table 2).

The expression of some of the representative genes was confirmed by real time PCR. As shown in Fig. 2a, the mRNA levels of TNF family genes Fas, Fas L, CD40 and CD40L were elevated by 1.4–2.4 fold in the retina in diabetes. In the same retina samples, mRNA levels

of caspase-3 and -9 were elevated by over 2.0 fold, Bax by 4.5 fold and NF- κ B1 by 2.0 fold compared to the values obtained from the age-matched normal rats (Fig. 2b).

Re-institution of good glycemic control for 6 months after 6 months of poor glycemic control in rats, however, did not provide any protection. Accumulation of apoptotic capillary cells in the retina continued; the number of TUNEL-positive cells in the retinal microvasculature in rats from PC-GC group remained significantly higher compared to the age-matched normal rats, and values were not different from those obtained from the rats that were maintained in poor glycemic control for the entire duration ($p = 0.497$; Fig. 1). Furthermore, all of the 24 retinal genes that were revealed by microarray experiments to be upregulated in the retina of rats diabetic for the entire 12 months (PC group) remained upregulated for at least 6 months after reversal of hyperglycemia, and the values remained significantly higher compared to those from the age-matched normal control rats (Table 2). Similarly, re-institution of good glycemic control failed to provide any benefit to the upregulation of the two classic anti-apoptotic genes (Akt1 and Atf5), and downregulation of five apoptotic genes (Casp4, Tnfrs1b, Dffa, trp73 and Trp53inp1) and one anti-apoptotic gene, Sphk2 (Table 3). Gene expressions (mRNA levels) of Fas, FasL, CD40, CD40L, caspase-3 and -9, Bax, and NF- κ B1 also remained elevated in the retina for at least 6 months after re-institution of good glycemic control in rats (Fig. 2).

4. Discussion

This is the first report showing that the retinal capillary cells, the cells that are the target of the pathology characteristic of diabetic retinopathy, continue to undergo apoptosis after good glycemic control is re-instituted in diabetic rats. An array of apoptosis-related genes belonging to TNF ligand and receptor, caspase, BCL-2 and cell death associated families that are upregulated in the retina in diabetes, remain upregulated for at least 6 months, thus suggesting that apoptosis of retinal cells is an important component of the metabolic memory phenomenon. In the same group of animals we have shown that the formation of acellular capillaries, a hallmark of diabetic retinopathy, does not halt after re-establishment of good glycemic control [15], and activation of retinal caspase-3, apoptosis execution enzyme, which begins early in the pathogenesis of diabetic retinopathy, does not reverse [21].

Apoptosis, a form of programmed cell death, involves a series of biochemical events leading to specific cell morphology characteristics and ultimately death of cells. In the pathogenesis of diabetic retinopathy, capillary cells are lost selectively via apoptosis before other histopathology is detectable, or loss of vision is evident in diabetes [27,31]. Diabetic retinopathy is a slow progressing disease, but apoptosis is a rapidly consummated phenomenon [34], and the small number of apoptotic capillary cells observed in diabetic retina are considered to have a major impact on the formation of acellular capillaries and pericyte ghosts [17,27,31]. Endothelial cell's replicative capacity is exhausted resulting in ischemia and, eventually in the development of acellular capillaries and pericyte drop-out, the characteristic features of diabetic retinopathy. Furthermore, appearance of apoptotic capillary cells in the retina is considered as a predictor of retinopathy in diabetes [17]. Failure of the apoptosis process halt in the retinal vasculature after reversal of hyperglycemia strongly suggests that continued activation of the apoptosis machinery is one of the participants in the resistance of diabetic retinopathy to arrest after re-establishment of good glycemic control.

We show that seven genes from TNF receptor and ligand family that are significantly upregulated in the retina in diabetes continue to remain upregulated in the rats from PC-GC group. These genes are associated with the trans-membrane pathway of apoptosis [4].

Apoptosis induced by TNF and its family members is postulated to be mediated through the production of reactive oxygen species (ROS), and elevated ROS play an important role in the development of diabetic retinopathy via activation of the major metabolic pathways implicated in the development of diabetic retinopathy [2]. TNF exerts many of its effects by binding to the cell membrane receptors that have extracellular domain. CD40, a member of the receptor family, is a surface receptor that can initiate multifaceted activation signals and induce apoptosis [35]. The interaction between CD40 and CD40L is considered as an initial event in the activation of several proinflammatory mediators [6], and diabetic retinopathy has many characteristics of a low-grade chronic inflammatory disease [16]. Data presented here show that both CD40 and CD40L are elevated in the retina in diabetes, and remain elevated even after reversal of hyperglycemia. This is consistent with the recent reports suggesting that dysregulation of epigenetic modification is important in the sustained proinflammatory phenotype of vascular smooth muscle cells from diabetic mice after being stimulated with TNF- α [39], and our data demonstrating that the levels of some of the major inflammatory mediators, including interleukin 1- β , vascular cell adhesion molecule 1 and TNF- α , fail to reverse in the retina after 6 months of good glycemic control that has followed 6 months of poor glycemic control [3]. Furthermore, our data also show that diabetes-induced elevation in retinal Fas and FasL also resist reversal after re-establishment of good glycemic control. Fas–FasL pathway plays an important role in the pathogenesis of diabetic retinopathy; endothelial cell apoptosis, which precedes the development of diabetic retinopathy, is postulated to be mediated via leukocyte–Fas–FasL-dependent pathway [13].

The receptors of TNF family are shown to interact with other molecules, including intracellular death domains and caspase recruitment domains (CARD), and this can further exacerbate the apoptosis process [32]. Here we show that upregulation of retinal Card 6 and Ripk3 genes of the CARD family and Dapk1 of the death domain family in diabetes is not attenuated by 6 months of good control that has followed 6 months of hyperglycemia. This suggests that these propaprotic genes continue to accumulate during normal glycemia state resulting in failure of apoptosis process initiated during prior poor glycemic condition [21], to halt after good glycemic control is re-instated. Binding of Fas to its ligand FasL recruits and activate zymogen forms of the cysteine protease caspases, particularly procaspase- or procaspase-10, and these molecules proteolytically activate downstream caspases that result in execution of apoptosis, such as caspase-3 [5]. Continued activation of caspase-8 and caspase-3 implies that this pathway has an important role in the continued apoptosis of retinal capillary cells.

Data presented here also suggest that in diabetes mitochondrial pathway of apoptosis also continues to progress in the retina, and this process is not altered by re-establishment of good control in diabetic rats. The pro-apoptotic members of the Bcl-2 family remain upregulated in the retina for a duration after re-establishment of good control. Increased levels of the pro-apoptotic protein Bax are observed in retinal pericytes in diabetes [33], and in isolated endothelial cells in high glucose [19]. Retina experiences mitochondrial dysfunction and the major steps of this pathway, the release of cytochrome *c* and mitochondrial membrane permeability are increased in diabetes [14,20]. The release of cytochrome *c* is shown to trigger the activation of caspase-9 to form an apoptosome, and this culminates in the initiation of the apoptosis [10,29,37]. Here we show that re-institution of good glycemic control fails to provide any benefit to the Bcl-2 family genes, Bad, Bax and Bcl210, and also caspase-9, these genes remain up-regulated in the retina even after hyperglycemia is terminated, suggesting that mitochondrial pathway continues to be dysfunctional during good glycemic control that has followed 6 months of poor glycemic control. In support, our recent results have shown continued retinal mtDNA damage 6 months after reversal of hyperglycemia [29], and others have shown that initiation of good

glycemic control after a period of hyperglycemia does not immediately reverse the ratio of Bax to Bcl-2 [10].

In addition to elevation in many apoptotic genes, we also show that two classic anti-apoptotic genes, Akt1 and Atf5, are elevated in the retina in diabetes. This is in contrast to the results obtained by other showing a reduction in protein expression of retinal Akt in diabetes [37]. The reason for such disparity is not clear, however, high glucose has been shown to activate PI3K/Akt1 pathway in retinal endothelial cells, and this is considered to play an important role in their enhanced migration [12].

The retina is a complex tissue with multiple layers and cell types, and cells other than microvascular cells (including ganglion cells and Muller cells) are also shown to undergo accelerated apoptosis in the pathogenesis of diabetic retinopathy [30]. We need to acknowledge that the microarray analysis (and their confirmation by RTPCR) was performed in the whole retina samples, and this did not allow us to quantify these apoptosis-related genes in the capillary cells. Our data showing a strong correlation between capillary cell apoptosis and retinal mRNA levels of the apoptosis related genes strongly suggest that the alterations in the mRNA levels observed in the whole retina samples are representative of microvasculature. Contribution of other cells (other than capillary cells) in the changes observed in the mRNA levels of the genes reported in this manuscript, however, cannot be ruled out.

In conclusion, we have provided strong evidence demonstrating that apoptosis of retinal capillary cells is an important contributor to the metabolic memory phenomenon; pro-apoptotic activation is sustained even after hyperglycemia is terminated.

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Fig. 1.

TUNEL-positive capillary cells in retinal trypsin digest: trypsin-digested microvessels were stained to detect TUNEL-positive cells using a kit from Roche Molecular Biochemicals. TUNEL-positive cells were identified in a masked fashion by scanning the trypsin-digested retinal microvessels under a Zeiss Axiophot photomicroscope. PC, poor control; PC-GC, poor control for 6 months followed by good control for 6 months; GC, good control for the entire 12 months; * $p < 0.05$ compared to normal and # $p < 0.05$ compared to PC.

Gene quantification by Q-RT-PCR. The Q-RT-PCR assay was conducted in 50 ng cDNA template using the ABI-7500 sequence detection system. Each sample was analyzed in triplicate, and B2 M was validated as an appropriate housekeeping gene. Data for the target genes were normalized with B2 M and the fold change in gene expression relative to normal was calculated using the ddCt method. Values are represented as fold change relative to those obtained from normal rat retina. Graph (a) has representative genes from TNF receptor and ligand family and (b) genes from caspase and BCL2 families and NFkB. Black bars represent rats in poor control for 12 months, and grey bars are from rats in poor control for 6 months followed by good control for 6 additional months.

Gene quantification by Q-RT-PCR. The Q-RT-PCR assay was conducted in 50 ng cDNA template using the ABI-7500 sequence detection system. Each sample was analyzed in triplicate, and B2 M was validated as an appropriate housekeeping gene. Data for the target genes were normalized with B2 M and the fold change in gene expression relative to normal was calculated using the ddCt method. Values are represented as fold change relative to those obtained from normal rat retina. Graph (a) has representative genes from TNF receptor and ligand family and (b) genes from caspase and BCL2 families and NFkB. Black bars represent rats in poor control for 12 months, and grey bars are from rats in poor control for 6 months followed by good control for 6 additional months.

Table 1

Severity of hyperglycemia in rats.

	Duration (months)	Body weight (g)	GHb (%)	Urine volume (ml)
Normal	12	525 ± 35	5.1 ± 0.9	9 ± 5
PC	12	310 ± 39*	11.6 ± 1.7*	115 ± 23
GC	12	474 ± 30	5.8 ± 0.7	19 ± 9
PC-GC	6-6	293 ± 21* -459 ± 25	12.1 ± 1.6* -6.1 ± 1.1	125 ± 30-27 ± 19

The values are mean ± SD of 7–9 rats in each group. Body weights were measured two times every week. Glycated hemoglobin was quantified every 3 months, and 24 h urine volume was measured on a daily basis.

* $p < 0.05$ compared to normal.

Table 2

Effect of glycemic control on retinal genes.

	Gene symbol	Description	Fold increase	
			PC	PC-GC
TNF receptor and ligand	Tnfrsf11b	TNF receptor superfamily, member 11b	7.7 ± 0.1	8.5 ± 0.9
	Tnfrsf12a	TNF receptor superfamily, member 12a	2.6 ± 0.1	3.1 ± 0.04
	Tnfrsf5/Cd40	TNF receptor superfamily, member 5	2.3 ± 0.5	2.1 ± 0.4
	Tnfrsf6 or Fas	TNF receptor superfamily, member 6	3.5 ± 0.7	3.4 ± 0.8
	Tnfrsf5/Cd40L	TNF receptor superfamily, member 5	2.9 ± 0.1	2.6 ± 0.4
	Tnfrsf6/FasL	TNF (ligand) superfamily, member 6	3.1 ± 0.5	2.6 ± 0.2
	Tnfrsf9	TNF (ligand) superfamily, member 9	8.4 ± 0.1	6.7 ± 0.1
Caspase	Casp3	Caspase 3	2.5 ± 0.3	2.5 ± 0.4
	Casp8	Caspase 8	2.5 ± 0.1	2.6 ± 0.2
	Casp9	Caspase 9	3.9 ± 0.3	2.0 ± 0.2
	Casp14	Caspase 14	4.9 ± 0.3	4.4 ± 0.3
Bcl2	Bad	Bcl2-associated death promoter	2.7 ± 1.0	2.0 ± 0.3
	Bax	Bcl2-associated X protein	3.0 ± 0.3	2.3 ± 0.6
	Bcl2110	B-cell CLL/lymphoma 10	2.6 ± 0.2	2.0 ± 0.4
Death domain	Dapk1	Death associated protein kinase 1	3.4 ± 0.2	2.2 ± 0.2
CIDE domain	Cidea	Cell death-inducing DNA fragmentation factor, α subunit-like effector A	5.1 ± 0.4	3.1 ± 0.5
CARD	Card6	Caspase recruitment domain family, member 6	6.7 ± 0.6	6.4 ± 0.2
	Ripk3	Receptor-interacting serine-threonine kinase 3	4.0 ± 0.3	3.5 ± 0.8
TRAF	Traip	TRAF-interacting protein	2.2 ± 0.7	2.1 ± 0.7
p53	Trp53bp2	Transformation related protein 53 binding protein 2	3.9 ± 0.2	3.7 ± 0.4
Anti-apoptosis	NF-kB1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	3.6 ± 0.4	3.1 ± 0.6
	Akt1	Thymoma viral proto-oncogene homolog 1	2.3 ± 1.1	2.7 ± 0.6
	Atf5	Activating transcription factor 5	4.6 ± 0.2	4.1 ± 0.2

The results are mean ± SD of 5 or more rats in each group. The values obtained from normal rat retina are considered as one.

Table 3

Effect of glycemic control on downregulation of retinal genes.

Gene family	Gene symbol	Description	Fold decrease	
			PC	PC-GC
Anti-apoptosis	Sphk2	Sphingosine kinase 2	2.1 ± 0.1	2.8 ± 0.6
Caspase	Casp4	Caspase 4	2.9 ± 0.1	2.8 ± 0.2
Death domain	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	2.9 ± 0.2	2.9 ± 0.2
CIDE domain	Dffa	DNA fragmentation factor, alpha subunit	2.1 ± 0.9	2.5 ± 0.5
DNA-damage-induced apoptosis	Trp63	Transformation related protein 63	3.0 ± 0.1	2.3 ± 0.5
	Trp53inp1	Transformation related protein 53 inducible nuclear protein 1	2.91 ± 0.1	3.71 ± 0.1