Published in final edited form as:
J Diabetes Complications. 2010 ; 24(1): 55–63. doi:10.1016/j.jdiacomp.2008.10.002.

Resistance of Retinal Inflammatory Mediators to Suppress After Re-institution of Good Glycemic Control: Novel Mechanism for Metabolic Memory

Pooi-See Chan, **Mamta Kanwar**, and **Renu A Kowluru***
Kresge Eye Institute, Wayne State University, Detroit, MI

Abstract

Diabetic retinopathy resists arrest of its progression after reestablishment of good glycemic control that has followed a profound period of poor glycemic control. This study is to elucidate the role of inflammation in the resistance of retinopathy to arrest after termination of hyperglycemia. Streptozotocin-diabetic rats were maintained either in poor glycemic control (PC, glycated hemoglobin, GHb>11%) or in good glycemic control (GC, GHb<7%) for 12 months, or allowed to be in PC for six months followed by GC for six additional months. At 12 months, retina was analyzed for pro-inflammatory mediators. Twelve months of PC increased retinal interleukin-1β (IL-1β) mRNA by 2-fold and its protein expressions by 25% compared to the values obtained from normal rat retina. Tumor necrosis factor-α (TNF-α) was elevated approximately 3 fold (both mRNA and protein), and the receptors for IL-1β and TNF-α were increased by 40% each. The concentrations of intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were elevated by 40% and 150% respectively, and iNOS transcripts by six fold. Six months of GC that followed six months of PC failed to reverse the elevations in IL-1β, TNFR1 and ICAM-1; and had some beneficial effects on TNF-α, iNOS and VCAM-I, but these mediators remained significantly elevated. However, GC group showed no significant changes in the retinal pro-inflammatory mediators compared to the normal rats. Failure to reverse retinal inflammatory mediators supports their important role in the resistance of retinopathy to arrest after cessation of hyperglycemia.

Keywords

Diabetic retinopathy; Glycemic control; Inflammation; Metabolic memory

INTRODUCTION

Retinopathy is a significant problem that affects nearly 90% patients with type I diabetes and more than 60% with type II diabetes for more than 20 years, and hyperglycemia is considered the initiating factor in its development (Engerman & Kern, 1986). Recent studies have suggested that diabetic retinopathy has many characteristics of chronic inflammation. The

*Corresponding Author Renu A. Kowluru, Ph.D., Kresge Eye Institute, Wayne State University, 4717 St. Antoine, Detroit, MI 48201, Telephone 313-993-6714, Fax 313-577-8884, rkowluru@med.wayne.edu.

levels of cytokines (e.g. interleukin-1β, IL-1β, and tumor necrosis factor-α, TNF-α), adhesion molecules (e.g. intercellular cell adhesion molecule, ICAM-1, and vascular cell adhesion molecule, VCAM-1) and inducible form of nitric oxide synthase (iNOS) are increased in the retina, vitreous and serum of diabetic patients and animals (Joussen et al., 2001, 2004 ; Yuuki et al., 2001 ; Matsumoto et al., 2002 ; Kowluru, 2003 ; Kowluru & Odenbach, 2004a; Vincent & Mohr, 2007; Kern, 2007; Adamis & Berman, 2008). The influx of leukocytes is increased, and chronic low-grade inflammation is considered responsible for vascular lesions that are hallmark of diabetic retinopathy (Joussen et al., 2004).

Clinical studies have shown that good glycemic control, instituted early in the course of diabetes, can 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, reestablishment of good control after a profound period of poor glycemic control does not immediately benefit the progression of retinopathy (DCCT, 1993; UKPDS, 1998; DCCT, 2002). The imprinted effects of prior glycemic control result either in the long lasting benefits of good glycemic control that follows, or in the resistance of retinopathy to halt (DCCT, 2002; LeRoith & Vinik, 2005). This phenomenon is commonly termed as ‘metabolic memory’. Animal models of diabetic retinopathy, including diabetic or experimentally galactosemic dogs and rats, also show this metabolic memory phenomenon (Kowluru & Koppolu, 2002; Kowluru, 2003; Engerman & Kern, 1987; Hammes et al., 1993; Kowluru, Chakrabarti & Chen S, 2004; Kowluru, Kanwar & Kennedy, 2007). We have shown that institution of good control soon after induction of diabetes in rats protects the retina from increases in oxidative stress and nitrative stress, and if poor glycemic control is maintained for two months before initiation of six months of good control, increases in retinal oxidative stress and nitrative stress are partially protected. However, if the duration of poor glycemic control is extended to six months, re-institution of good glycemic control for six additional months fails to provide the retina any protection from increased oxidative stress and nitrative stress (Kowluru, 2003), and its vasculature continues to accumulate elevated levels of nitrotyrosine (Kowluru, Kanwar & Kennedy, 2007). The exact mechanism responsible for this metabolic memory phenomenon, however, remains elusive.

Diabetic retinopathy shares many similarities with low grade chronic inflammatory diseases (Joussen et al., 2001, 2004 ; Yuuki et al., 2001 ; Matsumoto et al., 2002 ; Kowluru, 2003 ; Kowluru & Odenbach, 2004a and b; Vincent & Mohr, 2007; Kern, 2007; Adamis & Berman, 2008), and a redox-sensitive factor that regulates genes involved in the inflammatory responses, nuclear transcriptional factor (NF-*k*β), remains activated in the retina after reinstitution of good control in diabetic rats (Kowluru, Chakrabarti & Chen, 2004), suggesting that increased retinal inflammation that is induced in hyperglycemic conditions, could also be resisting reversal. The purpose of this study is to investigate the role of pro-inflammatory mediators that are suggested to play a role in the development of diabetic retinopathy, in the resistance of retinopathy to arrest after reestablishment of good glycemic control in diabetic rats, and to understand the mechanism/s involved in precipitating this resistance.

RESEARCH DESIGN AND METHODS

Animals

Lewis rats (male, 200g) were assigned to normal or diabetic groups. Diabetes was induced with streptozotocin (55mg/kg BW) and diabetic rats were randomly divided into three groups (15–20 rats/group). Rats in group one and two were in poor glycemic control (PC group, glycated hemoglobin, GHb>11%) or in good glycemic control (GC group, GHb<7%) for the entire 12 months respectively. The rats in group three were in poor glycemic control for six months followed by good glycemic control for six additional months (PC-GC group). A group of age-matched rats remained normal for the entire duration of the experiment. Some of the rats used

in the present study have been used by us in our previous studies (Kowluru, Kanwar & Kennedy 2007).

Glycemia

Diabetic rats received insulin (NPH) injections, and the dose and frequency of insulin were adjusted based on the desired severity of hyperglycemia. The rats in which poor glycemic control was intended received a single injection of insulin (1–2 units) four to five times a week to prevent ketosis and weight loss, and the rats in good glycemic control received insulin twice daily (6–8 units total) to maintain their blood glucose below 150 mg/dl (Kowluru, 2003; Kowluru, Kanwar & Kennedy, 2007). The entire rat colony was housed in metabolism cages; 24-hour urine samples were tested for glycosuria 2–3 times every week using quantitative methods (Glucose Kit, GAGO-20, Sigma-Aldrich Chemicals, St. Louis, MO, USA). Blood glucose was measured once a week (Glucometer Elite, Bayer Corporation), and GHb every three months using a kit from Helena Laboratories (Beaumont, TX, USA). All of the rats, including normal group, received powdered diet (Purina 5001), and the food consumption was measured once and body weights 2–3 times every week. These experiments conformed to the Association for Research in Vision and Ophthalmology's Resolution on Treatment of Animals in Research (National Institutes of Health). Twelve months after initiation of the experiment, the animals were sacrificed by an overdose of pentobarbital, and retina was isolated by gently separating sensory retina from choroid using a microspatula under a dissecting microscope and stored frozen at −80°C.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the retina by a method previously used by us (Kowluru, Menon & Gierhart, 2008). RNA (1μg) was converted to single stranded cDNA using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, ABI; Foster City, CA, USA). cDNA was synthesized in a total volume of 20μl using the GeneAmp PCR system 9700 thermal cycler (ABI). The single stranded cDNA was quantified spectrophotometerically, and diluted to 10ng/μl.

Quantitative real-time PCR (Q-RT-PCR) assay

Genes for IL-1β, TNF-α, ICAM-1, iNOS and COX-2 were quantified using the TaqMan Assays on Demand for the rat (ABI). The GenBank accession number for the ABI TaqMan assays (containing primers and probes) and the amplicon length are provided in Table I. The Q-RT-PCR assay was conducted with 50–200ng cDNA template in 96-well plates using the ABI-7500 sequence detection system as previously described (Kowluru, Menon & Gierhart, 2008). Each sample was analyzed in triplicate. Beta-2-macroglobulin (B2M) was validated as an appropriate housekeeping gene, and data for target genes were normalized with B2M. The fold change in gene expression relative to normal was calculated using the ddCt method. The values obtained from age-matched normal rat retina were considered as one.

Enzyme-linked immunosorbent assay (ELISA)

The amounts of ICAM-1 and VCAM-1 were quantified by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions using 50μg retinal protein for each assay. Briefly, for the ICAM-1 assay, standard or the sample was added in a 96-well plate that was pre-coated with a monoclonal antibody. The plate was incubated for 2 hours and after washing the plates, the samples were incubated with rat soluble ICAM-1 conjugate for another 2 hours. The unbound antibody-enzyme reagent was washed, followed by addition of the substrate solution. The intensity of the color was measured at 450nm.

Similarly for VCAM-1 assay, standard solution or the samples together with the soluble VCAM-1 conjugate were added in a 96-well plate that was pre-coated with a monoclonal antibody. After incubating for 1.5 hours, the plate was washed to remove the unbound antibody-enzyme reagent. The substrate solution was added, and the color produced was measured at 450nm. Results are reported as percentage elevation relative to the values obtained from normal rat retina.

Western blot analysis

Protein (20–30μg) was separated using 10–12% SDS-PAGE gels and transferred to nitrocellulose membranes following the western blot protocol. Each band was quantified using Un-Scan-It Gel digitizing software (Silk Scientific Inc, Orem, UT, USA), and the values in the histograms were presented as mean band density of the protein of interest adjusted by the intensity of β-actin in the same sample and expressed as percentage relative to the values obtained from normal rat retina.

Immunoprecipitation of cytokine receptors

The formation of protein-protein complexes between IL-1β and its receptor, IL-1 receptor I (IL-1RI), and also between TNF-α binding to its receptor, TNF receptor I (TNFRI), was assessed in the retina by immunoprecipitation methods. Retinal protein (100–120μg) was incubated overnight at 4°C with polyclonal anti-IL-1β or anti-TNF-α antibodies (Santa Cruz Inc, Santa Cruz, CA, USA). The mixture was then incubated with 20μl protein A/G PLUS-agarose beads (Santa Cruz Inc) at 4°C for 1 hour, followed by centrifugation at 1000xg for 5 minutes. The pellet was washed, and resuspended in the SDS sample buffer. The sample was boiled for 2 minutes; the immunoprecipitate was collected by centrifuging, and analyzed by western blot technique using anti-IL-1RI or anti-TNFRI antibodies.

Statistical analysis

Each measurement was made at least in duplicate, and the values are expressed as mean ± SD. Statistical analysis was performed using the nonparametric approach of Kruskal-Wallis followed by the Mann-Whitney test for multiple group comparisons employing the SAS statistical software. P value of <0.05 was considered statistically significant.

RESULTS

Severity of hyperglycemia in rats

The rats in PC group had GHb values above 11% throughout the 12 months duration of the study and those in GC group had GHb below 7%. GHb values in PC-GC group before the initiation of good control were similar to those in PC group (GHb>11%) but were reduced to below 7% two months after initiation of good glycemic control (the first measurement made after reinstitution of good glycemic control), and remained unchanged for the entire six months of good glycemic control (Table II). Body weight and daily food consumption in the PC-GC group before reinstitution of good control were similar to those in poor glycemic control, and these values became comparable to the GC group after initiation of good glycemic control. Similarly, 24 hour urine volumes of the rats in GC group were not different from those in age-matched normal rats, and were significantly lower than those in PC group (Table II).

Retinal cytokines and their receptors

Gene expression of IL-1β was increased by 2-fold in the retina of rats in PC group, and its protein expression by 25% as compared to values obtained from the age-matched normal rats (Figures 1a & b). Since IL-1β binds preferentially to IL-1RI for its activation, and immunoprecipitaion allows an indirect measurement of IL-1β to IL-1RI binding, the expression

of IL-1RI was quantified. Retinal IL-1RI was elevated by 40% in the rats maintained in poor glycemic control for the entire duration of the experiment (Figure 1c).

The transcript for TNF-α and its protein expression were increased approximately 3 fold in the retina of rats in PC group, and the expression of TNFRI, a receptor that TNF-α binds for its activation, was elevated by 40% (Figures 2a–c).

Six months of good glycemic control in rats that had followed six months of poor glycemic control failed to provide any protection to the increases in IL-1β (both, gene and protein expressions) and its receptor (Figures 1a–c). This six months of good control, however, had some beneficial effects on TNF-α, its gene and protein expressions were decreased by over 25% in PC-GC rats compared to PC rats, but the values remained significantly higher compared to the normal rats ($P<0.05$, Figures 2a&b). In contrast, diabetes-induced increase in the expression of TNFR1 was not affected by re-institution of good glycemic control in rats (Figure 2c)

Adhesion molecules

ICAM-1 concentration and its gene expression were elevated by 40% and over 500% respectively, and VCAM-1 concentration by 150% in the retina of rats in poor glycemic control for 12 months compared to the age-matched normal rats. Reversal of hyperglycemia after six months of poor glycemic control had no beneficial effect on retinal ICAM-1 concentration (Figure 3a), but had partial beneficial effect on its gene expression. In the same PC-GC animals, reversal of hyperglycemia had significant effect on retinal VCAM-1 levels ($P<0.05$ compared to PC), but VCAMI-1 remained 50% higher in PC-GC group compared to the normal rats (Figure 3b).

COX-2 and iNOS

As reported previously for protein expression of iNOS (10), its transcript was increased by 6-fold in the retina of rats in poor glycemic control for 12 months (Figure 4a). Reinstitution of good glycemic control (PC-GC group) resulted in partial, but significant, decrease in iNOS gene expression. However, iNOS mRNA remained significantly higher compared to those obtained from the age-matched normal rats.

Twelve months of diabetes increased the protein expression of retinal COX-2 by 70% in PC group rats (Figure 4b), and had some effect on its gene expression, but the values for COX-2 mRNA were not statistically different in PC, PC-GC and normal groups. Although reversal of hyperglycemia decreased diabetes-induced increase in the protein expression of COX-2 by about 50%, the values in PC-GC group remained high, and did not achieve any statistically significant reduction compared to those in PC group ($p=0.13$).

Initiation of good control soon after induction of diabetes and pro-inflammatory mediators

Maintenance of good glycemic control in rats for the entire duration of the experiment (GC group), prevented the retina from diabetes-induced increases in the cytokines (IL-1β and TNF-α), ICAMI-1 and iNOS (Figure 1–Figure 4) suggesting that increase in retinal inflammatory mediators in diabetes can be easily prevented if poor glycemic control is not allowed to sustain, and is intervened with good glycemic control very early in the pathogenesis of diabetic retinopathy. We need to acknowledge that due to some technical difficulties in preparing the samples for analysis we were unable to determine the protein expressions and concentrations of the proinflammatory mediators in the retina obtained from rats in GC group.

DISCUSSION

This is the first report demonstrating the role of inflammatory mediators in the metabolic memory phenomenon, the pro-inflammatory cytokines and adhesion molecules that are implicated in the development of diabetic retinopathy resist suppression after institution of good glycemic control for at least six months that has followed six months of poor glycemic control. Reversal of hyperglycemia fails to provide any significant effect on the development of histopathology that is characteristic of diabetic retinopathy and nitrotyrosine continues to accumulate in retinal capillary cells (Engerman & Kern, 1987; Hammes et al., 1993; Kowluru, Kanwar & Kennedy, 2007). Many retinal metabolic abnormalities, including oxidative stress, nitrative stress, advanced glycation end products, are shown to resist reversal after re-institution of good control in diabetic rats (Kowluru, 2003; Kowluru, Chakrabarti & Chen S, 2004; Kowluru, Kanwar & Kennedy, 2007; Genuth et al., 2005; Ihnat et al., 2007), and here we provide novel evidence that inflammatory mediators are also a major part of the metabolic memory puzzle.

IL-1β, a pro-inflammatory cytokine, binds to the cell membrane associated IL-1RI that eventually activates either NF-*k*B or proteins participating in translation of mRNA, modulating cell responses and gene expression (Sims et al., 1993; Chang & LoCicero, 2004). The IL-1RI and its activation are considered important in the pathogenesis of diabetic retinopathy (Vincet & Mohr, 2007). Although IL-1β is predominantly produced by macrophages, it is also secreted by the capillary cells; levels of IL-1β are elevated in the retina and its capillary cells in diabetes (Kowluru & Odenbach, 2004 a&b), and in here we show that IL-1β (mRNA and protein expression) remain elevated at duration of diabetes where histopathology can be observed. Normalization of glycemic control fails to prevent the elevation in retinal IL-1β and its receptor signifying that continued increase in IL-1β is important in the persistence of vascular lesions of diabetic retinopathy.

Similar to IL-1β, TNF-α is present in acute and chronic inflammatory conditions and its biological response is mediated via binding to TNF receptors. The induction of TNF-α expression observed in our poor glycemic control animals is in agreement with others (Joussen et al., 2002; Zheng, Gong, Hatala & Kern, 2007). Increase in TNF-α expression in the retina of diabetic rats is accompanied by elevation in its receptor, TNFRI. The activation of TNFRI stimulates pro-inflammatory pathway leading to the activation of NF-*k*B or in the apoptotic pathway. In support, our previous studies have shown that hyperglycemia activates retinal NF-*k*B and the apoptosis execution enzyme, caspase-3 and these abnormalities, likewise, resist arrest after reversal of hyperglycemia (Kowluru, Koppolu, Chakrabarti & Chen, 2003; Kowluru, Chakrabarti & Chen, 2004). Although we observed lower TNF-α mRNA levels in the retina from PC-GC group compared to PC group, the values remained significantly higher than those in normal rats or good glycemic control rats. Moreover, in the same rats six months of good glycemic control failed to provide any benefit to its receptor suggesting that the binding of TNF-α to TNFRI continues to remain abnormal even after reversal of hyperglycemia, and NF-*k*B and apoptotic pathways remain active.

In the pathogenesis of diabetic retinopathy increases in retinal ICAM-1 and VCAM-1 are implicated via activation of leukostasis, and these abnormalities are observed early in the development of diabetic retinopathy (Joussen et al., 2004; Tang, Le-Ruppert, Gabel, 1994). The failure to completely reverse increases of the ICAM-1 and VCAM-1 levels six months after reinstitution of good glycemic control suggests that the sustained elevation of these molecules is important in the resistance of retinopathy to reverse. The exact mechanisms of how ICAM-1 and VCAM-1 contribute to the resistance of diabetic retinopathy are not clear, but could include continued activation of leukostasis contributing to capillary cell loss.

iNOS is implicated in the inflammatory process in diabetes; diabetic mice with iNOS gene knocked out demonstrate reduction in retinal capillaries degeneration (Zheng et al., 2007). The protein expression of iNOS and levels of nitric oxide (NO) and nitrotyrosine are elevated in the retina of diabetic rats and humans (Abu El-Asrar et al., 2001; Du, Smith, Miller & Kern, 2002; Kowluru, 2003; Kowluru, Kanwar & Kennedy, 2007). Similar to its protein expression (Kowluru, 2003), here we show that iNOS transcript remains significantly elevated for at least six moths after reinstitution of good glycemic control in rats. This sustained increase in iNOS transcript could be sufficient to result in continued elevation in retinal NO and nitrotyrosine levels previously reported by us (Kowluru, 2003; Kowluru, Kanwar & Kennedy, 2007).

COX-2 modulates the levels of vascular endothelial growth factor and vascular permeability in the retina in diabetes (Joussen et al., 2002; Du, Sarthy & Kern 2004; Kern, 2007). Increased COX-2 mRNA expression is reported in rats as early as eight days after induction of diabetes (Ayalasomayajula & Kompella, 2003), and inhibitors of COX-2 decrease leukostasis in the retinal microvasculature (Joussen et al., 2002; Du, Sarthy & Kern 2004). Our failure to observe significant increase in COX-2 mRNA at 12 months of diabetes in rats could be due to the duration of diabetes or the increase in translation of the transcript that could have resulted in elevated COX-2 protein expression. However, we observed a significant increase in COX-2 protein expression at 12 months of diabetes, and this was only partly reversed in PC-GC rats. The mechanism by which sustained increase in retinal COX-2 protein could be contributing to the metabolic memory phenomenon is not clear but could include its sustained effects on increased vessel permeability and leukostasis, and continued retinal capillary cell apoptosis (Kern, 2007).

Clinical studies and animal models of diabetic retinopathy have demonstrated that the development of retinal vascular lesions are considered as the irreversible lesions, the ‘point of no return’, and the preexisting damage at the time of intervention is one of the primary factors in determining the outcome of a therapy (DCCT, 1995 and 1998; Robison et al., 1998; Kowluru, 2003). Here we show that if good glycemic control is initiated soon after induction of diabetes (GC group), retinal inflammatory mediators remain largely unchanged, but if poor glycemic control is maintained for six months before instituting good glycemic control, these abnormalities resist reversal suggesting that the damaging process of inflammation that starts early in the course of development of retinopathy in diabetes is not easily reversed by good glycemic control. These results are very significant because they suggest that during good glycemic control the rats did not experience major fluctuation in their glucose levels, and the effects of good glycemic control are not influenced by the high dose of insulin administered to maintain the desired glycemic control.

Clinical studies with diabetic patients have shown that a period of good control imprints a long term benefit on tissues even when followed by poor control. Our experimental plan, however, did not include a group of rats that were maintained in good control for six months followed by poor glycemic control for six additional months. Thus, the possibility that the retina of these rats could have lower pro-inflammatory mediators than PC-GC rats cannot be ruled out.

Diabetic retinopathy is a multi-factorial disease, and the process of inflammation can also interact with other metabolic abnormalities to affect the pathogenesis of diabetic retinopathy. NF-*k*B regulates the inflammatory response and mediators including all the markers assessed in the present study. Stimulation of IL-1β and TNF-α can lead to further NF-*k*B activation creating a continuous feedback loop (Chang, LoCicero, 2004). Oxidative stress can trigger and regulate vascular inflammation, while inflamed cells can stimulate the release of reactive oxygen species (ROS) (Kowluru & Odenbach, 2004b; Libby, 2007). ROS increase microvascular permeability, aid in the recruitment of neutrophils to the site of inflammation (Libby, 2007; Quan, He & Lai, 2003). Thus, the resistance of inflammatory markers to suppress

in the present study supports a possible link between inflammation, oxidative stress and NF-*k*B. However, it is difficult to predict which mediator is upstream in order to block the interrelated detrimental downstream events to diabetic retinopathy.

We have used the whole retina to analyze inflammatory mediators, and this approach did not allow us to identify the specific retinal cell types that continue to have increased mediators. However, in the same animals we have shown that reversal of hyperglycemia fails to inhibit the development of retinal histopathology (Kowluru, Kanwar & Kennedy, 2007), thus strongly suggesting that the inflammatory mediators continue to accumulate in the retinal vasculature even after hyperglycemia is terminated.

Diabetes Control and Complications Trial has shown that serum levels of ICAM-1 are reduced with intensive insulin therapy while C-reactive protein and soluble TNFRI do not benefit from intensive insulin therapy (Schaumberg et al., 2005). Further, recent reports have suggested that dysregulation of epigenetic modification could be responsible for sustained proinflammatory phenotype of the vascular smooth muscle cells from diabetic mice after being stimulated with TNF-α (Villeneuve et al., 2008). Our novel data demonstrate that the levels of these inflammatory mediators fail to reverse in the retina even after six months of good glycemic control that has followed six months of poor glycemic control. The inability to suppress retinal cytokines and adhesion molecules effectively at the transcriptional and translational levels implies that inflammation is one of the important pieces of the metabolic memory puzzle. Understanding the mechanism responsible for the tendency of diabetic retinopathy to progress after reestablishment of good glycemic control should help reveal targets for therapy to prevent its progression.

Acknowledgments

We would like to thank Divyesh Sarman for technical assistance. This study was supported in part by grants from the National Institutes of Health, Juvenile Diabetes Research Foundation, the Thomas Foundation, Midwest Eye-Banks and Research to Prevent Blindness.

REFERENCES

Abu El-Asrar AM, Desmet S, Meersschaert A, Dralands L, Missotten L, Geboes K. Expression of the inducible isoform of nitric oxide synthase in the retinas of human subjects with diabetes mellitus. American Journal of Ophthalmology 2001;132:551–556. [PubMed: 11589878]

Adamis AP, Berman AJ. Immunological mechanisms in the pathogenesis of diabetic retinopathy. Seminars in immunopathology 2008;30:65–84. [PubMed: 18340447]

Ayalasomayajula SP, Kompella UB. Celecoxib, a selective cyclooxygenase-2 inhibitor, inhibits retinal vascular endothelial growth factor expression and vascular leakage in a streptozotocin-induced diabetic rat model. European Journal of Pharmacology 2003;458:283–289. [PubMed: 12504784]

Chang CK, LoCicero J. Overexpressed nuclear factor [kappa]B correlates with enhanced expression of interleukin-1[beta] and inducible nitric oxide synthase in aged murine lungs to endotoxic stress. The Annals of thoracic surgery 2004;77:1222–1227. [PubMed: 15063239]

Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development of long-term complications in insulin-dependent diabetes mellitus. New England Journal of Medicine 1993;329:977–986. [PubMed: 8366922]

Diabetes Control and Complications Trial Research Group. Adverse events and their association with treatment regimens in the diabetes control and complications trial. Diabetes Care 1995;18:1415–1418. [PubMed: 8722064]

Diabetes Control and Complications Trial Research Group. Early worsening of diabetic retinopathy in the diabetes control and complication trial. Archives of Ophthalmology 1998;116:874–886. [PubMed: 9682700]

Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. Effect of intensive therapy on the microvascular complications of type 1 diabetes mellitus. The Journal of American Medical Association 2002;287:2563–2569.

Du Y, Smith MA, Miller CM, Kern TS. Diabetes-induced nitrative stress in the retina, and correction by aminoguanidine. Journal of Neurochemistry 2002;80:771–779. [PubMed: 11948240]

Du Y, Sarthy VP, Kern TS. Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats. American journal of physiology. Regulatory, integrative and comparative physiology 2004;287:R734–R741.

Engerman RL, Kern TS. Hyperglycemia as a cause of diabetic retinopathy. Metabolism 1986;35:20–23. [PubMed: 3083205]

Engerman RL, Kern TS. Progression of incipient diabetic retinopathy during good glycemic control. Diabetes 1987;36:808–812. [PubMed: 3556280]

Genuth S, Sun W, Cleary P, Sell DR, Dahms W, Malone J, Sivitz W, Monnier VM. Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the diabetes control and complications trial and epidemiology of diabetes interventions and complications participants with type 1 diabetes. Diabetes 2005;54:3103–3111. [PubMed: 16249432]

Hammes H-P, Klinzing I, Wiegand S, Bretzel RG, Cohen AM, Federlin K. Islet transplantation inhibits diabetic retinopathy in the sucrose-fed diabetic Cohen diabetic rat. Investigative Ophthalmology and Visual Science 1993;34:2092–2096. [PubMed: 8491559]

Ihnat MA, Thorpe JE, Kamat CD, Szabó C, Green DE, Warnke LA, Lacza Z, Cselenyák A, Ross K, Shakir S, Piconi L, Kaltreider RC, Ceriello A. Reactive oxygen species mediate a cellular 'memory' of high glucose stress signaling. Diabetologia 2007;50:1523–1531. [PubMed: 17508197]

Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP. Leukocyte-mediated endothelial cell injury and death in the diabetic retina. American Journal of Pathology 2001;158:147–152. [PubMed: 11141487]

Joussen AM, Poulaki V, Mitsiades N, Kirchhof B, Koizumi K, Dohmen S, Adamis AP. Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. The FASEB journal 2002;16:438–440. [PubMed: 11821258]

Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP. A central role for inflammation in the pathogenesis of diabetic retinopathy. The FASEB journal 2004;18:1450–1452. [PubMed: 15231732]

Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Experimental diabetes research 2007;2007:95103. [PubMed: 18274606]

Kowluru RA, Koppolu P. Termination of experimental galactosemia in rats, and progression of retinal metabolic abnormalities. Investigative Ophthalmology and Visual Science 2002;43:3287–3291. [PubMed: 12356836]

Kowluru RA. Effect of re-institution of good glycemic control on retinal oxidative stress and nitrative stress in diabetic rats. Diabetes 2003;52:818–823. [PubMed: 12606525]

Kowluru RA, Koppolu P, Chakrabarti S, Chen S. Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. Free Radical Research 2003;37:1169–1180. [PubMed: 14703729]

Kowluru RA, Chakrabarti S, Chen S. Re-Institution of good metabolic control in diabetic rats on the activation of caspase-3 and nuclear transcriptional factor (NF-kB) in the retina. Acta Diabetologica 2004;44:194–199. [PubMed: 15660203]

Kowluru RA, Odenbach S. Role of interleukin-1beta in the development of retinopathy in rats: effect of antioxidants. Investigative Ophthalmology and Visual Science 2004a;45:4161–4166. [PubMed: 15505070]

Kowluru RA, Odenbach S. Role of interleukin-1beta in the pathogenesis of diabetic retinopathy. British Journal of Ophthalmology 2004b;88:1343–1347. [PubMed: 15377563]

Kowluru RA, Kanwar M, Kennedy A. Metabolic memory phenomenon and accumulation of peroxynitrite in retinal capillaries. Experimental diabetes research 2007;2007:2196.

Kowluru RA, Menon B, Gierhart D. Beneficial effect of zeaxanthin on retinal metabolic abnormalities in diabetic rat. Investigative Ophthalmology and Visual Science 2008;49:1645–1651. [PubMed: 18385086]

LeRoith D, Fonseca V, Vinik A. Metabolic memory in diabetes--focus on insulin. Diabetes/metabolism research and reviews 2005;21:85–90. [PubMed: 15619272]

Libby P. Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutrition Reviews 2007;65:S140–S146. [PubMed: 18240538]

Matsumoto K, Sera Y, Ueki Y, Inukai G, Niiro E, Miyake S. Comparison of serum concentrations of soluble adhesion molecules in diabetic microangiopathy and macroangiopathy. Diabetic medicine 2002;19:822–826. [PubMed: 12358868]

Quan N, He L, Lai W. Endothelial activation is an intermediate step for peripheral lipopolysaccharide induced activation of paraventricular nucleus. Brain Research Bulletin 2003;59:447–452. [PubMed: 12576141]

Robison WG, Jacot JL, Glover JP, Basso MD, Hohman TC. Diabetic-like retinopathy: Early and late intervention therapies in galactose-fed rats. Investigative Ophthalmology and Visual Science 1998;39:1933–1941. [PubMed: 9727416]

Schaumberg DA, Glynn RJ, Jenkins AJ, Lyons TJ, Rifai N, Manson JE, Ridker PM, Nathan DM. Effect of intensive glycemic control on levels of markers of inflammation in type 1 diabetes mellitus in the diabetes control and complications trial. Circulation 2005;111:2446–2453. [PubMed: 15867184]

Sims JE, Gayle MA, Slack JL, Alderson MR, Bird TA, Giri JG, Colotta F, Re F, Mantovani A, Shanebeck K. Interleukin 1 signaling occurs exclusively via the type I receptor. Proceedings of the National Academy of Sciences (USA) 1993;90:6155–6159.

Tang S, Le-Ruppert KC, Gabel V-P. Expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on proliferating vascular endothelial cells in diabetic epiretinal membranes. British Journal of Ophthalmology 1994;78:370–376. [PubMed: 7517695]

UKPDS. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes. Lancet 1998;352:837–853. [PubMed: 9742976]

Villeneuve LM, Reddy MA, Lanting LL, Wang M, Meng L, Natarajan R. Epigenetic histone H3 lysine 9 methylation in metabolic memory and inflammatory phenotype of vascular smooth muscle cells in diabetes. Proc Natl Acad Sci U S A 2008;105:9047–9052. [PubMed: 18579779]

Vincent JA, Mohr S. Inhibition of caspase-1/interleukin-1beta signaling prevents degeneration of retinal capillaries in diabetes and galactosemia. Diabetes 2007;56:224–230. [PubMed: 17192486]

Yuuki T, Kanda T, Kimura Y, Kotajima N, Tamura J, Kobayashi I, Kishi S. Inflammatory cytokines in vitreous fluid and serum of patients with diabetic vitreoretinopathy. Journal of Diabetes and its Complications 2001;15:257–259. [PubMed: 11522500]

Zheng L, Du Y, Miller C, Gubitosi-Klug RA, Kern TS, Ball S, Berkowitz BA. Critical role of inducible nitric oxide synthase in degeneration of retinal capillaries in mice with streptozotocin-induced diabetes. Diabetologia 2007;50:1987–1996. [PubMed: 17583794]

Zheng L, Gong B, Hatala DA, Kern TS. Retinal ischemia and reperfusion causes capillary degeneration: similarities to diabetes. Investigative Ophthalmology and Visual Science 2007;48:361–367. [PubMed: 17197555]

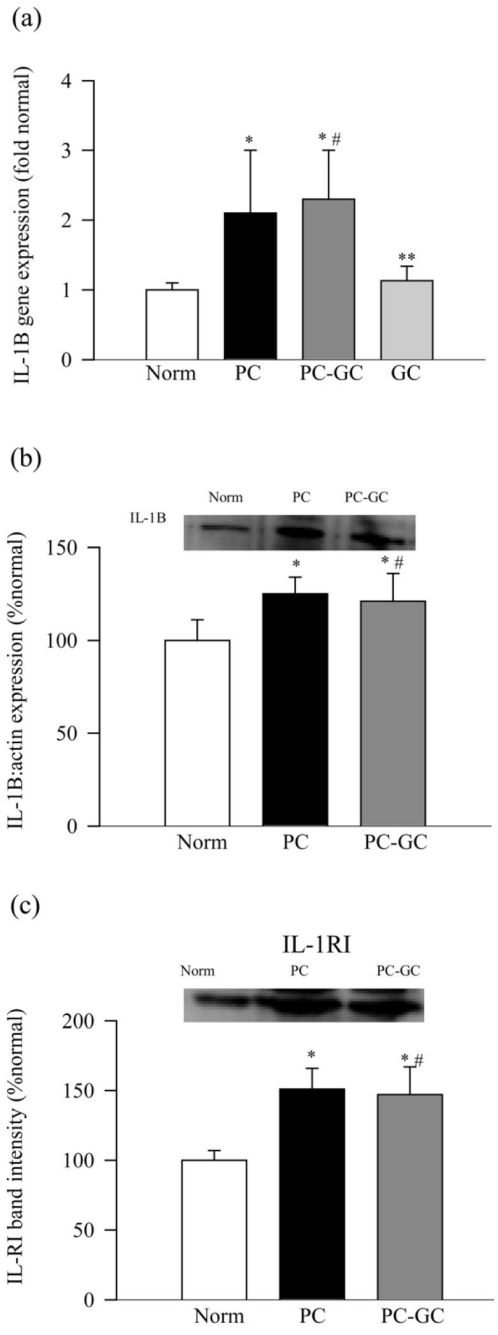

Figure 1.

Effect of reversal of hyperglycemia on IL-1β gene expression (a), protein expression (b) and its receptor, IL-1RI (c). Retinal gene expression was measured with Q-RT-PCR and the fold change for the retina of age-matched normal rats is considered one. Protein expression was assessed by western blot; the band intensities were adjusted to β-actin expression and the values obtained for normal rat retina are 100%. IL-1RI data was quantified by immunoprecipitation with IL-1β followed by western blotting with antibody for IL-1RI. Data for Q-RT-PCR represent the mean ± SD from 8–10 rats in each of the 4 groups. The Western blots are representative of at least 5 different rats in each group, and the bars represent the mean ± SD

of the adjusted band intensities obtained from those rats in each of the 4 groups (b & c). $^{*}P<0.05$ compared to normal, and $^{**}P<0.05$ and $^{\#}P>0.05$ compared to PC.

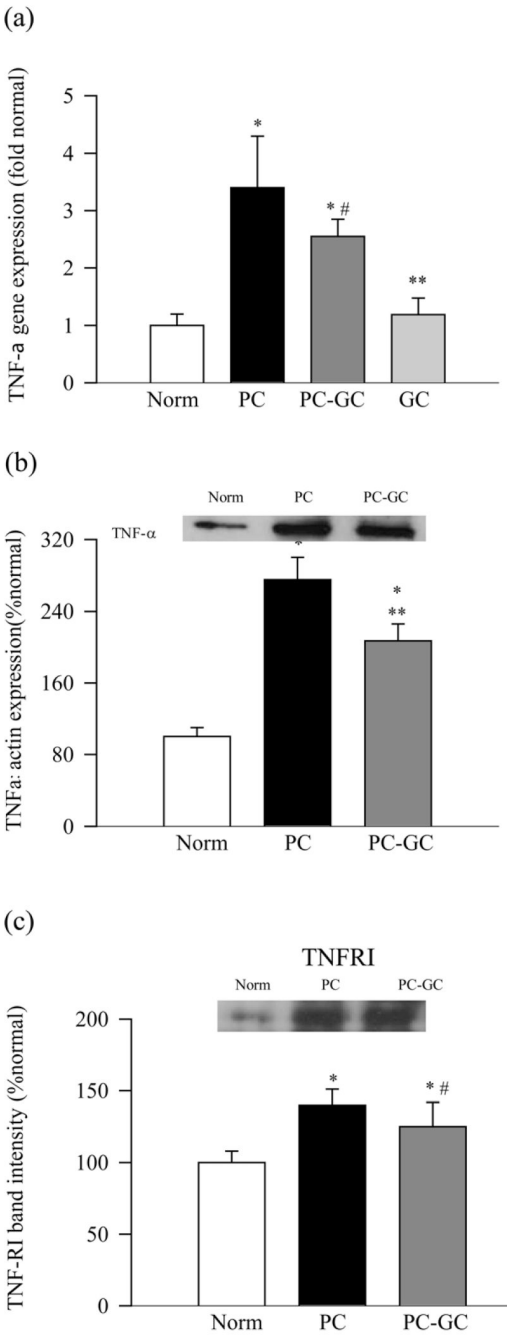

Figure 2.

Effect of reversal of hyperglycemia on retinal TNF-α gene expression (a), protein expression (b) and TNFRI (c). Gene expression was quantified with Q-RT-PCR and protein expression by western blot; the band intensities were normalized to β-actin expression and the values obtained for normal rats are considered 100%. TNFRI level was measured by immunoprecipitation. Data for Q-RT-PCR are mean ± SD from 8 or more rats in each group (a), and the western blots are representative of 5 different rats in each group $^{*}P<0.05$ compared to normal, and $^{**}P<0.05$ and $^{\#}P>0.05$ compared to PC.

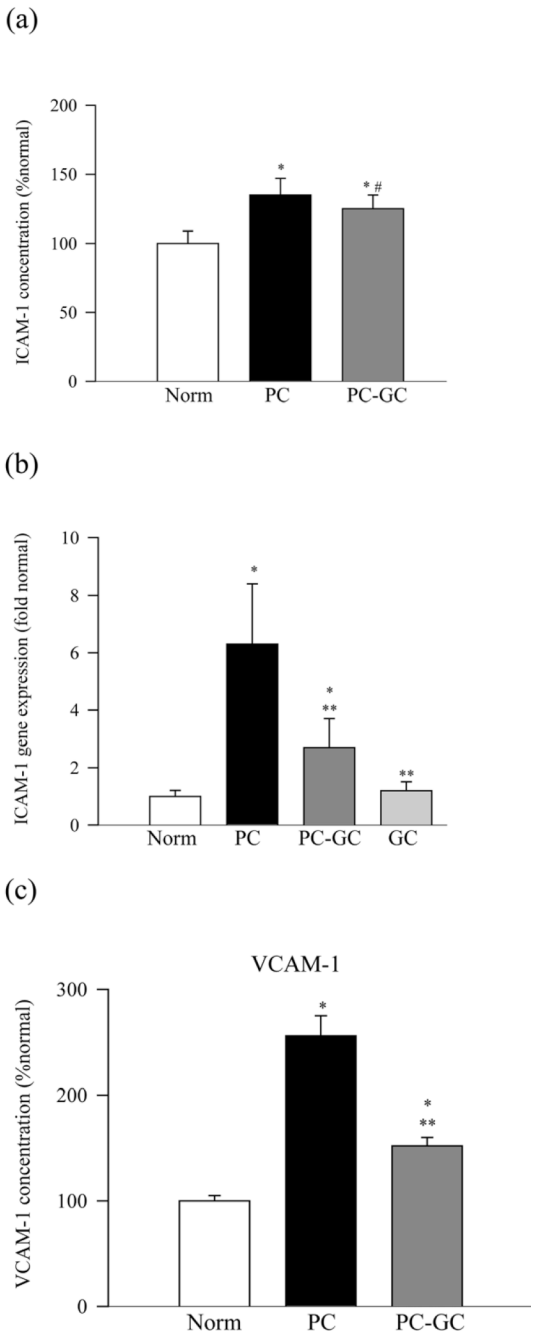

Figure 3.

Effect of re-institution of good glycemic control on retinal adhesion molecules. The mRNA levels of ICAM-1 (a) were determined by Q-RT-PCR. The concentration of ICAM-1 (b) and that of VCAM-1 (c) were determined by ELISA. For ELISA, the values obtained for normal rat retina are considered 100%. Data represent the mean ± SD from 8–10 each group for Q-RT-PCR and 5 rats each for ELISA. $^{*}P<0.05$ compared to normal, and $^{**}P<0.05$ and $^{\#}P>0.05$ compared to PC.

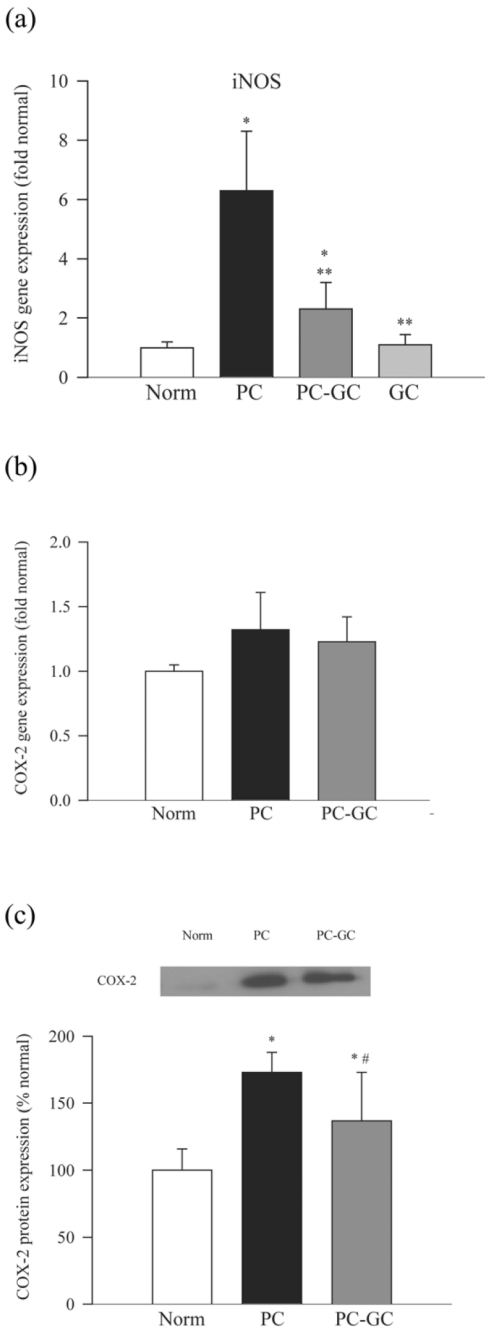

Figure 4.

Relative gene expression of iNOS (a) and COX-2 (b), and protein expression of COX-2 (c) in the retina of rats in glycemic control. Retinal gene expression data was obtained with Q-RT-PCR and compared with the age-matched normal rats. COX-2 protein expression for rat retina was measured with western blot; the band intensities were adjusted to β-actin expression and the values obtained for normal rats are 100%. $^{*}P<0.05$ compared to normal, and $^{**}P<0.05$ and $^{\#}P>0.05$ compared to PC.

Table 1

GenBank accession number and the amplicon length for the target genes and housekeeping gene used for Q-RT-PCR

Gene	GenBank Accession No.	Amplicon length (bp)
IL-1β	NM_031512.1	74
TNFα	NM_012675.2	108
ICAM-1	NM_012967.1	61
iNOS	NM_012611.2	77
COX-2	NM_017232.2	96
B2M	NM_012512.1	58

Table 2

Severity of hyperglycemia in rats assigned to poor or good glycemic control

	Duration (months)	Body weight (g)	Food (g/day)	GHb (%)	Urine Vol (ml)
Normal	12	405±17	23±3	6.5 ± 0.7	15±5
PC	12	291±33*	36±9*	12.9 ± 1.9*	97±35
GC	12	425±40	38±7	6.7±0.5	25±7
PC	6	283±11*	40±10*	13.5 ± 2.0*	110±29
↓	↓	↓	↓	↓	↓
GC	6	388±17	25±9	6.9 ± 1.1	27±10

The values are mean ± SD of 10 or more rats in each group. Body weights were measured two times and food intake once every week. Glycated hemoglobin was quantified every three months, and urine volume was measured on a daily basis.

*P<0.05 compared to normal.