The Effect of Hyperglycemia on Hypoperfusion-Induced Injury

Matthew C. Holman,¹,² Glyn Chidlow,¹,² John P. M. Wood,¹,² and Robert J. Casson¹,²

PURPOSE. Because of differences in energy metabolism between the brain and retina, the hypothesis for the study was that, in a model of ocular and cerebral hypoperfusion, the retina would be protected by short-term hyperglycemia, whereas brain injury would be exacerbated.

METHODS. Hyperglycemia was induced by intraperitoneal streptozotocin. An initial experiment determined the effect of hyperglycemia alone in sham-surgery rats. Simultaneous retinal and cerebral hypoperfusion was achieved by two-vessel occlusion (2VO; permanent ligation of both common carotid arteries). Hyperglycemia was induced 3 days before 2VO by streptozotocin injection. The rats were killed 7 days after 2VO or sham surgery. The retina of one eye was collected for histology/immunohistochemistry and the fellow retina was collected for real-time RT-PCR. The retinas were analyzed for neuronal and glial markers and heat shock protein-27. The brains were processed for histology and immunohistochemistry.

RESULTS. Short-term (approximately 10 days) hyperglycemia alone caused no discernible injury to the retina. The retinas of the normoglycemic 2VO animals showed a marked loss of retinal ganglion cells and horizontal cells, thinning of the inner retina, glial cell activation, and infiltration of macrophages. The hyperglycemic 2VO rats displayed remarkable protection of the retinal structure and reduced glial cell activation compared with the normoglycemic 2VO animals. There was a significantly greater number of heat shock protein-27–positive retinal ganglion cells in the normoglycemic animals than in the hyperglycemic ones. Brains of both the normoglycemic and hyperglycemic 2VO animals displayed scattered ischemic infarcts and mild white matter injury.

CONCLUSIONS. Short-term hyperglycemia affords robust protection against retinal hypoperfusion injury, but in the same animals, brain injury is not ameliorated. The mechanism of this retinal hyperglycemia-induced neuroprotection requires further study. (Invest Ophthalmol Vis Sci. 2010;51:2197–2207) DOI:10.1167/iovs.09-4191

The retina is derived from an outpouching of the diencephalon and is generally considered to be a specialized part of the central nervous system (CNS); however, although the retina is traditionally viewed as an “approachable part of the brain,”¹ there are important metabolic differences between these structures. The retina makes a considerable portion of its adenosine triphosphate (ATP) by glycolysis, even in the presence of oxygen (aerobic glycolysis).²⁻³ This is akin to the situation in some neoplasms and is known as the Warburg effect. In addition, by upregulating glycolysis, the retina can continue to make up to 70% of its ATP requirement in the absence of oxygen, as long as glucose is abundant—³—a manifestation of the Pasteur effect.⁴ The underlying mechanisms remain incompletely understood, but are thought to involve hypoxia inducible factor (HIF)-¹ and its effect on glycolytic enzymes and inhibition of substrate entry into the Krebs cycle.⁵ In contrast, the cerebrum relies on the continued presence of oxygen and oxidative phosphorylation (OXPHOS) to meet its energy demands.⁶

Through a variety of molecular mechanisms,⁸ hyperglycemia causes the end-organ pathologic effect of diabetes mellitus, including diabetic microangiopathy and neuropathy. Hyperglycemia is also known to worsen the outcome of most forms of stroke, both clinically and in animal models.⁹ However, our work has revealed the intriguing finding that short-term elevation of the glucose concentration within the eye produces a remarkable degree of protection against pressure-induced ischemic retinal injury (the acute model).¹⁰ We have further shown that hypoglycemia exacerbates ischemic retinal injury,¹¹ that retinal neurons preferentially metabolize glucose over lactate,¹² that intracellular lactate delivery is not protective,¹³ and that inhibition of lactate transport does not exacerbate ischemic retinal injury in vivo.¹⁴ Together, these findings indicate that glucose and not lactate is responsible for the protective effect. The immediacy of the protective effect makes trophic factor upregulation an implausible mechanism; moreover, given that blood flow is completely disrupted, and hyperglycemia in the posts ischemic reperfusion period is not protective, a glucose-dependent effect on blood flow is also an unconvincing explanation.

It is clear that underlying experimental hyperglycemia has contrasting effects on neuronal survival in different regions of the CNS subjected to acute injury paradigms. For these findings to have a genuine clinical relevance, however, it is desirable that they be replicated in chronic models of neuronal degeneration. To our knowledge, such studies have not been undertaken to date. The major goal of this study, then, was to determine the effect of experimental hyperglycemia on the retinal and brain outcomes after a common hypoperfusion insult. Because of the differences in energy metabolism between the retina and brain, furthermore, we were particularly eager to determine whether our data would indicate clear, opposing effects in the two tissues—namely, protection against retinal but not brain injury.

We tested this hypothesis in a model of global hypoperfusion by permanent bilateral ligation of the common carotid arteries in rats (two-vessel occlusion, 2VO).¹⁴ The attraction of this model is that it precipitates a severe retinal injury, making neuroprotection more difficult to achieve than in the pressure-
induced ischemia model, yet causes a relatively mild injury in the brain, thereby providing an interesting comparison with results from acute stroke models.

**MATERIALS AND METHODS**

**Animals and Procedures**

All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This project was approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science, Adelaide, and conforms to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2004. Adult Sprague-Dawley rats (220–350 g) were housed in a temperature- and humidity-controlled room with a 12-hour light:12-hour dark cycle and were provided with food and water ad libitum. Hyperglycemia was induced in age- and sex-matched rats with a 60-mg/kg intraperitoneal injection of streptozotocin; control animals received saline. Blood glucose levels were measured from tail vein blood 4 days later, before surgery, to ensure that hyperglycemia had been induced. 2VO was performed with the animals under isoflurane anesthesia. The common carotid arteries were identified after a midline neck incision and blunt dissection of strap muscles; the carotids were carefully separated from the vagus nerve and ligated with silk sutures. The sham-surgery (sham) rats underwent the same operation without occlusion of the vessels. The rats were killed 1 week after 2VO or sham surgery, and the retina of one eye was collected for histology/immunohistochemistry and the fellow retina for RT-PCR.

**Experimental Plan**

In preliminary experiments, we demonstrated that the retinal histology and immunohistochemistry of normoglycemic sham rats did not differ from the retinas of untouched control animals—that is, the sham surgery in normal rats did not cause any discernible changes in the retinal histology or immunohistochemistry (data not shown).

The principal hypothesis of interest (H1) was that 2VO hyperglycemic rats would have significantly less neuronal cell injury and death and significantly diminished glial cell activation than would 2VO normoglycemic rats (i.e., that hyperglycemia would protect against the hypoperfusion injury caused by 2VO). It was, however, important to determine whether the sham hyperglycemics would demonstrate any pathologic changes compared to the sham normoglycemics (i.e., whether the retina would be affected by ~10 days of hyperglycemia in the absence of any hypoperfusion insult). Our hypothesis (H2) was that hyperglycemia of this duration would produce no discernible changes. If this was not the case, then it would have been difficult to proceed with testing the principal hypothesis (H1). Hence, the data from the experiment to test H2 were analyzed first.

After the analysis of the data, a further experiment was performed to test the hypothesis (H3) that short-term induction of diabetes elicits a preconditioning effect in the retina that could contribute to the protective effect we observed against 2VO. In this experiment, the rats were given an injection of saline or streptozotocin (to induce hyperglycemia) and were analyzed after 4 days.

**Tissue Preparation**

Deeply anesthetized animals were transcardially perfused with physiological saline. After enucleation, whole eyes were postfixed in 10% neutral-buffered formalin and processed for routine paraffin-embedded sections. The eyes were embedded sagittally, and 5-μm serial sections were cut. The rat brains were cut into coronal slices by using a rat brain blocker and 2-mm coronal slices were taken, starting from the (PA001; Kopf Instruments, Tujunga, CA). The brains were positioned in

**Immunohistochemistry**

Tissue sections were deparaffinized, rinsed in 100% ethanol, and treated for 50 minutes with 0.5% H2O2 to block endogenous peroxidase activity. Antigen retrieval was achieved by microwaving the sections in 10 mM citrate buffer (pH 6.0). Tissue sections were then blocked in PBS containing 3% normal horse serum, incubated overnight at room temperature in primary antibody containing 3% normal horse serum (Table 1), followed by consecutive incubations with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and streptavidin–peroxidase conjugate (Pierce, Rockford, IL). Color development was achieved with 3′,3′-diaminobenzidine. The sections were counterstained with hematoxylin, dehydrated, and mounted. Specificity of antibody staining was confirmed by incubating adjacent sections with isotype controls (mouse IgG1 and IgG2a, 50878 and 553454; BD Pharmingen, Melbourne, VIC, Australia) for monoclonal antibodies, or normal rabbit/goat serum for polyclonal rabbit/goat antibodies.

For double labeling of heat shock protein (Hsp)-27 with β-tubulin, the sections were incubated overnight at room temperature with rabbit anti-Hsp27 (1:300) and mouse anti-β-tubulin (1:200), as described previously. On the following day, the sections were incubated with AlexaFluor donkey anti-mouse IgG 488 (1:250) together with AlexaFluor donkey anti-rabbit IgG 594 (1:250) for 1 hour. The sections were then mounted with antifade mounting medium (ProLong Gold; Invitrogen, Mulgrave, VIC, Australia) and examined with a confocal fluorescence microscope.

**Evaluation of Immunohistochemistry**

For each marker analyzed, immunostaining of sections from all four groups (sham and 2VO normoglycemic rats, and sham-surgery and 2VO hyperglycemic rats) was performed in a single batch, and each section was exposed to DAB color development for exactly 5 minutes. The number of rats in each group was as follows: normoglycemic 2VO, n = 15; hyperglycemic 2VO, n = 12; normoglycemic sham, n = 6; and hyperglycemic sham, n = 6.

**Retina.** Evaluations of the sections were performed by three independent observers blinded to the treatment group on a light microscope equipped with an ocular micrometer bar. To minimize sampling errors, all analyses were performed on sections taken at the level of the optic nerve head. In general, two sections per animal were immunostained for each marker. The resultant scores were pooled and averaged. The number of RGCs (Brn-3 and Islet-1) and horizontal cells (calbindin and protein gene product 9.5, PGP 9.5) were counted to a distance of 2 mm on either side of the optic nerve head, which equates to approximately 50% of the entire retina. Relative immunoreactivities for macroglia (GFAP and nestin) and microglia (Iba1 and ED1) were scored with a 4-point grading system after appraisal of the entire length of the retina.

**GFAP.** Normal staining of astrocytes and Müller cell endfeet; 1, normal staining of astrocytes, scattered Müller cell processes generally reaching only to the outer plexiform layer; 2, hypertrophied astrocytes, numerous Müller cell processes, some reaching to the outer limiting membrane; 3, hypertrophied astrocytes, numerous Müller cell processes, thicker in appearance, with most reaching to the outer limiting membrane.

**Nestin.** 0, blood vessels; 1, light staining of astrocytes and Müller cell endfeet, occasional thin processes; 2, stained astrocytes, many thin Müller cell processes but generally reaching only to the outer plexiform layer; 3, robust staining of astrocytes, numerous Müller cell processes, many reaching to the outer limiting membrane.

**Iba1** (ionized calcium-binding adapter molecule 1). 0, few ramified microglia within the inner retina; 1, numerous ramified microglia within the inner retina; 2, numerous microglia, some ramified, many amoeboid with retracted processes; and 3, numerous, hypertrophied microglia with few processes, some macrophages.

**ED1.** 0, fragments of blood vessels, no microglia; 1, light, wispy staining of some processes on a few microglia within the inner retina; 2, medium intensity staining of intermediate number of microglia within the inner retina; and 3, numerous, strongly stained microglia, some macrophages.
levels of each brain, corresponding to approximately analyzed for regions of focal injury. Tissue sections from four coronal

The scores from each pair of markers (e.g., Brn-3 and Islet-1 for RGCs, overall body of scientific evidence from which to draw conclusions.

changes were chosen to provide complementary data and produce an statistically significant. The statistical test was first applied to the data determine whether differences in the immunograde means were sta-

noted antibodies to select markers for their immunograde and neuronal changes were chosen to provide complementary data and produce an overall body of scientific evidence from which to draw conclusions. The scores from each pair of markers (e.g., Brn-3 and Islet-1 for RGCs, ED1 and APP) correlated highly because they are measuring the same effect; however, a P-value correction for each immunostain comparison was considered inappropriate (P < 0.05 only on one of a pair of markers would not have been considered supportive of any conclusion). However, because a statistically significant result in either glial activation or neuronal survival would have been considered important, we applied a Bonferroni correction for two comparisons. We believe that this provides a scientifically rigorous approach and minimizes the risk of both type I and type II statistical errors. Homocedasticity was checked with Bartlett's test, and when appropriate an approximate F-test with Welch's degrees of freedom was used for the analysis (Stata/IC ver. 10; StatSoft, College Station, TX).

Brain. Initially, gray matter and white matter from all brains were analyzed for regions of focal injury. Tissue sections from four coronal levels of each brain, corresponding to approximately +0.45, −1.33, −3.25, and −5.25 mm relative to the bregma, were evaluated. Every region of interest was photographed and imported into ImageJ (http://rsb.info.nih.gov/ij/) developed by Wayne Rasband, National Institutes of Health, Bethesda, MD), and image analysis was performed to measure the area. The total area per brain was then calculated.

Evaluations of immunohistochemical staining of brain sections were performed by three independent observers blinded to the treatment, on a light microscope equipped with an ocular micrometer bar. Astrocyte stress was evaluated by immunostaining for Hsp27 in the cerebral cortex; white matter damage was assessed by immunostaining for amyloid precursor protein (APP) and ED1 (markers of disrupted axonal flow and activated microglia, respectively) in the medial corpus callosum. Scores for ED1 and APP were combined and averaged, to provide an index of white matter injury. As was done for the retina, relative immunoreactivities were scored on a 4-point scale, based on intensity and frequency of immunostaining.

Statistical Analysis. Unpaired Student’s t-tests were used to determine whether differences in the immunograde means were statistically significant. The statistical test was first applied to the data from the test of H2 and then to the data from the test of H1. The eight test of H2 and then to the data from the test of H1. The eight scores from each pair of markers (e.g., Brn-3 and Islet-1 for RGCs, overall body of scientific evidence from which to draw conclusions.

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Real-Time RT-PCR

Reverse-transcription polymerization chain reaction (RT-PCR) studies were performed essentially as previously described. Briefly, total RNA was isolated from the entire retina and first-strand cDNA was synthesized from 2 μg DNease-treated RNA. Real-time PCR reactions were performed in 96-well optical reaction plates using the cDNA equivalent of 20-ng total RNA for each sample in a total volume of 25 μL, containing SYBR Green PCR master mix and forward and reverse primers at a final concentration of 400 nM. The thermal cycling conditions were 95°C for 3 minutes and 40 cycles of amplification comprising 95°C for 12 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds (see Table 1 for details and primer sequences). Results obtained from the real-time PCR experiments were quantified using the comparative threshold cycle (CT) method ($\Delta \Delta CT$) for relative quantitation of gene expression, corrected for amplification efficiency. All values were normalized using the endogenous housekeeping gene GAPDH and are expressed relative to the data for the shams. Statistical analysis was performed by ANOVA followed by the post hoc Tukey test. The null hypothesis tested was that CT differences between target and housekeeping genes would be the same in the sham, normoglycemic 2VO and hyperglycemic 2VO rats.

For conventional PCR, the individual cDNA species were amplified in a reaction containing the cDNA equivalent of 20 ng total RNA, 1× PCR buffer, MgCl₂, 0.8 mM each dNTP, the relevant sense and antisense primers at a final concentration of 4 ng/μL, and 0.5 U polymerase (AmpliTaq Gold; Applied Biosystems, Melbourne, Australia). PCRs (94°C, 12 seconds; annealing temperature, 50 seconds; 72°C, 30 seconds) were performed for a suitable number of cycles followed by a final extension at 72°C for 5 minutes. Primer sets used were as follows: FGF-2 (sense: 5'-CGTCAAACTACAGCATTCCAAGCAGA-3', antisense: 5'-GGATCGAGTTTATCGTCCGAGT3'), 52°C annealing temperature);
CNTF (sense: 5'-TGGCTAGCAGGAAGATTCGT-3', antisense: 5'-AG-GAAGTGGGATGGACCT-3', 60°C annealing temperature); TNFα (sense: 5'-TACGCACTGGGGGTATTGGTC-3', antisense: 5'-CAGCTTGTCCCGTTAGAAGAACC-3', 60°C annealing temperature); and iNOS (sense: 5'-CGCTACACTTCCAACGCAAC-3', antisense: 5'-AG-GAAGTGGGCTTGAAGAGAACC-3', 55°C annealing temperature). Prior experiments had determined the linear phase of amplification for each set of primers. All the PCR products yielded single bands corresponding to the expected molecular weights.

RESULTS

The Effect of Sham Surgery on the Retina and Brain

Sham surgery alone produced no discernible histologic change, nor any effect on neuronal or glial immunohistochemistry in either the retina or brain compared with the untouched controls (data not shown). In addition, real-time PCR showed that the retinal levels of mRNAs encoding neuronal or glial markers were unaltered after sham surgery alone compared with untouched controls (data not shown). Likewise, there was no difference in histology, neuronal counts, glial grading, or retinal levels of mRNAs encoding neuronal or glial markers in the sham normoglycemics compared with the sham hyperglycemics (data not shown).

Retinal Histology and Neuronal Survival in Normoglycemic 2VO and Hyperglycemic 2VO Rats

Representative histologic images of tissue sections from the sham, normoglycemic 2VO, and hyperglycemic 2VO rats are shown in Figure 1. Normoglycemic 2VO rats showed marked degenerative changes, with a reduced number of neuronal cell bodies and thinner retinas compared with that in sham animals (Figs. 1A, 1B). In contrast, in the retinas of the hyperglycemic 2VO rats, hematoxylin and eosin (H&E) staining revealed little evidence of neuronal degeneration (Fig. 1C). These findings prompted a quantitative evaluation of the effect of hypergly-
Neurons were counted to a distance of 2 mm either side of the optic nerve head. In all cases, values represent the mean ± SEM, where n = 12–13. NG, normoglycemic; HG, hyperglycemic.

* P < 0.001, † P < 0.01 by unpaired *t* test (hyperglycemic versus normoglycemic).

In addition to histologic methods, reliable and sensitive assessment of neuronal injury or glial cell activation can be achieved by measurement of the total retinal content of mRNAs encoding cell-specific markers. Quantification of the levels of certain mRNAs provides information that is complementary to that obtained from immunohistochemistry. Advantages of mRNA analysis include the fact that the entire retina is analyzed, that real-time PCR is a sensitive and precise quantitative tool, and that downregulation of mRNA synthesis precedes cell death and can be viewed as an early marker of neuronal injury. In the present study, we examined the levels of mRNAs specific to RGCs, horizontal cells and macroglia in sham-surgery, normoglycemic 2VO, and hyperglycemic 2VO rats.

In the normoglycemic rats, 2VO followed by a 7-day recovery, caused an 85% reduction in the total retinal level of neurofilament-light (NF-L) mRNA PCR and a 74% reduction in the total retinal level of calbindin when measured by real-time RT-PCR compared to that in the sham animals (Table 4). In contrast, in hyperglycemic 2VO rats, the total retinal levels of NF-L and calbindin were reduced by 50% and 29%, respectively; thus, hyperglycemia elicited approximately 35% and 45% protection against loss of these mRNAs, values that were statistically (P < 0.001) significant. With regard to glial activation, 2VO induced a statistically significant fourfold increase in nestin synthesis in the normoglycemic rats compared with the sham animals, but only a nonsignificant twofold elevation in the hyperglycemic rats (Table 4).

**The Effect of 2VO on Hsp27 Expression in Retinas of Normoglycemic 2VO and Hyperglycemic 2VO Rats**

Quantitative evaluation of neuronal survival is necessarily of paramount interest in neuroprotection studies. In the context of a chronic injury like 2VO, however, it is also of real importance to ascertain whether neurons that survive the time course of the experiment are healthy or compromised. Analysis of Hsp27 provides such information. Hsp27 is not constitutively expressed by RGCs, but within an ongoing pathologic setting is persistently upregulated. Expression of Hsp27 by such cells is thought to be induced by cellular stress and functions to assist refolding of misfolded and aggregated proteins. It can, therefore, be considered a marker of ongoing RGC stress/injury. We analyzed sham, normoglycemic 2VO, and hyperglycemic 2VO rats for the number of Hsp27-positive RGCs.

Negligible Hsp27 immunoreactive RGCs were observed in the sham rats (Fig. 3A). In both normoglycemic and hyperglycemic 2VO rats, Hsp27-positive cells were identified within the GCL (Figs. 3B, 3C, respectively). These cells were clearly identified as RGCs, since double-labeling immunohistochemistry demonstrated a clear co-localization of Hsp27 with specific RGC markers, such as β-tubulin (Figs. 3D–F). Quantitative evaluation of the number of Hsp27-labeled RGCs in the three groups revealed that there were twice as many cells in the retinas of the normoglycemic...
mic 2VO rats as in the hyperglycemic 2VO rats, despite the lower total number of surviving RGCs in the normoglycemic retinas (Fig. 3G). When expressed as a fraction of the total number of surviving RGCs (Fig. 3G), ~50% of the surviving RGCs in the normoglycemic 2VO rats were Hsp27-positive compared with only 13% in the hyperglycemic 2VO rats.

Involvement of Preconditioning in the Neuroprotective Effect of Hyperglycemia against 2VO

In the present study, hyperglycemia (via injection of streptozotocin) was induced several days before carotid artery ligation. As such, it is possible that the resultant short-term diabetes stimulated a retinal preconditioning response that contributed to the neuroprotection against 2VO. Although the precise mechanisms of preconditioning remain incompletely characterized, upregulation of trophic factors, inducible Hsps, and certain proinflammatory mediators, have all been purported to be involved. To assess the possible involvement of preconditioning, we measured the expression of two inducible Hsps (Hsp27 and -70), two trophic factors (fibroblast growth factor 2 [FGF-2] and ciliary neurotrophic factor, [CNTF]), and two

Table 3. Effect of Hyperglycemia on 2VO-Induced Glial Activation in the Rat Retina

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Microglia Response</th>
<th>Astrocyte/Müller Cell Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED1</td>
<td>Iba1</td>
</tr>
<tr>
<td>Sham</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>NG 2VO</td>
<td>2.3 ± 0.6</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>HG 2VO</td>
<td>0.9 ± 0.2†</td>
<td>1.1 ± 0.2‡</td>
</tr>
</tbody>
</table>

Glial cell-marker immunostaining was scored on a 4-point grading system (0–3). In all cases, values represent mean ± SEM, where n = 12–13.

* P < 0.001; † P = 0.05, by unpaired t-test (hyperglycemic versus normoglycemic).
proinflammatory mediators (inducible nitric oxide synthase [iNOS]; and tumor necrosis factor α [TNF-α]) in saline-injected control rats versus rats made diabetic several days previously. The results showed that there was no discernible upregulation of FGF-2, CNTF, iNOS, or TNF-α mRNAs in the diabetic rats compared with that in the saline-injected animals (Figs. 4E–H). There was also no alteration in the expression of Hsp27 or -70 immunoreactivities in the diabetic rats (Figs. 4B, 4D) compared with that in the control animals (Figs. 4A, 4C). Hsp27 mRNA levels were also measured in the control and diabetic rats by real-time PCR. The results showed that the Hsp27 mRNA level (1.30 ± 0.31) was slightly, but not significantly (P > 0.26; n = 5), higher in the diabetic rats than in the control rats.

Brain Injury in Sham, Normoglycemic 2VO, and Hyperglycemic 2VO Rats

Two different types of brain injury can occur after 2VO: early, stroke-like events, such as sudden death and focal ischemic injury,28–30 and slow, progressive damage to gray matter and white matter tracts.14 We determined whether hyperglycemia exacerbates or ameliorates either type of brain injury.

Acute Effects. Most strikingly, the peri- and postoperative mortality rates in the hyperglycemic rats (68%) was over twice that in the normoglycemic rats (32%) (Table 5), indicating that hyperglycemia increased the likelihood of lethal brain damage. With regard to focal ischemic injury, we evaluated the total area of damage in four coronal planes of each brain, representative images of which are shown in Figure 5. The results showed that the occurrence of ischemic neuronal or axonal death was slightly greater in the hyperglycemic than in the normoglycemic rat brains (Table 5). Certainly, and in contrast to the retina, there was no evidence that hyperglycemia protected the brain from ischemic infarcts.

Chronic Effects. Astrocyte stress in the cerebral cortex was evaluated by immunostaining for Hsp27; white matter tract damage was assessed by immunostaining for APP and ED1 (markers of

Table 4. Effect of Hyperglycemia on 2VO-Induced Gene Expression Alterations in the Rat Retina

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Cell Types</th>
<th>Sham</th>
<th>NG 2VO</th>
<th>HG 2VO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin</td>
<td>Horizontal</td>
<td>1.00 (0.94–1.06)</td>
<td>0.26 (0.22–0.32)†</td>
<td>0.71 (0.68–0.74)‡</td>
</tr>
<tr>
<td></td>
<td>RGC</td>
<td>1.00 (0.95–1.05)</td>
<td>0.15 (0.13–0.16)§</td>
<td>0.50 (0.42–0.59)†‡</td>
</tr>
<tr>
<td>Nestin</td>
<td>Glial</td>
<td>1.00 (0.85–1.17)</td>
<td>4.37 (3.58–5.35)§</td>
<td>2.06 (1.50–2.82)</td>
</tr>
</tbody>
</table>

In all cases, values (represented as the mean ± SEM range) are normalized for GAPDH and expressed relative to the pooled sham group, where n = 9–10. RGCs, retinal ganglion cells; NF-L, neurofilament light; NG, normoglycemic; HG, hyperglycemic.

* P < 0.001, † P < 0.01, by Tukey’s test (versus sham).
‡ P < 0.001 by Tukey’s test (NG 2VO versus HG 2VO).

FIGURE 3. Representative images of Hsp27 expression in the retinas of sham, normoglycemic 2VO, and hyperglycemic 2VO rats. In the sham rats, Hsp27 immunoreactivity (A) was expressed at low levels by astrocytes within the nerve fiber layer. 2VO induced a strong expression of Hsp27 by cells within the GCL in the normoglycemic rats (B). The number of Hsp27-positive cells was markedly lower in the retinas of the hyperglycemic 2VO rats (C). Double-labeling immunofluorescence of β-tubulin (D, green) with Hsp27 (E, red) in the retina of a normoglycemic 2VO rat revealed a clear co-localization in some RGCs, as shown in (F), the merged image. Scale bars: (A–C) 30 μm; (D–F), 50 μm. (G) The number of Hsp27-labeled RGCs in the sham, normoglycemic 2VO, and hyperglycemic 2VO rat retinas. For each retina, the number of Hsp27 RGCs was counted to a distance of 2 mm on either side of the optic nerve head. Left: the absolute number of Hsp27-positive RGCs in each group. Right: for each group, the fraction of surviving RGCs that were Hsp27-positive, calculated by dividing the number of Hsp27 RGCs by the total number of Islet-1 RGCs. Values on the left are the mean ± SEM, where n = 12–13.
disrupted axonal flow and activated microglia, respectively) in the corpus callosum. In the sham rats, negligible expression of Hsp27 was observed (Figs. 6A, 6C, Table 5). In both the normoglycemic and hyperglycemic 2VO rats, widespread localization of Hsp27 was apparent throughout the cortical layers (Figs. 6B, 6D) and was of a similar magnitude (Table 5). Similarly, in the sham rats, no disrupted axon transport or microglial activation was noted in the corpus callosum (Figs. 6E, 6F), but in both the normoglycemic and hyperglycemic 2VO rats, infrequent APP-positive axons and ED1-positive microglia were observed (Figs. 6G, 6H, Table 5). Overall, hyperglycemia appeared to have no obviously favorable or adverse effect on chronic injury caused by hypoperfusion (Table 5).

**DISCUSSION**

It has become clinical dogma that hyperglycemia exacerbates ischemic CNS injury and is neurotoxic to the retina. In this study, hyperglycemia protected the retinal neurons against hypoperfusion injury. This result was found despite severe degeneration of the retina in normoglycemic animals; in fact, the rescue of retinal neurons by hyperglycemia in this hypoperfusion injury model is arguably one of the strongest neuroprotective effects observed in the CNS, given the severity of the insult. 2VO is a model of global cerebral and ocular ischemia that has been well described.14–17 In the brain, it induces gradual white matter degeneration,15 complicated by scattered ischemic infarcts in both gray and white matter,28–30 whereas in the retina it causes extensive neurodegeneration within a fairly short time.17 Consistent with the recent study of Yamamoto et al.,17 we noted a marked loss of RGCs and horizontal cells, a thinning of the IPL, but sparing of the outer horizontal cells, a thinning of the IPL, but sparing of the outer

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**TABLE 5.** Effect of Hyperglycemia on 2VO-Induced Mortality, Focal Ischemic Injury, and Chronic Hypoperfusion Injury in the Rat Brain

<table>
<thead>
<tr>
<th>Measure of Injury</th>
<th>Region</th>
<th>Sham Group</th>
<th>Normoglycemic</th>
<th>Hyperglycemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortal</td>
<td>—</td>
<td>0</td>
<td>32%</td>
<td>68%</td>
</tr>
<tr>
<td>Focal injury</td>
<td>—</td>
<td>0</td>
<td>68.9 ± 36.8</td>
<td>74.6 ± 33.3</td>
</tr>
<tr>
<td>Astrocyte stress</td>
<td>Cerebral cortex</td>
<td>0</td>
<td>2.0 ± 0.3</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>White matter damage</td>
<td>Corpus callosum</td>
<td>0</td>
<td>0.9 ± 0.4</td>
<td>0.8 ± 0.3</td>
</tr>
</tbody>
</table>

Regions of focal ischemic injury were measured within four coronal planes of each brain and are expressed as area in pixels (×10⁴). Astrocyte stress was evaluated by immunostaining for Hsp27; white matter damage was assessed by immunostaining for APP and ED1 (markers of disrupted axonal flow and activated microglia, respectively). Both measures of injury were scored on a 4-point grading system (0–3). In all cases, values represent mean ± SEM, where n = 11–13.
RGCs that had survived the time course of the experiment in each group were metabolically compromised. Such information is of real importance in a chronic model of injury. The results showed that approximately half of the surviving RGCs in normoglycemic rats, yet less than 15% in hyperglycemic rats, were stressed (i.e., Hsp27-positive), presumably indicating better relative RGC health in the hyperglycemic 2VO retinas. There was of course variability between the rats, which can be explained by appreciation of what the data represent. In some rats, mostly the normoglycemic ones, few RGCs survived, but those that did were compromised and expressed Hsp27; in other rats, both normoglycemic ones and hyperglycemic ones, more RGCs survived, but most of these were still unhealthy and expressed Hsp27; in a third cohort of rats, mostly hyperglycemic ones, many RGCs survived and the majority of these were healthy and did not express Hsp27. The stress threshold needed for Hsp27 expression by RGCs is unknown, and, to our knowledge, no other neuroprotective studies have assessed RGC health by this method. Thus, it is difficult to know whether similar variability would occur with other published neuroprotectants, but in the light of the current results, we advocate the use of Hsp27 in future neuroprotection studies.

Investigation of the changes in cerebral blood flow during 2VO have indicated the existence of an acute, ischemia/hypoxia-like phase lasting 2 to 3 days, followed by a chronic, oligemic phase lasting up to 3 months. Accordingly, two different types of brain injury can occur: early, strokelike events, such as sudden death, seizures and focal ischemic infarcts, and progressive damage to gray matter and white matter tracts which proceeds over many weeks. In the present study, we determined whether hyperglycemia exacerbated or ameliorated either type of brain injury. With regard to the ischemic phase of injury, the results showed that the hyperglycemic 2VO rats fared slightly less well than the normoglycemic 2VO rats. Significantly, however, mortality was far higher in the hyperglycemia group, suggesting that many of these animals suffered irreparable brain damage. The difficulty in ascertaining whether hyperglycemia worsened or ameliorated the outcome of the slow, progressive damage that

![Figure 5](https://www.iovs.org/content/51/4/2205.s4)

**Figure 5.** Representative histology and immunohistochemistry of ischemic infarcts in rat brains after 2VO. In both normoglycemic and hyperglycemic 2VO rats, heterogeneous areas of intense focal damage were visible, which were most frequently located in the gray matter (A–D), but sometimes in the white matter (E–H). Representative areas from both the gray matter (top row) and the white matter (bottom row) are shown: H&E (A, E), microtubule-associated protein 2 identifying neuronal loss (B), amyloid precursor protein revealing disrupted axonal transport (F), ED1 (C) and Iba1 (G) showing macrophage infiltration, heat shock protein 27 (D, H) showing reactive astrocytes in the penumbra. Scale bars: (A–C) 500 µm; (D–F) 60 µm.

![Figure 6](https://www.iovs.org/content/51/4/2205.s5)

**Figure 6.** Representative immunohistochemistry of chronic hypoperfusion injury in rat brains after 2VO. In the normoglycemic and hyperglycemic 2VO rats, widespread activation of astrocytes throughout the cortical layers was apparent, as illustrated by expression of Hsp27 (B, D), which was not evident in the sham rats (A, C). Limited white matter injury was observed in the corpus callosum by staining for amyloid precursor protein (F) and ED1 (H), respectively, markers of disrupted axonal flow and activated microglia. No damage was evident in the sham rats (E, G). Scale bars: (A, B) 150 µm; (C, D) 30 µm; (E–H) 20 µm.
occurs in the cerebral cortex, hippocampus, and white matter tracts is that the rats were killed at an early time point (7 days), at which time the injury is mild. This scenario was unavoidable for two reasons: First, increasing the duration of hyperglycemia introduces interpretive complications relating to disease caused by diabetes, per se; second, the magnitude of retinal degeneration at 7 days is already substantial, potentially overwhelming even the most robust neuroprotection. Nevertheless, we used two conventional methods of assessing slow, progressive injury in 2VO rats: measurements of astrocytic stress in the cerebral cortex and of axonal injury in the corpus callosum. The pathologic changes observed were similar in the hyperglycemic and normoglycemic 2VO rats, showing that hyperglycemia had no beneficial effect.

There is no doubt that hyperglycemia is eventually deleterious to the retina. In fact, retinal neuronal apoptosis has been detected as early as 2 weeks after the onset of streptozotocin-induced diabetes in rats.34 How can diabetes possibly be protective? In fact, there are several biologically plausible explanations in the current model. Quigley35 recently outlined possible reasons for diabetes to be protective against glaucoma. Although the current model is not glaucoma, it bears similarities, and three possible explanations for the protective effect observed in the current model are:

1. Glucose may have increased blood flow to the retina and/or choroid during the period of ischemia and/or reperfusion. This explanation was rejected because (1) in the previously published acute model,10 blood flow was totally disrupted during the ischemia, (2) hyperglycemia in the reperfusion period was not protective,16 and (3) the current chronic model had no reperfusion period.

2. Given that the streptozotocin was injected several days before the carotid artery ligation, it is possible that it induced a form of preconditioning. Preconditioning in the retina refers to the situation in which a brief, relatively mild insult affords neuroprotection against a subsequent robust insult. Ischemic preconditioning in the retina has been well described,96 as have other forms of conditioning-type injuries. Although the precise mechanisms remain incompletely characterized, up-regulation of trophic factors, such as FGF-2 and CNTF, inducible HSPs, principally Hsp27 and -70,43,58,59, and certain proinflammatory mediators, notably iNOS and TNF-α,10,41 have all been purported to be involved. Thus, hyperglycemia and/or streptozotocin may have caused a preconditioning-related neuroprotection of the retina, with energy metabolism playing little or no role. Ergul et al.42 in a recent review of the literature on this topic, pointed out that most studies describe a worsening of the cerebral injury in the presence of hyperglycemia. However, there are contrasting study findings that imply that hyperglycemia may protect neurons close to infarcted brain regions. Ergul et al. speculated that such contrasting data probably derive from the means of inducing neuronal damage or indeed of increasing blood glucose. They also pointed out that this necessarily emphasized the complexity of stroke pathophysiology and the need for a more systematic experimental approach to delineate the roles of hyperglycemia and diabetes. They go on to suggest that diabetes is associated with a preconditioning effect that can, under some circumstances, ameliorate stroke. The available evidence, however, argues against such a mechanism. First, in the present study we found no evidence of upregulation of Hsps, trophic factors, and proinflammatory mediators in sham diabetic rats; second, in the previously published acute model,10 the fact that neuroprotection could be replicated by directly elevating vitreal glucose via intraocular glucose injection immediately before induction of ischemia, eliminates the possibility of increased protein expression as a route for promoting neuronal survival.

3. There is evidence that lactate is actually the preferred substrate of neurons; hence, it was actually the conversion of glucose to lactate that provided the neuroprotection. This notion is rejected because in the acute model, delivery of lactate alone provided no neuroprotection.

We believe that the convergence of evidence suggests a metabolic mechanism. The most likely possibilities are a combination of the following three mechanisms: (1) that abundant glucose provides sufficient retinal ATP production for cell survival via glycolysis during an ischemic period—a manifestation of the retinal Pasteur effect; (2) that abundant glucose increases nicotinamide adenine dinucleotide phosphate (NADPH) and cellular reducing power via the pentose phosphate pathway, reducing oxidative damage and inhibiting cytochrome c-induced apoptosis33; and (3) that pyruvate dehydrogenase kinase 1 (PDK1) is a key mediator of glucose-induced neuroprotection.

This third explanation is based on the mitochondrial-associated link between energy metabolism and cell death. Pyruvate dehydrogenase (PD) is a regulatory enzyme that converts pyruvate to Acetyl CoA (a key substrate for OXPHOS via the Krebs cycle). When O2 is limited, hypoxia inducible factor (HIF)-1 is activated, upregulating glycolytic enzymes and PDK1, which inactivates PD. Hence, the formation of Acetyl CoA from pyruvate is inhibited, and substrate entry into OXPHOS is reduced. Details of mitochondrial-induced cell death remain unclear and controversial, but OXPHOS-induced reactive oxygen species (ROS) are indisputably responsible for a plethora of interrelated damaging cellular events,44 including impairing the efficiency of the electron transport chain (ETC), causing a decrease in the mitochondrial membrane potential (Ψm) and release of cytochrome c and proapoptotic factors. Hence, dichloroacetate (DCA) which inhibits PDK1, favoring Acetyl CoA production and increasing OXPHOS, increases cell death in tumors.45 The converse effect has recently been demonstrated.46–48 ROS production is paradoxically increased under hypoxic conditions47 but is substantially decreased when Acetyl CoA production is inhibited by PDK1. Hence, upregulation of PDK1 has been shown to inhibit hypoxia-induced cell death.6,46 This effect occurs even in the absence of HIF-1.46

**Conclusions**

We believe that these findings can be adapted to retinal research and we hypothesize that a combination of these metabolic factors provides a mechanistic explanation for the powerful neuroprotection we have observed. This effect requires further research and could lead to novel treatment strategies for common blinding retinal disease where energy failure is part of the pathogenesis, including ischemic retinopathies, age-related macular degeneration, and glaucoma.

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**References**


Hyperglycemia Protection of the Retina

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