Expression of Apoptosis Regulatory Genes by Retinal Pericytes after Rapid Glucose Reduction

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PURPOSE. Retinal capillary pericytes underwent apoptosis in vitro after they had been exposed long-term to high levels of glucose followed by a rapid glucose reduction. The present work was designed to study the expression of bcl-2 family members and apoptosis regulatory genes and to determine the status of oxidative stress induced by high concentrations of glucose in this in vitro apoptosis model.

METHODS. Pericytes were grown in normal or high glucose concentrations (5, 20, 30, and 40 mM) for 10 days and then exposed to a rapid reduction of glucose to 0.5 mM or 5 mM. Pericyte cell death was evaluated by determining the loss of cell viability and the fragmentation of DNA using agarose gel electrophoresis. In parallel, the quantitative reverse transcription-polymerase chain reaction technique was used to determine the expression of bcl-2, bax, p53, and glutathione peroxidase (GSH-Px) genes. The intracellular level of glutathione (GSH) and the DNA fragmentation were determined simultaneously for pericytes treated with or without exogenous GSH monoethylester. Retinal capillary endothelial cells, experiencing the same glucose variation, were studied as a comparison.

RESULTS. For pericytes, downregulation of bcl-2 was observed as early as 24 hours after rapid glucose reduction, whereas DNA fragmentation was not detectable at that time. After 72 hours, a decreased protein ratio of Bcl-2 to Bax was concomitant to evident loss of pericyte viability. During the period of high glucose and the following glucose reduction, p53 expression essentially was unchanged. Decreased levels of GSH induced by high concentrations of glucose (>30 mM) became further depleted when the glucose levels were rapidly reduced. Addition of GSH monoethylester to the medium restored the level of GSH in pericytes and prevented pericyte apoptosis induced by glucose variation. Moreover, the mRNA levels of GSH-Px were significantly elevated. By contrast, with the same glucose reduction endothelial cells did not undergo apoptosis. Their mRNA levels of bcl-2, bax, and GSH-Px essentially were unchanged.

CONCLUSIONS. High levels of glucose and rapid reduction of glucose modulate the expression of bcl-2 family genes in retinal pericytes. Upputation of GSH-Px and depletion of GSH indicate a reparative process of accelerated elimination of reactive oxygen species following rapid glucose reduction. These findings indicate that the aggravated oxidative stress and the weakened antioxidant defense induced by the combined effects of high levels of glucose and subsequent rapid glucose reduction cause pericyte apoptosis. Prevention of DNA fragmentation of pericytes by exogenous GSH further supports this notion. Because endothelial cells did not show similar pathologic changes, this proposed mechanism seems to be specific to pericytes. (Invest Ophthalmol Vis Sci. 1998;39:1535–1543)

Selective dropout of retinal capillary pericytes is one of the earliest pathologic changes of diabetic retinopathy.1 In experiments in which diabetic eyes from eye banks were used, it has been proven that the type of cell death causing pericyte dropout in vivo is apoptosis.2,3 An in vitro study has demonstrated that pericytes undergo apoptosis after an abrupt reduction of extracellular glucose levels from the hyperglycemic range.4 In this in vitro model, de novo protein synthesis is necessary for pericyte apoptosis because cycloheximide, a protein synthesis inhibitor, protects pericytes against cell death.4 Newly synthesized proteins, specific products of apoptosis regulatory genes, actually exist in all cells in healthy animals; the question is, Why do some cells die, and others survive? The answer appears to be that a balance between cell-death inducer or inhibitor genes may shift with the influence of environmental factors. The best characterized group of apoptosis regulatory genes in mammals is the proto-oncogene bcl-2 family.5 Therefore, in the present study, the expression of bcl-2 genes was determined in stressed pericytes that were exposed to high levels of glucose followed by glucose reduction. Because the glucose variation may result in a change of cellular redox state,6,7 the oxidative stress and the

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Supported by grants from the National Eye Institute, Bethesda, Maryland, EY06563; the American Diabetes Association, Alexandria, Virginia; the Frank E. Snider Trust Fund, Phoenix, Arizona; the Alcon Laboratory, Fort Worth, Texas; the Fuller Memorial Diabetes Fund, Philadelphia, Pennsylvania; and the Chinese National Natural Science Foundation, Beijing, China (WI).

Submitted for publication November 18, 1997; revised April 21, 1998; accepted May 4, 1998.

Proprietary interest category: N.

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function of antioxidant defense of pericytes under these conditions also were evaluated. To illustrate that the proposed mechanism is specific for pericytes, retinal capillary endothelial cells (RCEC) were studied as a comparison.

**MATERIALS AND METHODS**

**Cell Culture under High-Glucose and Glucose-Reduction Conditions**

Bovine retinal capillary pericytes and endothelial cells (RCEC) were cultured as previously described. To ensure the purity of these two types of cells, morphologic and biochemical markers (including Factor VIII-related antigen, intermediate density lipoprotein incorporation, angiotensin-converting enzyme, and the reaction of angiotensin monocomonoclonal antibody 3G5, for pericytes) were examined. Only the batches that met the standard criteria for purity were used. In the present study, both types of cells at the second passage were routinely used.

Because the present study focused on the effect of glucose variation on cell death in culture, other factors such as concentrations of fetal calf serum (FCS) and Dulbecco modified Eagle’s medium (DMEM)-based growth medium have been reduced to minimal levels that can sustain cells. The regular medium initially for pericytes was DMEM and 2% FCS; for RCEC, it was a mixture of 50% serum-free endothelial medium (GIBCO, Gaithersburg, MD) and 50% DMEM supplemented with 2% FCS. RCEC growth required a fibronectin-coated surface (1 μg/cm²). In the study of glucose reduction, the regular media of pericytes and RCEC (10⁵ cells/cm² for pericytes and 1.5 × 10⁵ cells/cm² for RCEC at early confluence) were replaced by a modified medium supplemented with different concentrations of glucose (5, 20, 30, or 40 mM). The modified medium is a Locke’s solution—based medium that has been used in the study of glucose withdrawal in neuron cultures. Locke’s solution consisted of 154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM HEPES, pH 7.4. For pericytes, the modified medium was comprised of glucose-free DMEM (10%), Locke’s solution (90 vol/vol), and FCS (2%), referred to as the “experimental medium for pericytes. RCEC were maintained in a serum-free endothelial medium (10%), Locke’s solution (90 vol/vol), and 2% FCS, referred to as “the experimental medium for RCEC.” The media were changed every other day. Both experimental media were able to sustain cells for 3 to 4 weeks.

The equal osmolarity of all cultures was maintained by adding mannitol (5-35 mM). Extracellular glucose concentrations were monitored by the method previously reported. Glucose-reduction experiments were performed with pericytes for 10 days or with RCEC for 6 days in preexisting high concentrations of glucose.

Abrupt glucose reduction was achieved by removing the culture media and washing cell layers three times with glucose-free Locke’s solution. Afterward, Locke’s solution supplemented with 2% FCS and different concentrations of glucose (5, 20, 30, or 40 mM) was added to pericyte or RCEC dishes, and they were left for the required period (0-3 days).

**Determination of DNA Fragmentation**

Genomic DNA of cells was isolated as previously described. DNA was precipitated, collected, and redissolved in Tris/EDTA buffer. The concentration of DNA from each sample was determined against a DNA standard curve to control the quantity of loading. To detect DNA fragments, isolated DNA was separated on 1.5% agarose gels. Gels were stained with ethidium bromide (10 μg/100 ml), and the molecular weight of each DNA band was compared with a DNA marker.

**Viability Assay**

Pericytes treated by the same procedure as the experimental and control groups were seeded in 96-well microplates. At different time intervals, the cells were labeled with the lysosomally accumulated vital dye, neutral red, for 2 hours at 37°C. The cells were washed with 0.1 M phosphate-buffered saline, pH 7.4, and were lysed with 100 μl per well of 50% ethanol with 1% glacial acetic acid. Viability was calculated as the optical density at 540 nm, with 650 nm as a reference wavelength, using a microtiterplate reader (Bio-Tex Instrument, Winooski, VT).

**Cellular RNA Preparation and cDNA Synthesis**

Retinal capillary pericytes or RCEC (10⁷ cells/sample) were harvested for total RNA isolation at day 1 or day 3 after glucose reduction. Total RNA was isolated using a single-step RNA isolation kit (RNAzol; Tel-Test, Friendswood, TX), originally described by Chromczynski and Sacchi. The resultant RNA sample was resuspended in 75% ethanol, sedimented, dried, and then dissolved in 50 μl RNase-free water. At this point the RNA concentration was determined spectrophotometrically at 260 nm and 280 nm; 16 μg to 20 μg total RNA was recovered from 10⁷ cells. The integrity of the isolated RNA was assessed by agarose gel electrophoresis and by staining with ethidium bromide. Only samples with intact ribosomal RNA bands were used for further study.

cDNA synthesis was performed in a 30-μl reaction solution containing 1 μg denatured RNA (heating to 70°C for 5 minutes followed by fast cooling to 4°C), 1X first strand buffer (GIBCO Laboratory, Grand Island, NY), 0.5 mM dNTP (dATP, dCTP, dGTP, and dTTP), 10 mM dithiothreitol, 1 μg oligo(dT), 3 μg bovine serum albumin, 2 U/μl human placenta RNAse inhibitor (RNasin; Promega, Madison, WI), and 10 U/μl M-MLV reverse transcriptase (GIBCO Laboratory). The reaction was carried out at 42°C for 60 minutes and stopped by incubation at 95°C for 10 minutes. Negative controls of cDNA synthesis were carried out under the same experimental condition in the absence of reverse transcriptase.

**Semiquantitative Polymerase Chain Reaction**

The primer pairs for p53 and glutathione peroxidase (GSH-Px) were synthesized based on bovine p53 and GSH-Px cDNA sequences obtained from GenBank, respectively. The resultant polymerase chain reaction (PCR) products were cloned with the TA cloning kit (Invitrogen, San Diego, CA) and sequenced by the dideoxy chain termination method, using a DNA sequencing kit (Sequenase version 2.0; United States Biochem, Cleveland, OH) to confirm the detection (Table 1). Because the complete cDNA sequence of bcl-2 or bax was not available for bovine species when we conducted
TABLE 1. Oligonucleotides as Primer Pairs for Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Size of PCR Products (bp)</th>
<th>Annealing Temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bax</td>
<td>Forward 5'-GGA ATT CGC GGT GAT GGA CGG GTC CGG-3'</td>
<td>579</td>
<td>65</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GGA ATT CTC AGC CCA TCT TCT TCC AGA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td>Forward 5'-GTG GAG AGC GTC AAC CGG-3'</td>
<td>229</td>
<td>65</td>
<td>15, 16</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGG CAC CCA GGG TGA TGC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Forward 5'-GGA GCA CTA AGC GAG CAC-3'</td>
<td>275</td>
<td>65</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCA GTC TGA GTC AGG CCC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH-Px</td>
<td>Forward 5'-TTG GAA AAC AGG AAC CAG GA-3'</td>
<td>257</td>
<td>65</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CCC CAC CAG GAA CTT CTC A-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-AGA AGG TGG TGA AGC AGG CGT CG-3'</td>
<td>228</td>
<td>65</td>
<td>OPERON (1995)</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CCT TGG AGG CCA TGT GGG CC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction.

Primer pairs for detecting bcl-2, bax, p53, GSH-Px, and GAPDH were designed as described in the Materials and Methods section. The base pairs of individual PCR products were confirmed by sequence analysis as described in the Materials and Methods section.

In this experiment, multiple pairs of oligonucleotide primers were designed, initially based on the homology of rodent and human cDNA sequence of individual genes obtained from GenBank. The initial PCR products from amplification of bovine total cDNA were at the range of 550 bp to 650 bp. These PCR products were cloned and sequenced as described above. Internal primer pairs were then designed from the obtained sequences (Table 1). The internal primers were used for semiquantitative PCR. The final PCR products were at the range of 220 bp to 600 bp (Table 1). The resultant bovine PCR products had 94.8% sequence homology compared with human bcl-2 cDNA from 506 bp to 734 bp and 94.6% sequence homology to human bax cDNA, respectively. During the revision period the bovine sequences of bcl-2 (partial) and bax cDNA were released from GenBank (U92434, U92569). Our PCR products of bcl-2 and bax had 100% sequence homology with these released sequences.

To quantify the expression of specific genes, internal standards called PCR MIMIC were prepared using the Clontech PCR MIMIC construction kit according to the manufacturer’s instructions. To construct a PCR MIMIC, two rounds of PCR amplification were performed. In the first reaction, two composite primers were used, each with the target gene primer sequence attached to a short 20-nucleotide stretch designed to hybridize to opposite strands of the heterologous DNA fragment. A dilution of the first PCR then was amplified using only the gene-specific primers. The resultant PCR MIMIC was purified and diluted to 100 attomole/μl. bcl-2, bax, p53, GSH-Px, or GAPDH primers were spiked with experimental cDNA samples and twofold dilution series of PCR MIMIC. PCR MIMIC competes with sample cDNA for the same target gene primers. Because the amount of PCR MIMIC added is known, when the product of the PCR MIMIC is equal to the product of the sample cDNA, as determined by the Bio-Rad Image Analysis System (Bio-Rad), the cDNA of a specific gene can be calculated.

PCR amplification of cDNA was performed in a DNA thermal cycler (Perkin Elmer Cetus, Branchburg, NJ). The reaction solution contained 1X PCR buffer, 2.5 mM MgCl₂, 0.25 mM dNTPs, 1.5 μM of each primer, 1 U Taq polymerase (Perkin Elmer), and 2.5 μl cDNA in a final volume of 25 μl. The amplification protocol was as follows: 20 to 30 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. Negative controls (see above) were introduced in each experiment to ensure that only specific cDNA was amplified.

Bcl-2/Bax Protein Ratio Determined by Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays were performed as previously reported with several modifications. Pericytes were lysed. The lysate (from 10⁶ cells) was pipetted into each well of microplates precoated with rabbit polyclonal antibodies against Bcl-2 or Bax proteins (Calbiochem, La Jolla, CA). Polyclonal antibodies against Bcl-2 or Bax conjugated with fluorescein isothiocyanate (FITC) then were added to each well. After a 2-hour incubation at room temperature, the microplates were washed three times to
remove unbounded proteins. Bound Bcl-2 and Bax proteins were detected by a horseradish peroxidase-labeled sheep anti-FITC IgG conjugate and the substrate. Based on the "standard curves" constructed by commercial Bcl-2 and Bax peptides (Amersham Life Science, Arlington Heights, IL), the protein ratios of Bcl-2/Bax were calculated.

**Determination of Glutathione Content**

Glutathione (GSH) content was determined by a method previously reported.\(^ {19}\) Trypsinized pericytes were washed and resuspended in phosphate-buffered saline. An aliquot was removed for the determination of cell number with a Coulter counter (Coulter Electronics, Luton Beds, England); the remainder was pelleted and cells were homogenized in 1 M formic acid. Aliquots of the extracts recovered by centrifugation were reacted with HCHO to eliminate interference from histidyl compounds and treated with o-phenalddehyde. Fluorescence was read at 345 nm excitation and 425 nm emission, and GSH levels were calibrated against a standard curve and expressed as nanomoles per milligram of protein.

In the experiment of glucose variation from 40 mM to 5 mM, pericytes were treated with or without 0.5 mM GSH monoethylester (Sigma Chemical, St Louis, MO) in the experimental medium. After 3 days, a fraction of cells was collected to determine intracellular GSH, and another fraction of cells was used to detect DNA "laddering" on agarose gels.

**Statistical Analysis**

For quantitative studies, the obtained data were expressed as mean ± SD of four to five separate cultures each performed in duplicate. The mean values were analyzed by the Student's t-test to examine the statistical significance at \( P < 0.05 \).

**RESULTS**

**Effect of Preexisting High Levels of Glucose and Rapid Glucose Reduction on Pericyte DNA Fragmentation**

Figure 1 is a representative agarose gel electrophoresis that shows internucleosomal DNA cleavage of pericytes after abrupt glucose reduction for 72 hours. For pericytes (lane 2, glucose reduction from 40 mM to 5 mM), 9 distinct bands of this lane, corresponding to the molecular weight standards, were multiples of approximately 180 bp. In lane 3, DNA-extracted from pericytes experiencing glucose-reduction from 30 mM to 5 mM also demonstrated a "ladder" pattern of DNA fragmentation but with relatively weak signals in comparison with lane 2. Lanes 4 and 5 were samples of pericytes that experienced 20 mM to 5 mM and 5 mM to 0.5 mM glucose reductions. In these latter two lanes, characteristic DNA fragmentation did not occur. Lanes 6 and 7 show the samples extracted from pericytes kept at constant levels of glucose (5 mM and 40 mM, respectively). No internucleosomal DNA fragmentation was observed under these conditions.

In a separate experiment, DNA laddering was observed in pericytes experiencing glucose variation from 40 mM to 5 mM without exogenous GSH (lane 8 equivalent to lane 2). In contrast, the addition of 0.5 mM GSH monoethylester to the medium during glucose reduction completely prevented DNA fragmentation of pericytes (lane 9).

**Altered Expression of mRNA Levels in Apoptotic Pericytes But Not in RCEC**

Pericytes expressed constitutive bcl-2, bax, p53, and GSH-Px genes. The impact of high levels of glucose followed by a rapid glucose reduction (from 40 mM to 5 mM) on the expression of bcl-2, bax, p53, and GSH-Px genes by pericytes is shown in Figures 2 and 3. At day 1 after the glucose reduction, only the expression of bcl-2 was slightly decreased (2 ± 0.5-fold, \( P < 0.05, n = 6 \)) (Fig. 3A), whereas neither DNA fragmentation (data not shown) nor loss of cell viability was found at this point (Fig. 4). After 3 days, pericytes kept constant at high levels of glucose (40 mM) slightly downregulated bcl-2 (<2-fold decrease, \( P < 0.05, n = 6 \)) (Fig. 3A) and slightly upregulated bax (<2-fold increase, \( P < 0.05, n = 6 \)) (Fig. 3B) in comparison with pericytes incubated with a normal level of glucose (5 mM, control in Figs. 3A, 3B). Pericytes experiencing a rapid glucose reduction demonstrated a significant decrease in...
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**Figure 2.** Representative electrophoresis of altered mRNA expression by pericytes after glucose reduction. Pericytes were exposed to a constant glucose level of 40 mM (designated lane 1), to 40 mM glucose for 10 days and then glucose reduction to 5 mM for 3 days (designated lane 2), and to glucose reduction to a constant level of 5 mM (designated lane 3, as control). Total RNA was isolated as described in the Materials and Methods section. Expression of bcl-2, bax, p53, Gp (glutathione peroxidase), and Ga (GAPDH) was determined by semiquantitative reverse transcription-polymerase chain reaction technique as described in the Materials and Methods section. The cDNA quantity was expressed as relative area in Figure 3.

*bcl-2 (4 ± 0.6-fold decrease, \( P < 0.01, n = 6 \)) (Figs. 2, 3A) and a dramatic increase in bax (8 ± 1.1-fold increase, \( P < 0.01, n = 6 \)) (Figs. 2, 3B) as compared with the controls. No evident change in the mRNA levels of p53 was observed within 3 days in any experimental condition (Fig. 2). The similar expression pattern of GAPDH, a housekeeping gene, in different samples (Fig. 2) ensured equal loading. There was no significant change of bcl-2, bax, or p53 expression in any RCEC groups after glucose variation (data not shown).

**Correlation of Bcl-2/Bax Ratio with Pericyte Viability**

Because nonspecific binding may arise from the reaction of polyclonal antibodies against purified Bcl-2 or Bax peptides, the exact quantity of Bcl-2 or Bax protein had not been derived from the "standard curves" constructed by using peptides, although the color reaction was proportional to the amount of bound Bcl-2 or Bax peptide, ranging from 8 ng to 40 ng. When \( 10^6 \) pericytes growing in constant normal

**Figure 3.** Altered expression of *bcl-2*, *bax*, and *glutathione peroxidase* (GSH-Px) genes after the rapid glucose reduction. Pericytes were exposed to a constant glucose level of 40 mM for 13 days (group 1), to 40 mM glucose for 10 days and then experienced glucose reduction to 5 mM for 3 days (group 2), and to a constant glucose level of 5 mM for 13 days (group 3, as control). After 10 days at constant glucose levels (day 0), the glucose levels were either kept unchanged in groups 1 and 3 or reduced in group 2 for 1 day (day 1) and 3 days (day 3), respectively. Each gene expression was determined by quantitative reverse transcription-polymerase chain reaction (PCR) with PCR MIMIC technique as described in the Materials and Methods section. The cDNA quantity was expressed as relative area compared with the data of group 3 (pericytes kept at normal glucose level, 5 mM) by the Bio-Rad Image Analysis System. Each value is the mean ± SD of four to five separate experiments (each performed in duplicate).
Glutathione Content of Pericytes That Experienced High Levels of Glucose and Glucose Reduction

Glutathione levels were essentially unchanged when pericytes were growing at constant glucose levels (5 mM or 20 mM) (Fig. 5). Glutathione levels of pericytes that had been kept in high (30 mM or 40 mM) glucose for 10 days were moderately lower than the control tissues (at 5 mM glucose). In the high-glucose group (40 mM), 3 days after glucose reduction, GSH became essentially depleted (Fig. 5). However, the addition of 0.5 mM GSH monoethylester in the culture medium completely restored the intracellular GSH level (Fig. 5).

Upregulation of GSH-Px mRNA in Pericytes under Conditions of High Levels of Glucose and Rapid Glucose Reduction

As shown in Figures 2 and 3C, the high glucose level (40 mM) induced a twofold increase in GSH-Px mRNA expression by pericytes (2 ± 0.6-fold, n = 6, P < 0.05), and rapid glucose reduction (from 40 mM to 5 mM) upregulated GSH-Px mRNA to a much higher extent (8 ± 1.2-fold, n = 4, P < 0.01). After glucose reduction (from 40 mM to 5 mM), GSH-Px expression was essentially unchanged in RCEC (data not shown).

DISCUSSION

A slight downregulation of bcl-2 and a small elevation of bax expression could be detected after long-term high-glucose incubation, whereas an evident decrease in bcl-2 and a dramatic increase in bax expression occurred only when the high glucose levels were rapidly dropped. These findings suggest that the combined stresses of preexisting high glucose level and the subsequent abrupt glucose reduction induce an altered expression of the bcl-2 gene family. Most members of the bcl-2 gene family are capable of blocking apoptosis, whereas some members can promote cell death. For instance, bcl-2 inhibits cell death and bax promotes apoptosis. The bcl-2 gene product, Bcl-2, dimerizes with Bax. In some respects, cells with a deficiency of Bcl-2 represent a rheostat reset to excess of Bax homodimers Bax/Bax. A stoichiometric competition between Bcl-2 and Bax determines whether or not a cell enters apoptosis or remains viable. The present data revealed an apparent correlation between a decreased protein ratio of Bcl-2 to Bax and a loss of pericyte viability after rapid glucose reduction. This finding also agrees with the pattern of mRNA expression of bcl-2 family genes (i.e., downregulation of bcl-2 and upregulation of bax). The mechanism by which Bcl-2 regulates apoptosis is not completely understood. A proposed mechanism is that decreased levels of Bcl-2 may disturb the function for scavenging oxygen free radicals. There is accumulating evidence that the Bcl-2 family also may act upstream of the cell death proteinase cascade such as interleukin-1β-converting enzyme (ICE) family (e.g., CPP32). The molecular identity of other factors that work together with Bcl-2 family members to protect cells against oxidative stress remains to be determined.

Correlatively, significant pericyte DNA fragmentation and loss of cell viability occurred in the same temporal sequence of glucose reduction, indicating that the rapid glucose shift aggravated the preexisting high-glucose toxicity. Regarding high-glucose stress, previous experiments demonstrated that peri-
Pericyte proliferation was inhibited by high levels of glucose in the culture medium. A variety of adverse effects of high glucose also have been observed in other types of cells. The present data emphasize how oxidative stress induced by high levels of glucose is aggravated during the transition stage from the preexisting high glucose level to the subsequent reduced glucose level. There is evidence that the further breakdown of antioxidant defense brought about during the rapid shift of glucose concentrations may be one such adverse effect. Under physiological conditions glucose auto-oxidizes. The term auto-oxidation describes the capacity of glucose to enolize, thereby reducing molecular oxygen and producing free radicals. Moreover, glucose auto-oxidation is specifically accelerated under hyperglycemic conditions. The resultant free radicals in turn inhibit pericyte proliferation or alter cellular functions in vitro. High levels of glucose also increase oxidative stress by raising the NADH-to-NAD⁺ ratio, an imbalance of glucose-induced redox, which is referred to as hyperglycemic pseudohypoxia. Increased NADH/NAD⁺ stimulates the production of reactive oxygen species. After pseudohypoxia, if glucose levels abruptly decrease, the unavailability of glucose to pericytes may occur, producing a condition called "experimental ischemia." As a matter of fact, high-glucose concentrations have been shown to selectively downregulate glucose transport activity and GLUT1 content in retinal capillary pericytes, so that intracellular glucose concentrations of pericytes were depleted after rapid reduction of extracellular glucose from preexisting high glucose levels. It is possible that when superimposed on preexisting pseudohypoxia induced by high glucose, relatively mild ischemia transforms into a more serious imbalance of redox that is enough to cause tissue dysfunction. A period of ischemia itself without reperfusion also can produce a significant amount of reactive oxygen species.

The present study did not determine individual species of such free radicals generated under the conditions of either pseudohypoxia or experimental ischemia. However, the basic antioxidant defense system has been studied here under both conditions, because presumably the generation of free radicals may not necessarily cause cell damage unless the antioxidant defense is broken down. For instance, it has been shown that in normal rat thyroid follicular cells, glucose-dependent cell damage is related to GSH production, a basic element of antioxidant defense. The key issue is at which stage (the high-glucose or the subsequent glucose-reduction stage), the capacity of scavenging oxygen free radicals is more seriously diminished. In the present study, when constant glucose concentrations were below 20 mM, cellular GSH levels remained unchanged. Constant high levels of glucose (30 or 40 mM) caused moderately decreased GSH levels. Only rapid glucose reduction following the long-existing high levels of glucose (30 mM or 40 mM) led to GSH depletion. These data indicate that the increased susceptibility of pericytes to oxidative stress was determined by the exacerbated imbalance between pro-oxi-
Ulcerated factors and those factors that scavenge them. When nat-
ural antioxidant defense was further depressed, pericyte death
ensued. Conversely, when the decreased intracellular GSH was
restored by the addition of exogenous GSH in culture medium,
pericyte cell death was prevented.

Upregulation of \( \text{GSH-Px} \) mRNA during the glucose-reduc-
tion period also is an indicator of a disturbance of antioxidant
defenses. Glutathione peroxidase requires more reduced-GSH
to perform its function to convert any peroxide and to inacti-
vate hydroxyl radicals.\(^{36}\) The overexpression of \( \text{GSH-Px} \) may
lead to an exhaustion of the GSH supply. However, the status
of GSH synthesis and regeneration power under the stress of
high levels of glucose or glucose-reduction has not been deter-
mined in the present study. Nevertheless, the simultaneous
overexpression of \( \text{GSH-Px} \) and the depletion of GSH indicated
a compensatory process of accelerated elimination of reactive
oxygen species.

The present data reveal that the quantity of preexisting
glucose (which determines the magnitude of glucose reduc-
tion) determines the extent of final DNA fragmentation of
pericyte chromatin. This study also demonstrates that rapid
glucose reduction is the direct trigger of pericyte apoptosis,
because the abrupt glucose reduction leads to a significant
profile change in the expression of the \( bcl-2 \) gene family as
well as the breakdown of antioxidant defenses. The changed
Bcl-2/Bax ratio favors apoptosis.\(^{41}\) Moreover, the depletion of
GSH levels and the decompensation of its regeneration
mechanism combine to further precipitate pericyte cell
death.

In the comparative study, under the same experimental
conditions RCEC did not undergo apoptosis and did not show
similar changes in \( bcl-2 \) and \( \text{GSH-Px} \) gene expression, indicat-
ing that the mechanism by which pericytes undergo apoptosis
after glucose variation is cell specific. Therefore, we will pur-
sue further comparative study of the underlying mechanisms
that regulate apoptosis when pericytes and RCEC are exposed
to oxidative stress induced by high glucose.

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ANNOUNCEMENTS

Association for Ocular Pharmacology and Therapeutics

The 4th Annual AOPT Meeting will be held January 28 to 31, 1999, at The Marriott, Irvine, CA. Emphasis of the meeting is current advances in pharmacology as they relate to the eye and vision. For further information contact: Achim Krauss, Allergan, Inc., 2525 Dupont Dr., Irvine, CA 92713 (tel: 714-246-4842; fax: 714-246-5578; e-mail: krauss_achim@allergan.com).