Glyoxalase I Is Critical for Human Retinal Capillary Pericyte Survival under Hyperglycemic Conditions*

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Retinal capillary pericytes undergo premature death, possibly by apoptosis, during the early stages of diabetic retinopathy. The α-oxoaldehyde, methylglyoxal (MGO), has been implicated as a cause of cell damage in diabetes. We have investigated the role of MGO and its metabolizing enzyme, glyoxalase I, in high glucose-induced apoptosis (annexin V binding) of human retinal pericyte (HRP). HRP incubated with high glucose (30 mM d-glucose) for 7 days did not undergo apoptosis despite accumulation of MGO. However, treatment with a combination of high glucose and S-p-bromobenzylglutathione cyclopentyl diester, a competitive inhibitor of glyoxalase I, resulted in apoptosis along with a dramatic increase in MGO. Overexpression of glyoxalase I in HRP protected against S-p-bromobenzylglutathione cyclopentyl diester-induced apoptosis under high glucose conditions. Incubation of HRP with high concentrations of MGO resulted in an increase of apoptosis relative to untreated controls. We found an elevation of nitric oxide (NO) in HRP that was incubated with high glucose when compared with those incubated with either the l-glucose or untreated controls. When HRP were incubated with an NO donor, DETANONOATE ([(Z)-1-[(2-(2-aminoethyl)-N-(2-ammonioethyl)amino)]diazene-1-ium-1,2-diolate), we observed both decreased glyoxalase I expression and activity relative to untreated control cells. Further studies showed that HRP underwent apoptosis when incubated with DETANONOATE and that apoptosis increased further on co-incubation with high glucose. Our findings indicate that glyoxalase I is critical for pericyte survival under hyperglycemic conditions, and its inactivation and/or down-regulation by NO may contribute to pericyte death by apoptosis during the early stages of diabetic retinopathy.

Diabetic retinopathy is one of the leading causes of blindness in the working age population (1). Loss of pericytes from the retinal microvasculature is considered one of the earliest hallmarks of diabetic retinopathy (2, 3), and it is thought to occur via an apoptotic pathway (4, 5). Once pericytes are eliminated from retinal capillaries, endothelial cell death follows with the subsequent formation of acellular capillaries (6). In the proliferative form of the disease, areas containing acellular capillaries become ischemic and subsequently non-perfused. Non-perfused regions of the retina suffer hypoxia, which causes the expression of a number of angiogenic factors including vascular endothelial growth factor (7, 8). New blood vessels that grow toward the vitreous are fragile and prone to rupture, resulting in retinal detachment.

In both diabetic humans and animal models of diabetes, retinal capillary pericytes and endothelial cells die, possibly by apoptosis, as indicated by observations of terminal dUTP nick-end labeling-positive cells in capillaries (9) and activation of key apoptotic proteins and caspases in the retina (10). One recent study suggested that mitochondrial dysfunction causes apoptosis of both endothelial cells and pericytes in retinas of diabetics (11). Other studies documented activation of pro-apoptotic caspases and other apoptotic pathway proteins in the diabetic retina (12, 13). Some investigators proposed that the frequency of early apoptosis in retinal pericytes was the major determinant for development of diabetic retinopathy (14). Exactly how pericytes undergo apoptosis is still uncertain, although evidence suggests various biochemical mechanisms (5, 13, 15, 16), including triggering of a pro-apoptotic program by activation of NF-κB in response to hyperglycemia (16).

Cultured pericytes undergo apoptosis in the presence of high concentrations of glucose (11), implying that glucose-driven processes cause or enhance their death. MGO is an α-oxoaldehyde that is highly reactive with lysine or arginine residues in proteins, modifying them at their amine moieties to form AGEs (17, 18). Elevated levels of both MGO and AGEs have been observed in serum and various tissues of diabetic patients, respectively (19, 20), and both AGEs and MGO cause pericyte apoptosis (21, 22). Apoptosis prompted by this mechanism is probably due to enhanced oxidative stress within the cells. In fact, antioxidants inhibit apoptosis caused by MGO and AGEs (22, 23). We showed that extracellular matrix proteins modified by dicarboxyls induce pericytes apoptosis as well (24). In addition, activation of the polyol pathway is implicated in pericyte apoptosis (25, 26). Retinal capillary cells produce insulin-like growth factor-I, and a recent study related loss of retinal pericytes to excessive levels of insulin-like growth factor-I (27).

Until now, studies were concerned with damage of pericytes by high glucose, but there was little emphasis on how high glucose stress influences enzymes within these cells. Glyoxalase I is an integral part of the cellular machinery for removal of MGO. The glyoxalase system is composed of two enzymes: glyoxalase I, which metabolizes MGO to S-d-lactoylglutathione, and glyoxalase II, which converts S-d-lactoylglutathione to d-lactate. Work by Shinohara et al. (28) demonstrated that overexpression of glyoxalase I in bovine endothelial cells reduced intracellular AGEs when the cells were cultured in the presence of high glucose. In addition, a series of studies indicated that certain tumor primary cultures and cell lines overexpress glyoxalase I (29, 30), suggesting that increased amounts of this enzyme prevent tumor cell apoptosis (31), possibly by limiting MGO production. These findings spurred us

* This work was supported by National Institutes of Health Grants R01EY-09912, R01EY-016219, and R21DK-068045 (to R. H. N.) and P30EY-11373 (to the Visual Sciences Research Center, Case Western Reserve University) and grants from Research to Prevent Blindness and the Ohio Lions Eye Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: MGO, methylglyoxal; glu, glucose; AGE, advanced glycation end product; BBGC, bromobenzylglutathione cyclopentyl diester; DAPI, 4,6-diamidino-2-phenylindole; DETANONOATE, [(Z)-1-[(2-(2-aminoethyl)-N-(2-ammonioethyl)amino)]diazene-1-ium-1,2-diolate]; HRP, human retinal pericytes; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
to determine whether impaired function of glyoxalase I could result in HRP apoptosis in diabetes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modification of Eagle’s medium (with 5 mmol/liter D-glucose), 0.25% trypsin, 0.1% EDTA, and Hank’s balanced salt solution were purchased from Mediatech (Herndon, VA). Fetal bovine serum and antibiotic/antimycotic solution were purchased from Invitrogen. Endothelial growth supplement, insulin-transferrin-sodium selenite (ITS), porcine esterase, nitrate reductase, and MGO (40% solution) were purchased from Sigma. MGO was purified by distilling twice under low pressure and temperature. The TACS-annexin apoptosis detection kit was purchased from R&D Systems Inc. (Minneapolis, MN). Annexin-V-647 was obtained from Invitrogen. Bromobenzylglutathione cyclopentyl diester (BBGC) was synthesized as previously described (32). The NO· donor DETANONOATE was purchased from Cayman Chemical Co. (Ann Arbor, MI).

**Isolation, Culture, and Characterization of HRP**—HRPs were isolated by the method of Grant and Guay (33), with minor modifications. Two sets of eyes from two non-diabetic donors (aged 41 and 72) were received 23 h after death from the Cleveland Eye Bank. Retina were detached from the pigmented layer, placed on 55-μm nylon mesh, and macerated while flooding with 2% bovine serum albumin in Hank’s balanced salt solution. The vasculature was transferred to a flask containing 5 mg/ml collagenase (Type I, Worthington Biochemical, Lakewood, NJ) and stirred for 30–45 min at 37 °C. Collagenase was inactivated by the addition of growth media (1:1 Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 10% fetal bovine serum, 15 μg/ml endothelial cell growth supplement, 1× ITS, antibiotic/antimycotic (10 units of penicillin, 10 μg of streptomycin, 25 μg of amphotericin)), and the homogenate was centrifuged at 1300 rpm for 5 min. The pellet was washed once with 5 ml of growth medium and centrifuged again. The final pellet was resuspended in 10 ml of growth medium and seeded onto 2×25 cm² flasks pre-coated with gelatin. The cultures were maintained at 37 °C in 5% CO₂ with a change of medium every 2–4 days until the cells attained near confluence. Cells were detached by washing with PBS then covered with 0.25% trypsin, 0.1% EDTA for 60–90 s. Fresh medium was added, and the cells were centrifuged at 1300 rpm for 5 min. The pellet was resuspended in growth media, and cells were plated in 10-cm² dishes. Positive staining for both smooth muscle actin a submin. The supernatant was then added to 80% confluent cultures. After 24 h the cells were supplied with fresh media containing either MGO or glyoxal and 35 μM BBGC in Me₂SO or Me₂SO alone. The cells were then incubated for 24 h longer.

**Measurement of Glyoxalase I Activity**—Cells were detached with trypsin-EDTA for 5 min, washed with PBS, and harvested by centrifugation at 13,000 rpm for 5 min at 4 °C. The pellet was suspended in 10 mM Tris-HCl, pH 7.4, containing 1:100 diluted protease inhibitor mixture (Sigma), subjected to 3 freeze/thaw cycles in liquid nitrogen, and sonicated for 20 s on ice. The lysate was centrifuged at 20,000 × g for 20 min at 4 °C and extensively dialyzed against PBS overnight at 4 °C, and the supernantant was tested for glyoxalase I activity as previously described (28). One unit is defined as the amount of enzyme required to produce 1 mmol of S-lactoylglutathione/min/mg of protein. Protein concentration of lysates was measured using the Bio-Rad protein assay solution (Bio-Rad). For BBGC de-esterification, BBGC dissolved in 10% Me₂SO was added to 0.5 units of esterase in 50 mM Tris-HCl, pH 8.0, and incubated for 2 h at room temperature. Controls contained esterase or BBGC alone in either 10% Me₂SO or 50 mM Tris-HCl. After incubation for 2 h at room temperature, 50 μg of lysate was added, and the mixtures were incubated 1 h longer. Glyoxalase I activity was measured as described above.

**Flow Cytometry**—After exposure of cells to BBGC and DETANONOATE, the incubation media along with any detached HRP was removed and retained. The adherent cells were detached with trypsin, mixed with the retained media, and centrifuged at 1000 rpm for 5 min. The cell pellet was washed with Hank’s balanced salt solution and then incubated with fluorescein isothiocyanate-conjugated annexin V and propidium iodide according to the manufacturer’s instructions. Apoptosis was quantified using a Beckman Coulter XL flow cytometer (Fullerton, CA) equipped with an argon-ion laser for excitation at 488 nm. Fifteen thousand cells were analyzed per sample. HRP treated with 1 μM staurosporine (Kamiya Biomedical Co., Seattle, WA) for 1.5 h were positive controls for apoptosis. We used the same procedure for transfected cells (see below), except annexin V-647 was substituted for annexin V-fluosothiocyanate. The samples were analyzed with a BD Biosciences LSR I flow cytometer (San Jose, CA) using 2 laser excitations; a 488-nm argon laser for propidium iodide and a 633 nm helium/argon laser for annexin V-647 fluorescence. At least 20,000 events were collected for transfection experiments.

**Quantification of MGO in HRP**—HRP cultures were washed with PBS, the cells were detached with trypsin, pooled with the media in which they had been incubated, and collected by centrifugation at 13,000 rpm for 5 min. The cell pellets were resuspended in 10% trichloroacetic acid and centrifuged at 13,000 rpm for 5 min at 4 °C. The resulting supernatant was derivatized with 7 mM 6-hydroxy-2,4,5-triaminoypyrimidine at 60 °C for 45 min. The subsequent pterin adduct (6-methylpterin) was measured by high pressure liquid chromatography as previously described (34).

**Glyoxalase I Expression after Treatment with DETANONOATE**—Cells were treated with various concentrations of DETANONOATE as described for the apoptosis experiments. Total RNA was extracted using Trizol reagent (Invitrogen), and 0.5 μg of RNA was subjected to reverse transcription-PCR using the SuperScript First Strand Synthesis
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system for reverse transcription-PCR (Invitrogen), according to the manufacturer’s instructions. The resulting cDNA was diluted 10× in distilled H₂O. Ten µl of cDNA was added to the appropriate primers (final concentration of primers was 1 µM). The human glyoxalase I forward primer was 5-CCGCCCATTGACCATTTGAT-3, and the reverse primer was 5-GTTGGCGATGCCCTTCTCA-3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalize expression. Human GAPDH M33197 forward primer (5-ACCCAACCTCCACCTTTGAT-3) and reverse primer (5-CTGTTGTGGTGATGCCAATTTGCT-3) were added to cDNA in parallel. Twelve µl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA) was added to these mixtures, and quantitative PCR was done with an ABI PRISM 7000 sequence detection system (Applied Biosystems). The relative -fold difference in expression was calculated using the 2^-ΔΔCT method (35).

Transient Transfection of HRP with Vector Harboring Glyoxalase I—The human glyoxalase I sequence (accession number NM_006708) was PCR-amplified from a pUC 19 vector harboring the gene (gift from Dr. Sulabha Ranganathan, Fox Chase Cancer Center, Philadelphia, PA). We introduced restriction sites Nhel and KpnI and subcloned the sequence into pCMS-EGFP (Clontech, Mountain View, CA) to form pCMS-EGFP-glyI. Next, we used 10 µg of either pCMS-EGFP or pCMS-EGFP-glyI to transfect 8 × 10⁶ HRP in 100 µl of Basic Nucleofector™ kit for Primary Smooth Muscle Cell solution (Amaxa, Gaithersburg, MD). Mock-transfected cells were prepared by substituting sterile distilled H₂O for DNA. The remainder of the transfection procedure was done according to the manufacturer’s instructions. Electroporation was carried out with a Nucleofector II apparatus (Amaxa) using the P-13 program. After electroporation, each sample containing 8 × 10⁵ cells was incubated in 500 µl of RPMI (Cambrex, Pittsburgh, PA) for 15 min and then diluted 1:7 in HRP media in a 60-mm dish. Transfected cells were incubated between 48–72 h with a change of medium every day. The percentage of cells transfected was estimated by counting green fluorescent protein-expressing cells through a Nikon Eclipse TS100 light microscope (Melville, NY) fitted with an epifluorescence attachment and camera (Digital Sight DS-L1, Nikon). For apoptosis experiments immediately after transfection, the cells were incubated for 48 h in media supplemented with either 30 mM D-glu or 25 mM L-glu media alone. Controls were incubated in media without additions. Then all samples were treated for 24 h with either 10 µM BBGC or 50% Me₂SO in the presence of the appropriate glucose isomer or H₂O.

Measurement of NO Concentration in HRP—Cell lysates were prepared as described for the glyoxalase I assay, except that the culture media from the cells was pooled with the trypsinized cells. After sonication and centrifugation, the samples were passed through 10-kDa cutoff filters (Millipore, Billerica, MA). Samples (125 µl) were treated with nitrate reductase to reduce nitrates to nitrite (36). Twenty µl of Griess reagent (Griess reagent kit for nitrite determination, Invitrogen) was then added, and absorbance of the resulting azo compound was measured at 570 nm in a microplate reader (Dynex Technologies, Chantilly, VA). The concentration was determined by comparison with a standard curve constructed from known standards of sodium nitrite.

Statistical Analyses—All experiments were performed independently at least twice, with triplicate samples for each intervention within the experiment. Data were assessed for normal distribution and then compared by either Student’s t test (parametric) or a Mann-Whitney U test (non-parametric). A p value of less than 0.05 was considered statistically significant.

RESULTS

Pericytes isolated from human retina by the procedure described were 90–95% positive for the purported pericyte markers, smooth muscle α-actin and NG-2 proteoglycan (Fig. 1). This procedure was used previously to isolate human retinal endothelial cells (33). The absence of endothelial growth factors in the culture medium allowed us to select for pericytes over endothelial cells.

Our first experiments sought to determine whether incubation in a high glucose environment increased glyoxalase I activity in HRP. Glyoxalase I activity was assessed by measuring the product, S-d-lactoylglutathione. Control cell lysates had 1.64 units of glyoxalase I activity (Fig. 2A). The enzyme activities in cells incubated with either D-glu or L-glu (final concentration = 30 mM) were not significantly different from controls (Fig. 2A).

To determine whether glyoxalase I protects HRP from apoptosis, we used BBGC, a competitive inhibitor of glyoxalase I. We first needed to confirm that BBGC inhibits the HRP glyoxalase I under our experimental conditions. We showed that incubation of HRP with BBGC results in a concentration-dependent increase in the un-metabolized substrate, MGO. Fig. 2B shows that MGO increases ~4-fold compared with untreated controls at the highest concentration (200 µM) of BBGC. These data confirm inhibition of HRP glyoxalase I activity by BBGC, as determined by increased intracellular MGO.

We confirmed that BBGC inhibited glyoxalase I in a second experiment. HRP cell lysates were incubated with de-esterified BBGC, the form required for its inhibitory capacity (37). Fig. 2C shows that glyoxalase I activity in cell lysates incubated with de-esterified BBGC decreased ~45% compared with lysates treated with esterase or BBGC alone.

Because we found an increase in MGO with increasing concentrations of BBGC, we wanted to determine whether elevation of MGO caused pericyte apoptosis. We defined early apoptosis by quantifying cells that bind annexin V-fluoroisothiocyanate in the process of early apoptosis, phosphatidylserine is exposed to the outer leaflet of the plasma membrane, which then binds annexin V-fluoroisothiocyanate in the presence of Ca²⁺. Late apoptosis is defined by the binding of propidium iodide; this reagent intercalates with DNA only after permeabilization of the cell membrane.

Treatment of HRP with BBGC results in a concentration-dependent increase in both early and late apoptosis (up to 50 µM, Fig. 3). Fifty µM BBGC appears to be the limit for early apoptosis (22%), with little increase at
either 100 or 200 μM. However, 100 μM BBGC increased the late apoptotic population of HRP as much as 45% relative to the untreated control. Our results, thus, link inhibition of HRP glyoxalase I to pericyte apoptosis. To our knowledge this is the first report of such a correlation.

We wanted to determine whether glyoxalase I contributes to pericyte dropout during diabetic retinopathy. Accordingly, we investigated whether a high glucose environment increased apoptosis in cultures of retinal pericytes and whether the addition of BBGC exacerbates this effect. A high glucose environment can elevate MGO in a number of tissues (38), apparently by increasing the metabolic flux of glucose through the glycolytic pathway (38) (which is the major route) and also through glucose autoxidation, degradation of Amadori products, acetone and threonine metabolism (a minor route). Experiments with HRP cultured for as long as 7 days in a high concentration of glucose (30 mM) failed to show statistically significant increases in early apoptosis relative to either the L-glu control or the untreated control (Fig. 4). We noted a statistically significant increase in late apoptosis compared with the L-glu control, but not the untreated control. A dramatic increase in both early and late apoptosis occurred when cells cultured in media containing 30 mM D-glu were also exposed to BBGC. Both early and late apoptosis increased in both the osmotic control co-incubated with BBGC (L-glu plus BBGC) and cells treated with BBGC alone, although not to the same degree as the D-glu plus BBGC samples (Fig. 4). These findings indicate that as long as glyoxalase I remains functional, HRP remain viable, even in a high glucose environment. However, once this enzyme is inhibited, apoptosis ensues.

To address whether apoptosis was due to an elevation of intracellular MGO due to glyoxalase I blockade and additional synthesis of MGO as a result of the high glucose environment, we measured MGO levels in BBGC and D-glu-treated cells. After 7 days of culture in media contain-
ing 30 mM D-glucose, the intracellular MGO of HRP increased relative to untreated controls (to 89 pmol of MGO/mg of protein). There was a slight but significant increase in MGO concentration in BBGC-treated cells (p = 0.059) and l-glucose plus BBGC-treated cells (p = 0.053) relative to controls. When HRP were co-incubated with D-glucose and BBGC, the intracellular MGO increased significantly (121 pmol/mg of protein, p < 0.01) (Fig. 5).

To confirm that accumulated MGO caused the cells to undergo apoptosis, HRP were incubated for 48 h with 175 μM MGO. This MGO concentration is less than that previously reported to cause retinal pericyte apoptosis (22). Fig. 6 shows that 175 μM MGO increased early apoptosis by almost 3.5-fold and late apoptosis by 5.7-fold relative to untreated controls. Inclusion of BBGC with MGO did not exacerbate this effect in early apoptosis but resulted in a significant increase in late apoptosis. We incubated HRP with the same concentration (175 μM) of glyoxal to determine whether other α-oxoaldehydes caused the same response, but we found no increase in HRP apoptosis (data not shown).

Next, we wanted to establish whether overexpression of glyoxalase I could protect HRP from BBGC-induced apoptosis. We determined that the transfection efficiency of HRP with pCMS-EGFP-glyI (the vector containing glyoxalase I) after 48 h was 20–30%, as judged by green fluorescent protein fluorescence (Fig. 7, A versus B). Despite this low transfection efficiency, glyoxalase I activity increased ~5.5-fold compared with the vector control (Fig. 7C). We noted an increase in early apoptosis when HRP were transiently transfected with either glyoxalase I vector or an empty vector and also in mock-transfected cells (cultures that did not receive DNA) (Fig. 7D). On inspection of our late apoptotic data, we noted similar levels of apoptosis in glyoxalase I- and vector-only-treated cells (Fig. 7E). When transfected cells exposed to vector-only were treated with high glucose and BBGC, apoptosis almost doubled. Interestingly, cells transfected with the active glyoxalase I enzyme (pCMS-EGFP-glyI) did not show such a large increase in apoptosis. These results confirm our earlier findings that overexpression of glyoxalase I protects HRP against apoptosis caused by the combination of BBGC and a high glucose environment.

Once we found that glyoxalase I was important for preventing apoptosis, we wanted to understand how this mechanism impacts diabetic retinopathy. Nitrosative stress occurs during diabetes (40, 41), and it has been suggested that NO is involved in the retina stimulates vascular endothelial growth factor production, thus promoting angiogenesis. NO is also thought to modify anti-oxidant proteins and impair the mitochondrial respiration chain (40), which could result in increased superoxide production with subsequent apoptosis or necrosis. We designed experiments to determine whether NO decreased glyoxalase I expression and/or activity in HRP.

We first assessed the generation of NO by incubating HRP in a high glucose environment. NO in l-glucose-treated cells was 1.56 nmol/mg of protein and 1.17 nmol/mg of protein in control cells (Fig. 8). Incubation of HRP with 30 mM D-glucose resulted in a modest but statistically insignificant elevation of NO (2.12 nmol/mg of protein).

Next, we wanted to determine whether NO inhibited glyoxalase I. Fig. 9A shows a concentration-dependent decrease in glyoxalase I activity when HRP were incubated for 24 h with up to 1 mM DETANONOATE. These results agree with those of Mitsumoto et al. (42) who demon-
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strated inhibition of glyoxalase I through post-translational modification by NO\(^-\) in human endothelial cells. However, the regulation of glyoxalase I expression by NO\(^-\) has not to our knowledge been examined. Accordingly, we used quantitative PCR to examine how DETANONOATE affects expression levels of glyoxalase I mRNA. We found a concentration-dependent down-regulation of glyoxalase I when HRP were incubated with DETANONOATE for 24 h (Fig. 9B).

Because we had found that glyoxalase I can be impaired by incubation with a NO\(^-\) donor, we wanted to explore a possible connection to HRP apoptosis. Fig. 10 shows that incubation of HRP with DETANONOATE alone increases early apoptosis nearly 30\% beyond that in untreated controls. Incubation of HRP with a combination of D-glucose and DETANONOATE resulted in a greater increase (25\%) than in samples combining DETANONOATE and L-glucose samples. We noted a 20\% increase in cells treated with DETANONOATE compared with untreated cells. We observed less late apoptosis than in our experiments with BBGC, and we recorded no significant differences between any of the treatments. Our results confirm that NO\(^-\) has the capacity to induce apoptosis in HRP and that the addition of glucose exacerbates this effect. We can speculate that elevated NO\(^-\) causes apoptosis by inactivating glyoxalase I and/or down-regulating its expression and that the addition of D-glucose provides additional substrate MGO. The rise in MGO enhances apoptosis because the metabolite cannot be detoxified due to impaired or decreased levels of glyoxalase I.

DISCUSSION

The biochemical pathways that lead to pericyte death during diabetic retinopathy are not yet fully understood. Modulation of critical detoxifying enzymes in retinal capillary pericytes may indeed prove to be a key element in this process. Because glyoxalase I protects tumor cells against apoptosis, we suspected that this enzyme could protect retinal pericytes as well, particularly those exposed to hyperglycemic conditions.

Our study for the first time links inhibition of glyoxalase I in human retinal capillary pericytes to apoptosis. Others reported that BBGC treatment of lung cancer cell lines that overexpress glyoxalase I activates caspases and the subsequent c-Jun NH\(_2\)-terminal kinase and p38 MAPK kinase pathways (43). Our experimental data indicate that elevated MGO causes apoptosis as a result of glyoxalase I inhibition (Scheme 1). First, treatment of pericytes with BBGC, an inhibitor of glyoxalase I, elevated MGO levels and subsequently increased apoptosis. Second, co-incubation with both high glucose (30 mM) and BBGC further augmented apoptosis. Finally, MGO (175 \(\mu\)M) added directly to HRP caused apoptosis. We used this high concentration of MGO to offset binding to proteins in the culture media (44). MGO concentrations higher than 175 \(\mu\)M have been reported in mammalian cells (45). Inclusion of BBGC with the added MGO failed to augment early apoptosis, which may reflect saturation of the early apoptotic pathways. However, further elevation of MGO through BBGC increased late apoptosis. Glyoxal is another dicarbonyl and substrate of glyoxalase I that can modify proteins to form AGEs. Although glyoxal reportedly caused apoptosis in a lung epithelial cell line (46), we did not find that it caused HRP apoptosis.

We planned transfection experiments to determine whether glyoxalase I conferred a protective effect against HRP apoptosis, but we had some difficulties. First, we found that all interventions, including the mock-transfected control, increased early apoptosis, and we considered that the transfection procedure caused the cells to become apoptotic. However, late apoptosis was significantly reduced in cells that were transfected with glyoxalase I cDNA and then exposed to the combination of high glucose and BBGC. These results indicate that glyoxalase I protects against high glucose and BBGC-induced apoptosis.

Glyoxalase I activity was unchanged by exposure to a high glucose environment that mimics hyperglycemia, presumably because there is sufficient enzyme to cope with the amount of MGO generated under these conditions. HRP appear to be relatively resistant to MGO since we found that as much as 89 pmol/mg provoked little apoptosis. This agrees with the report by Podesta et al. (5) who found only subtle, non-significant increases in apoptosis in bovine retinal pericytes after 1 week of incubation with 30 mM D-glucose.

Various mechanisms likely contribute to MGO-induced apoptosis. Kim et al. (22) report that MGO-mediated apoptosis in retinal pericytes may occur via an oxidative stress mechanism involving NF-\(\kappa\)B (22). Du et al. (47, 48) likewise suggest a role for oxidative stress generated by incubation of Jurkat cells with MGO, which then triggers c-Jun NH\(_2\)-terminal kinase activation and subsequent apoptosis. Inactivation of GAPDH can elevate levels of MGO because MGO is formed...
after incubation of HRP in a high glucose environment. NO’ has been suggested to promote diabetic retinopathy by stimulating production of the powerful angiogenic factor vascular endothelial growth factor and also by nitration of retinal proteins to cause neurotoxicity (40). Elevated levels of NO’ during hyperglycemia have been attributed to inducible nitric-oxide synthase induction through the action of NF-κB (16, 56). Our findings support previous reports of elevated NO’ and inducible nitric-oxide synthase in the diabetic rat retina (41). Overproduction of NO’ is also reported to cause loss of pericytes from the retina (40). Kim et al. (57) measured inducible nitric-oxide synthase (iNOS) in retinal pericytes and showed that both iNOS and NO’ decrease in the presence of 25 mM glucose (57). This is in contrast to our findings but may be explained by the fact that Kim et al. (57) measured extracellular levels of NO’, whereas we measured intracellular levels.

Our data confirm previous reports that NO’ inhibits glyoxalase I activity (42, 58), and we show for the first time that glyoxalase I expression can be down-regulated by a NO’ donor. We found that the addition of DETANONOATE (100 μM) to HRP markedly decreased transcription, although enzyme activity is retained. Because the expression trends do not mirror precisely the profiles obtained from activity experiments, we suspect that NO’ decreases glyoxalase I activity by a combination of transcription down-regulation and post-translational modification.

The precise pathways of NO’-mediated apoptosis in HRP remain to be established; consequently, it is difficult to determine whether nitrosative stress has a direct role in apoptosis, e.g. by inactivating glyoxalase I. Although we noted increased NO’ formation in d-glu-treated cells, apoptosis did not occur. We recognize the amount of NO’ produced under our experimental conditions may be insufficient to induce apoptosis. Alternatively, defense mechanisms of HRP may be adequate to metabolize rising levels of NO’. Despite lack of evidence for direct activation of apoptosis by NO’, results of our experiments indicate a role for nitrosative stress in pericyte dropout (Scheme 1). Notably, we found 1) an increase in NO’ on incubation of HRP with D-glu, 2) an NO’ donor down-regulated glyoxalase I activity and expression, and 3) inhibition of glyoxalase I or incubation of HRP with an NO’ donor both increased apoptosis. Other effects of NO’ cannot be excluded. For example, nitrosylation could inactivate GAPDH (39), which would result in elevated amounts of MGO and further inactivation of this enzyme as discussed earlier.
In summary, we showed for the first time that inhibition of glyoxalase I in HRP cultured under high glucose conditions results in apoptosis. However, because a high glucose environment alone does not induce apoptosis, we believe glyoxalase I to be critical for maintaining HRP viability. Even under hyperglycemic conditions, presumably decreases glyoxalase I activity by down-regulation and inactivation of glyoxalase I. Under conditions of metabolic stress found in uncontrolled diabetes, this dual effect would increase formation of MGO within HRP and eventually cause death by apoptosis. Our findings underscore the complex nature of pericyte apoptosis and indicate potentially important mechanisms for pericyte dropout during the early stages of diabetic retinopathy.

Acknowledgments—We thank Dr. Yuming Zhang (Department of Chemistry, Case Western Reserve University) for preparation of BBGC and MGO along with Mike Sramkowski and staff (Comprehensive Cancer Center Flow Cytometry Core, Case Western Reserve University) for advice on flow cytometry experiments. We also acknowledge Maria Codispoti for immunohistochemical experiments. We also acknowledge Maria Codispoti for immunohistochemical staining of HRP. We also thank Dr. Bondi Gong for assistance with primer design for quantitative PCR experiments.

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