Poly(ADP-Ribose)Polymerase Inhibition Counteracts Cataract Formation and Early Retinal Changes in Streptozotocin-Diabetic Rats

Viktor R. Drel,1 Weizheng Xu,2 Jie Zhang,3,4 Tayyeba K. Ali,5 Jebo Shin,1 Ulrich Julius,6 Barbara Slusher,2 Azza B. El-Remessy,5 and Irina G. Obrosova1

PURPOSE. This study evaluated the role for poly(ADP-ribose) polymerase (PARP) in diabetes-induced cataractogenesis and early retinal changes.

METHODS. Control and streptozotocin (STZ)-diabetic rats were treated with or without the PARP inhibitors 1,5-isoquinolinediol (ISO; 5 mg kg-1 d-1 intraperitoneally) and 10-(4-methyl-piperazin-1-ylmethyl)-2H-7-oxa-1,2-diaza-benzof[de]anthracen-3-1 (GPI-15427, 30 mg kg-1 d-1 orally) for 10 weeks after the first 2 weeks without treatment. Lens clarity was evaluated by indirect ophtalmoscopy and slit lamp examination, and retinal changes were evaluated by immunohistochemistry and Western blot analysis. In in vitro studies, cultured human lens epithelial cells and bovine retinal pericytes and endothelial cells were exposed to high glucose or palmitate.

RESULTS. PARP is expressed in lens, and poly(ADP-ribose)-labeled proteins are primarily localized in the 38- to 87-kDa range of the protein spectrum, with several minor bands at 17 to 38 kDa. The 38- to 87-kDa and the 17- to 38-kDa poly(PARP)-labeled protein expression increased by 74% and 275%, respectively, after 4 weeks of diabetes and by approximately 65% early after exposure of lens epithelial cells to 30 mM glucose. Both PARP inhibitors delayed, but did not prevent, the formation of diabetic cataract. The number of TUNEL-positive nuclei in flatmounted retinas increased approximately 4-fold in STZ diabetic rats, and this increase was prevented by ISO and GPI-15427. Both PARP inhibitors reduced diabetes-induced retinal oxidative-nitrosative and endoplasmic reticulum stress and glial activation. GPI-15427 (20 μM) prevented oxidative-nitrosative stress and cell death in palmitate-exposed pericytes and endothelial cells.

CONCLUSIONS. PARP activation is implicated in the formation of diabetic cataract and in early retinal changes. These findings provide a rationale for the development of PARP inhibitors for the prevention of diabetic ocular complications. (Invest Ophthalmol Vis Sci. 2009;50:1778–1790) DOI:10.1167/iovs.08-2191

Growing evidence suggests that the activation of poly(ADP-ribose) polymerase (PARP), the enzyme that cleaves nicotinamide adenine dinucleotide (NAD+) with the formation of nicotinamide and poly(ADP-ribose) polymer, is an important event in the development of cardiovascular disease, cancer, and diabetes mellitus.1,2 PARP activation contributes to NAD+ depletion and energy failure,1,2 changes in transcriptional regulation and gene expression,1,2,3 impaired signal transduction,5 and, in extreme cases, necrosis and apoptosis.1,2 In the past several years, it has been shown that PARP activation plays a key role in diabetes-associated endothelial and myocardial dysfunction,1,6,7 peripheral and autonomic neuropathy,3,8,9 and nephropathy.10

The role of PARP in diabetic ocular complications deserves thorough evaluation considering that PARP-1 is abundantly expressed in lens11 and retina.12,13 PARP activation contributes to the formation of pericyte ghosts and acellular capillaries,13 increased leukocyte adhesion to endothelial cells,13,14 and vascular endothelial growth factor (VEGF) formation15 and angiogenesis.16,17 The role of PARP activation in diabetes-associated cataractogenesis remains unexplored. The consequences of retinal PARP activation at the early stages of diabetes and the relations of this mechanism to other stresses (oxidative-nitrosative stress, endoplasmic reticulum stress, neuroglial activation, and premature neural retinal apoptosis) in the diabetic retina have not been evaluated. Furthermore, although it has been established that PARP activation mediates high glucose-induced apoptosis in retinal endothelial cells,13 its role in premature cell death caused by other factors in the diabetic milieu, particularly free fatty acids,18 is unknown.

The present study was aimed at evaluating the role of PARP activation in cataract formation and early retinal changes associated with diabetes through the use of animal and cell culture models and a pharmacologic approach with two structurally unrelated PARP inhibitors, 1,5-isoquinolinediol (ISO) and 10-(4-methyl-piperazin-1-ylmethyl)-2H-7-oxa-1,2-diaza-benzof[de]anthracen-3-one (GPI-15427).

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma Chemical (St. Louis, MO). GPI-15427 was obtained from MGI Pharma (Baltimore, MD). Rabbit polyclonal anti-nitrotyrosine (NT) antibody and mouse monoclonal anti-NT antibody, clone IAI6, were purchased from Upstate (Lake Placid, NY), and mouse monoclonal anti-poly(ADP-ribose) antibody was purchased from Trexgen, Inc. (Gaithersburg, MD). Mouse monoclonal anti-BiP (immunoglobulin heavy-chain binding protein)/GRP78 (78kDa glu-
cose-regulated protein) antibody was purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal GRP94 antibody was purchased from Abcam Inc. (Cambridge, MA). Secondary Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse antibodies, antifade reagent (Prolong Gold), 4’,6-diamidino-2-phenylindole (DAPI), and hydroxyethidine were purchased from Invitrogen (Eugene, OR). Biotinylated anti-rabbit and anti-mouse antibody, avidin/biotin blocking kit, ABC kit (Vectorstain Elite; Standard), and DAB substrate kit were obtained from Vector Laboratories (Burlingame, CA). Mouse monoclonal anti-gliial fibrillary acidic protein (GFAP) antibody and two in situ apoptosis detection kits (ApopTag Plus Fluorescein and ApopTag Peroxidase; Chemicon International, Temecula, CA). A caspase assay kit (EnzChek Caspase-3) was purchased from Invitrogen (Carlsbad, CA). Mounting medium (Micromount) was purchased from Surgipath Medical (Richmond, IL). Other reagents for immunohistochemistry were purchased from Dako Laboratories (Santa Barbara, CA).

Animals

Experiments were performed in accordance with regulations specified by the National Institutes of Health Principles of Laboratory Animal Care (1985 revised version), the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Pennington Biomedical Research Center Protocol for Animal Studies. Male Wistar rats (Charles River, Wilmington, MA), body weight 250–300 g, were fed a standard diet (PMI Nutrition International, Brentwood, MO) and had access to water and ad libitum. Streptozotocin (STZ) diabetes was induced as described. Blood samples for glucose measurements were taken from the tail vein approximately 48 hours after STZ injection and the day before study termination. All rats with blood glucose levels ≥ 13.8 mM were considered diabetic. The experimental groups were composed of control and diabetic rats treated with or without the PARP inhibitors, ISO (3 mg kg⁻¹ d⁻¹ intraperitoneally) or GPI-15427 (formulated as mesylate salt, 30 mg kg⁻¹ H₁₁₀₀₂) were composed of control and diabetic rats treated with or without the PARP inhibitors, ISO (3 mg kg⁻¹ d⁻¹ intraperitoneally) or GPI-15427 (formulated as mesylate salt, 30 mg kg⁻¹ d⁻¹, in the drinking water), for 10 weeks after the first 2 weeks without treatment. At the end of the 12-week study, lens changes were evaluated by indirect ophthalmoscope and portable slit lamp (Kowa, Tokyo, Japan). Evaluations were preceded by mydriasis with topical 1% tropicamide hydrochloride. Cataracts were scored as follows: 1, no cataract (clear lenses); 2, equatorial vacuoles; 3, cortical opacities; 4, mature cataract when the whole lens becomes opaque. Control rats and rats with STZ diabetes of 4 weeks’ duration were used for assessment of lens PARP and poly(ADP-ribosyl)ated protein expression.

Anesthesia, Euthanization, and Tissue Sampling

Animals were sedated by CO₂ and immediately killed by cervical dislocation. One eye from each rat was enucleated and fixed in normal saline before study termination. All rats with blood glucose levels ≥ 13.8 mM were considered diabetic. The experimental groups were composed of control and diabetic rats treated with or without the PARP inhibitors, ISO (3 mg kg⁻¹ d⁻¹ intraperitoneally) or GPI-15427 (formulated as mesylate salt, 30 mg kg⁻¹ d⁻¹, in the drinking water), for 10 weeks after the first 2 weeks without treatment. At the end of the 12-week study, lens changes were evaluated by indirect ophthalmoscope and portable slit lamp (Kowa, Tokyo, Japan). Evaluations were preceded by mydriasis with topical 1% tropicamide hydrochloride. Cataracts were scored as follows: 1, no cataract (clear lenses); 2, equatorial vacuoles; 3, cortical opacities; 4, mature cataract when the whole lens becomes opaque. Control rats and rats with STZ diabetes of 4 weeks’ duration were used for assessment of lens PARP and poly(ADP-ribosyl)ated protein expression.

## Table 1. Initial and Final Body Weights and Blood Glucose Concentrations in Control and Diabetic Rats Maintained with and without PARP Inhibitor Treatment

<table>
<thead>
<tr>
<th>Body Weight (g)</th>
<th>Blood Glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Control</td>
<td>291 ± 8</td>
</tr>
<tr>
<td>Control + GPI-15427</td>
<td>299 ± 23</td>
</tr>
<tr>
<td>Control + ISO</td>
<td>296 ± 11</td>
</tr>
<tr>
<td>Diabetic</td>
<td>288 ± 17</td>
</tr>
<tr>
<td>Diabetic + GPI-15427</td>
<td>297 ± 16</td>
</tr>
<tr>
<td>Diabetic + ISO</td>
<td>298 ± 17</td>
</tr>
</tbody>
</table>

Data are mean ± SD; n = 12–20 per group. * P < 0.01 vs. controls.

### Specific Methods Used in Animal Studies

#### Immunohistochemical Studies

All flattened retinas were processed by a single investigator and evaluated blindly. The rate of apoptosis was quantified with an in situ apoptosis detection kit (ApopTag Peroxidase; Chemicon International), as described previously, with a minor modification. NT, poly(ADP-ribose), GFAP, BiP/GRP78, and GRP94 immunoreactivities in retinal sections were assessed by conventional immunohistochemistry. At least 10 fields of each section were examined to select one representative image. Low-power observations of retinal sections stained for NT, poly(ADP-ribose), GFAP, BiP/GRP78, and GRP94 were made with a fluorescein microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY). Color images were captured with a CCD camera at 1300 × 1030 resolution (Axiocam HRC; Carl Zeiss, Inc.). Low-power images were generated with a 40× acroplan objective using the automatic capturing feature of the software (Axiovision, version 3.1.2.1; Carl Zeiss, Inc.). Low-power observations of retinal sections stained for TUNEL-positive cells (ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit; Chemicon International) were made with an imaging microscope (Axioplan 2; Carl Zeiss, Inc.). Fluorescent images were captured with a CCD camera (CoolSNAP HQ; Photometrics, Tucson, AZ) at 1392 × 1040 resolution. Low-power images were generated with a 40× acroplan objective with image-acquisition software (RS Image 1.9.2; Photometrics).

#### Western Blot Analyses

Western blot analyses of poly(ADP-ribosyl)ated and nitrated proteins, BiP/GRP78, and GRP94 in individual retinas (one retina from each rat) were performed as described previously. Protein bands were visualized (BiM Chemiluminescence blotting Substrate; Roche, Indianapolis, IN). Membranes were then stripped and reprobed with β-actin antibody to confirm equal protein loading. Data were quantified by densitometry (Quantity One 4.5.0 software; Bio-Rad Laboratories, Richmond, CA).

### Cell Culture Studies

#### Human Lens Epithelial Cells

HLE cells, passages 6 to 10, were supplied by the laboratory of Usha Andley at Washington University (St. Louis, MO). In this laboratory, HLE cells were isolated from adult lenses obtained from MidAmerica Eye Bank (St. Louis, MO). The use of human tissue for research purposes conformed to the tenets of the Declaration of Helsinki. Lenses were processed less than 24 hours after surgical removal. Cells were cultured in HBSS (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂.
after death. The capsule epithelium from each lens was dissected and divided into two or three pieces. Primary cultures were initiated in 35-mm tissue culture plates and subcultured after confluence was achieved, usually in 7 to 10 days. Cells were cultured on tissue culture plasticware (Corning, Corning, NY or Falcon Plastics, Cockeysville, MD) in Eagle minimum essential medium (EMEM) containing 50 mg/mL gentamicin and 20% fetal bovine serum. After confluence was attained, cells were passaged using Trypsin-EDTA (T3924; Sigma), frozen, and shipped to Pennington Biomedical Research Center. There, HLE cells were cultured in 6-well plates (well diameter, 3.5 cm) at a cell density of 2.0 x 10^5 cells/mL. 2.0 x 10^5 cells/mL. 

**TABLE 2. Lens Clarity in Control Rats and Rats with 12-Week Duration of STZ-Induced Diabetes Maintained with and without PARP Inhibitor Treatment**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>C + GPI</th>
<th>C + ISO</th>
<th>D</th>
<th>D + GPI</th>
<th>D + ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n)</td>
<td>12</td>
<td>12</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Clear lenses (n)</td>
<td>12</td>
<td>12</td>
<td>14</td>
<td>1</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Vacuolar stage (n)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Opacities (n)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Mature cataract (n)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cataract score*</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.125 ± 0.34</td>
<td>2.88 ± 0.89†</td>
<td>2.0 ± 1.16‡</td>
<td>2.31 ± 1.08‡</td>
</tr>
</tbody>
</table>

* Values are mean ± SD. Clear lenses were scored as 1, those with vacuoles were scored as 2, those with cortical opacities were scored as 3, and those with mature cataract were scored as 4.
† P < 0.1 vs. controls.
‡ P < 0.01 vs. untreated diabetic rats.

**FIGURE 1. Left:** representative Western blot analyses of PARP-1 (A) and poly(ADP-ribosyl)ated proteins (C, E) in the lenses of rats with STZ diabetes of 4 weeks’ duration. **Right:** PARP-1 (B) and poly(ADP-ribosyl)ated protein (D, F) contents (densitometry) in control and diabetic rats. Equal protein loading was confirmed with β-actin antibody. Poly(ADP-ribosyl)ated protein content in control rats is taken as 100%. M, standards of PARP-1 (A) and poly(ADP-ribosyl)ated proteins (C, E); C1, C2, C3, lenses from control rats; D1, D2, D3, lenses from diabetic rats. Mean ± SD. n = 3 per group. *P < 0.05 vs. controls; **P < 0.01 vs. controls.
density of approximately $5 \times 10^4$/well, at $37^\circ$C, in a humidified atmosphere consisting of air/CO$_2$ (19:1) for 48 hours in EMEM containing either 5 or 30 mM glucose. HLE cells were used for Western blot analyses of PARP-1 and poly(ADP-ribosyl)ated proteins performed as described previously.

**Bovine Retinal Pericytes and Endothelial Cells. Cell Preparation.** Primary bovine retinal pericyte and endothelial cell cultures were established from fresh cow eyes as described previously. Passages 4 to 6 were used for all experiments. Purity of the cultures was confirmed by characteristic pericyte and endothelial cell morphology and by the use of specific pericyte ($\alpha$-smooth muscle actin) and endothelial cell (von Willebrand factor) markers. On average, in pericyte experiments, 98.8% ± 1.4% of the isolated cells were identified as pericytes. In endothelial cell experiments, 99.9% ± 1.1% of the isolated cells were identified as endothelial cells.

To dissect the effects of palmitate (prepared as described) and PARP inhibition, pericytes and endothelial cells were cultured in the Dulbecco modified Eagle medium containing 20% serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and, for endothelial cells only, 50 μg/mL endothelial growth supplement. At least three 6-well plates were used per experimental condition. Cells were placed on round glass coverslips and coated with gelatin or fibronectin (for pericyte and endothelial cells, respectively). At 80% confluence, pericyte and endothelial cell cultures were transferred for 48 hours to the media without palmitate and without GPI-15427, with 0.6 mM palmitate and without GPI-15427, or with 0.6 mM palmitate and with 20 μM GPI-15427.

**Assessment of Apoptosis.** The rate of cell death was quantified at the end of exposure (ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit; Chemicon International). Parallel cultures from each group were trypsinized and used for assessment of caspase activity. DEVD (rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)-specific protease, that is, primarily those of caspase-3 and caspase-7, activity measurements were based on monitoring increases in fluorescence caused by conversion of the nonfluorescent bisamide substrate to the fluorescent monoamine and even more fluorescent derivative of rhodamine 110 and were performed with a caspase assay kit (EnzChek Caspase-3; Invitrogen). Increases in fluorescence were measured spectrofluorometrically at $\lambda$ excitation/496 nm and $\lambda$ emission/520 nm (LS 55 Luminescence Spectrometer).

**FIGURE 2.** Left: representative Western blot analyses of PARP-1 (A) and poly(ADP-ribosyl)ated proteins (C, E) in HLE cells cultured for 48 hours in 5 mM and 30 mM glucose. Right: PARP-1 (B) and poly(ADP-ribosyl)ated protein (D, F) contents (densitometry) in HLE cells cultured for 48 hours in 5 mM and 30 mM glucose. Equal protein loading was confirmed with $\beta$-actin antibody. Poly(ADP-ribosyl)ated protein content in HLE cells cultured in 5 mM glucose is taken as 100%. M, standards of PARP-1 (A) and poly(ADP-ribosyl)ated proteins (C, E). Mean ± SD. $n = 3$ per group. **P < 0.01 vs. controls.

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/932957/ on 06/06/2017
equipped with microplate reader; Perkin Elmer, Waltham, MA). After spectrofluorometry, the cells were counted, and caspase activity was calculated in relative fluorescence units per $10^4$ cells during 30 minutes of reaction.

**Superoxide Detection.** For superoxide production measurements, pericytes and endothelial cells were cultured in 6-well plates in media containing 0, 0.2, 0.4, 0.6, or 0.8 mM palmitate. Culture media were then aspirated, and the cells were washed with PBS. Two milliliters of serum-free medium containing 50 µL of 10 mM hydroethidine was added per well at 37°C for 30 minutes. Then the cells were washed with PBS and trypsinized, and ethidium fluorescence, an index of superoxide generation, was measured with the use of spectrofluorometry at λ excitation/465 nm and λ emission/630 nm (LS 55 Luminescence Spectrometer equipped with microplate reader; Perkin Elmer). After spectrofluorometry, the cells were counted, and fluorescence intensity was expressed per $10^4$ cells.

**Immunocytochemical Assessment of Nitrotyrosine and Poly(ADP-ribose).** Coverslips with pericyte or endothelial cells were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes. Fixed cells were washed in PBS and preincubated with 0.2% Triton X-100 in PBS for 15 minutes. Coverslips were blocked with 1% BSA containing 10% goat serum for 1 hour. Then the cells were treated with either mouse monoclonal anti–poly(ADP-ribose) antibody (1:100 dilution) or rabbit polyclonal anti–NT antibody (1:200 dilution). Secondary Alexa Fluor 488 goat anti–mouse antibody or Alexa Fluor 488 goat anti–rabbit antibody was applied in working dilutions of 1:200. Primary antibody was omitted in negative controls. Coverslips were mounted in antifade reagent (Prolong Gold; Invitrogen) and placed on a slide. Images of immunostained cells were captured with a CCD camera (Photometric CoolSNAP HQ) at 1392 × 1040 resolutions. Fluorescence was quantified with Image 1.32 software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Seven to 10 images were quantified per experimental condition, and the average per cell was calculated.

**Statistical Analysis**

Results are expressed as mean ± SD. Data were subjected to equality of variance $F$ test and then to log transformation, if necessary, before one-way analysis of variance. Where overall significance ($P < 0.05$) was attained, individual between-group comparisons were made using the Student-Newman-Keuls multiple-range test. Significance was defined at $P ≤ 0.05$. When between-group variance differences could not be normalized by log transformation (data sets for body weights and plasma glucose), the data were analyzed by nonparametric Kruskal-Wallis one-way analysis of variance, followed by Bonferroni/Dunn or Fisher PLSD test for multiple comparisons.

**RESULTS**

Initial (before STZ administration) body weights were similar in control and diabetic rats treated with or without ISO or GPI-15427. Final body weights were similarly reduced in untreated and PARP inhibitor–treated diabetic rats compared with the control group (Table 1). Initial blood glucose concentrations were 4.5-, 4.6-, and 4.5-fold higher in untreated, ISO-, and GPI-15427–treated diabetic rats, respectively, than in non-diabetic controls. Similarly, final blood glucose concentrations...
were 4.7-, 4.5-, and 4.6-fold higher in untreated, ISO-, and GPI-15427–treated diabetic rats than in nondiabetic controls. PARP inhibition did not affect weight gain or blood glucose concentration in nondiabetic rats.

The average cataract score was 2.88-fold higher in rats with STZ diabetes of 12 weeks’ duration than in nondiabetic controls (Table 2). Twenty-five percent of eyes of untreated diabetic rats displayed the vacuolar stage of cataract, 44% had cortical opacities, and 25% had mature cataract. PARP inhibition counteracted, but did not prevent, diabetes-associated cataractogenesis. Clear lenses were detected in 50% and 31% of eyes in diabetic rats treated with GPI-15427 and ISO, respectively, compared with 44% in diabetic rats. Mature cataract (the whole lens was opaque) was detected in 12.5% of eyes of diabetic rats treated with ISO or GPI-15427 compared with 25% of eyes in the untreated diabetic group.

PARP-1 expression was similar in the lenses of nondiabetic control rats and rats with STZ diabetes of 4 weeks’ duration (Figs. 1A, 1B). Poly(ADP-ribosyl)ated proteins were detected primarily in the 38- to 87-kDa range of the lens protein spectrum (Figs. 1C, 1D), with several minor bands at 17 to 38 kDa (Figs. 1E, 1F). Both 38- to 87-kDa and 17- to 38-kDa poly(ADP-ribosyl)ated protein expression was increased in rats with 4-week STZ diabetes compared with nondiabetic controls.

In a similar fashion, PARP-1 expression was indistinguishable between HLE cells cultured in 5 mM or 30 mM glucose for 48 hours (Figs. 2A, 2B). Poly(ADP-ribosyl)ated proteins were abundantly expressed in the 17- to 38-kDa range of the lens protein spectrum, whereas the upper part of the spectrum...
displayed less manifest poly(ADP-ribosyl)ation (Figs. 2C, 2D). Poly(ADP-ribosyl)ated protein expression in the upper and lower parts of the lens protein spectrum was increased by approximately 65% early (48 hours) after exposure of HLE cells to 30 mM glucose.

Retinal poly(ADP-ribose) immunoreactivity was increased in diabetic rats compared with nondiabetic controls, and this increase was essentially prevented by ISO and GPI-15427 (Fig. 3A). Poly(ADP-ribose) positive nuclei were localized primarily in the ganglion cell layer but were also detectable in other parts of the retina. Poly(ADP-ribosyl)ated protein expression, quantified by Western blot analysis, was increased by 41% in untreated diabetic rats compared with nondiabetic controls but remained essentially unchanged from the control level in diabetic rats treated with ISO or GPI-15427 (Figs. 3B, 3C).

Increased nitrotyrosine immunoreactivity in all retinal layers was detected in untreated diabetic rats compared with nondiabetic controls, and this increase was essentially prevented by ISO and GPI-15427 (Fig. 4A). Poly(ADP-ribose) positive nuclei were localized primarily in the ganglion cell layer but were also detectable in other parts of the retina. Poly(ADP-ribosyl)ated protein expression, quantified by Western blot analysis, was increased by 53% in untreated diabetic rats compared with controls (Figs. 4B, 4C). ISO and GPI-15427 counteracted the accumulation of diabetes-associated retinal nitrotyrosine.

Retinal GFAP immunoreactivity was increased in diabetic rats compared with controls, and this increase was counteracted by PARP inhibitors (Fig. 5). Immunoreactive BIP/GRP78 (Fig. 7A) and GRP94 (Fig. 8A) were identified in the retinas of control rats. GRP94 immunoreactivity displayed a uniform distribution among all retinal layers, whereas BIP/GRP78 distribution was less homogeneous, and expression in inner plexiform and outer nuclear layers was faint. BIP/GRP78 expression was increased by 37% in untreated diabetic rats compared with controls, indicative of ER stress (Figs. 7B, 7C). ISO and GPI-15427 counteracted diabetes-induced BIP/GRP78 expression. Conversely, GRP94 expression showed a minor (9%) induction in untreated diabetic rats compared with nondiabetic controls (Figs. 8B, 8C) and remained in the nondiabetic range in ISO- and GPI-15427-treated diabetic rats.

A 48-hour exposure to palmitate caused a dose-dependent increase in superoxide production in retinal pericytes and endothelial cells (Figs. 9A, 9B), with enhanced oxidative stress after exposure to 0.6 mM palmitate.

Poly(ADP-ribosyl)ated protein fluorescence was increased in retinal pericytes (Figs. 10A, 10B) and endothelial cells (Figs. 10C, 10D) cultured with the addition of 0.6 mM palmitate compared with those cultured without palmitate. The PARP inhibitor GPI-15427 prevented the accumulation of poly(ADP-ribosyl)ated proteins in palmitate-exposed retinal microvascular cells.

Nitrotyrosine fluorescence was increased in 0.6 mM palmitate-exposed cultured retinal pericytes (Figs. 11A, 11B) and endothelial cells (Figs. 11C, 11D). Palmitate-induced nitrosative stress in both cell types was reduced though not completely blunted by GPI-15427.
FIGURE 7. (A) Representative microphotographs of retinal BiP/GRP78 immunostaining in control and diabetic rats maintained with or without PARP inhibitor treatment. n = 6 to 10 per group. Magnification, ×40. (B) Representative Western blot analyses of retinal BiP/GRP78 in control and diabetic rats maintained with and without PARP inhibitor treatment. (C) Retinal BiP/GRP78 contents (densitometry) in control and diabetic rats maintained with and without PARP inhibitor treatment. Equal protein loading was confirmed with β-actin antibody. BiP/GRP78 content in control rats is taken as 100%. Mean ± SD. n = 6 per group. **P < 0.01 vs. controls; #P < 0.05 vs. untreated diabetic group.

FIGURE 8. (A) Representative microphotographs of retinal GRP94 immunostaining in control and diabetic rats maintained with or without PARP inhibitor treatment. n = 6 to 10 per group. Magnification, ×40. (B) Representative Western blot analyses of retinal GRP94 in control and diabetic rats maintained with and without PARP inhibitor treatment. (C) Retinal GRP94 contents (densitometry) in control and diabetic rats maintained with and without PARP inhibitor treatment. Equal protein loading was confirmed with β-actin antibody. GRP94 content in control rats is taken as 100%. Mean ± SD. n = 6 per group.
**FIGURE 9.** Dose-dependent increase in superoxide fluorescence in cultured retinal pericytes (A) and endothelial cells (B) exposed to 0, 0.2, 0.4, 0.6, and 0.8 mM palmitate. RFU, relative fluorescence units. Mean ± SD. n = 4 per group. *P < 0.05 and **P < 0.01 vs. cells cultured in 5 mM glucose.

**FIGURE 10.** Left: representative microphotographs of poly(ADP-ribose) fluorescence (green) in retinal pericytes (A) and endothelial cells (C) cultured for 48 hours without 0.6 mM palmitate or GPI-15427 (C), with 0.6 mM palmitate and without GPI-15427 (P), and with 0.6 mM palmitate and with GPI-15427 (P+GPI). Magnification, ×100. Blue fluorescence corresponds to 4',6-diamidino-2-phenylindole-stained nuclei. Right: poly(ADP-ribose) fluorescence (relative fluorescence units [RFU] per cell) in retinal pericytes (B) and endothelial cells (D) cultured without 0.6 mM palmitate or GPI-15427 (C), with 0.6 mM palmitate and without GPI-15427 (P), and with 0.6 mM palmitate and with GPI-15427 (P+GPI). Mean ± SD. n = 4 per group. **P < 0.01 vs. cells cultured without palmitate and without GPI-15427; ##P < 0.01 vs. cells cultured with palmitate and without GPI-15427.
Palmitate exposure (0.6 mM) was associated with augmented cell death manifested by increased numbers of TUNEL-positive cells (Figs. 12A–D) and elevated caspase activity (Figs. 12E, 12F) in retinal pericytes and endothelial cells. PARP inhibition counteracted palmitate-induced increases in TUNEL positivity and caspase activation in both cell types.

**DISCUSSION**

The findings described herein provide the first evidence of early PARP activation in the lenses of diabetic rats and high glucose-exposed HLE cells and of the contribution of this mechanism to the formation of diabetic cataract. Multiple mechanisms have been implicated in diabetes-associated and other types of cataractogenesis,25 but no anticataract agent is available for use in humans. Although numerous findings in diabetic animal models25–28 and gene polymorphism studies in human subjects with type 2 diabetes29 point to the key role for the first enzyme of the sorbitol pathway, aldose reductase (AR), several AR inhibitors (ARIs) have been withdrawn from diabetic complication–related clinical trials because of low efficacy (carboxylic acid–derived ARIs) or adverse effects (hydantoin ARIs). Evidence of participation of nonenzymatic glycooxidation, another important mechanism in diabetic complications,30 in cataractogenesis in animals and humans with diabetes is controversial.25,31,32 Long-term consumption of vitamin C and vitamin E supplements reduced the development of age-related lens opacities in humans.33,34 Note, however, that the results of clinical trials of conventional antioxidants in human subjects with diabetic complications have been inconclusive.35,36 In the present experimental study, PARP inhibitors delayed rather than prevented diabetic cataract formation (i.e., efficacy was comparable to that of conventional antioxidants in other reports).25,37
15427 have been used at lower doses; therefore, dose-response studies are needed for full assessment of the anticataractogenic potential of PARP inhibitors. Taking into consideration the multiple consequences of PARP activation in tissue sites for diabetic complications, it is probably important to control this enzyme activity from an early stage of diabetes to prevent the development of cataract.

Our findings also demonstrate that PARP inhibition counteracts numerous changes characteristic of early diabetic retinopathy. In particular, PARP inhibition alleviated oxidative-nitrosative stress in the retina of STZ diabetic rats and palmitate-exposed cultured retinal pericytes and endothelial cells. Until recently, PARP activation was regarded as a phenomenon arising from free radical- and peroxynitrite-induced DNA single-strand breakage. However, recent studies reveal that in some tissues of diabetic animals PARP activation may lead to rather than result from oxidative-nitrosative stress and that PARP activation does not necessarily require DNA single-strand breakage and may occur because of enzyme phosphorylation by ERK.

**Figure 12.** (A, C) Representative microphotographs of TUNEL-positive cells in retinal pericytes (A) and endothelial cells (C) cultured for 48 hours without 0.6 mM palmitate or GPI-15427 (C), with 0.6 mM palmitate and without GPI-15427 (P), and with 0.6 mM palmitate and with 20 μM GPI-15427 (P+GPI). Magnification, ×100. Blue fluorescence corresponds to 4',6-diamidino-2-phenylindole-stained nuclei. (B, D) Percentage of TUNEL-positive cells in retinal pericyte (B) and endothelial cell (D) cultured for 48 hours without 0.6 mM palmitate or GPI-15427 (C), with 0.6 mM palmitate and without GPI-15427 (P), and with 0.6 mM palmitate and with 20 μM GPI-15427 (P+GPI). Mean ± SD. **P < 0.01 vs. cells cultured without palmitate and without GPI-15427; #P < 0.01 vs. cells cultured with palmitate and without GPI-15427.** (E, F) Caspase activities in retinal pericytes (A) and endothelial cells (C) cultured for 48 hours without 0.6 mM palmitate or GPI-15427 (C), with 0.6 mM palmitate and without GPI-15427 (P), and with 0.6 mM palmitate and with 20 μM GPI-15427 (P+GPI). Caspase activity in cells cultured without 0.6 mM palmitate and without GPI-15427 is taken as 100%. Mean ± SD. n = 3 to 6 per group. **P < 0.01 vs. cells cultured without palmitate and without GPI-15427; #P < 0.05 and ##P < 0.01 vs. cells cultured with palmitate and without GPI-15427.
PARP, Diabetic Cataract, and Early Retinal Changes 1789

ribosylated proteins accumulated in cells containing DNA breaks and in those with preserved DNA integrity. The latter is consistent with current findings suggesting that the relations between diabetes-associated oxidative-nitrosative stress and PARP activation in retina and retinal capillary cells are bidirectional rather than unidirectional. Increased formation of reactive oxygen and nitrogen species leads to PARP activation and vice versa.

PARP inhibition also counteracted diabetes-induced retinal glial activation manifest in GFAP accumulation and neural retinal apoptosis. Similar effects on both phenomena have been reported for two ARIs, sorbinil and ARI-809. It has been hypothesized that retinal neurodegenerative changes, including increased glial cell reactivity and microglial activation, together with altered glutamate metabolism and premature apoptosis, are critical components of diabetic retinopathy. However, a recent study in the STZ diabetic mouse model has shown that diabetes-induced degeneration of retinal capillaries can develop independently of neuronal loss or chronic GFAP upregulation in glial cells.

The frequency of early apoptosis in retinal capillary cells has been reported to predict the development of the histologic lesions of retinopathy in diabetes and galactosemia. In addition to high glucose, other factors in the diabetic milieu, particularly fatty acids, cause premature apoptosis of retinal pericytes and endothelial cells. In the present study, the number of apoptotic cells increased more than 3-fold in retinal pericytes and endothelial cells shortly (48 hours) after exposure to 0.6 mM palmitate, the concentration in the circulation of diabetic rodents. These findings are consistent with caspase activation in both cell types. PARP inhibition counteracted fatty acid-induced increases in TUNEL positivity and caspase activation in retinal capillary cells. Taking into consideration that a PARP inhibitor treatment has previously been reported to counteract high glucose-induced endothelial cell apoptosis, it is reasonable to suggest that PARP activation expedites the mechanism(s) involved in hyperglycemia and elevated fatty acid-induced cell death.

Recent reports suggest that the accumulation of unfolded or misfolded proteins that cause ER stress and the unfolded protein response play important roles in diabetes-associated β-cell dysfunction. The role for ER stress in diabetic complications remains unexplored, though a recent study has implicated this phenomenon in lens epithelial cell apoptosis and cataract formation in galactose-fed rats. The present study showed that modest retinal ER stress, manifested by the presence of ER-mediated chaperones BiP/GRP78 and GRP94 (two proteins containing ER stress response element in their promoters), is identifiable by immunohistochemistry and Western blot analysis in nondiabetic and diabetic rats. Furthermore, a slight, but statistically significant, increase in BiP/GRP78 expression, primarily confined to inner and outer plexiform and ganglion cell layers of diabetic rats, indicated that a weak induction of retinal ER stress was present at a very early stage of diabetes. Another ER chaperone, GRP94, showed only a trend toward an increase, suggesting that diabetes may affect a recently identified ER stress sensor in the retina. Recent studies have shown that diabetes may affect a recently identified ER stress sensor in the retina.

In conclusion, PARP activation is involved in cataractogenesis, retinal oxidative-nitrosative and ER stresses, neuroglial activation, and retinal neural and capillary cell apoptosis in diabetic rats. These findings, consistent with previous reports on PARP contribution to the formation of acellular capillaries and pericyte ghosts, leukostasis, and VEGF formation, provide a rationale for the development of PARP inhibitors to prevent and slow the progression of diabetic retinopathy and cataract formation.

Acknowledgments

The authors thank Usha P. Andley for providing human lens epithelial cells and valuable recommendations regarding their use.

References

1790  Drel et al.


29. Lee YK, Chung SK, Chung SS. Demonstration that polyol accumulation is responsible for diabetic cataract by the use of transgenic mice expressing the aldose reductase gene in the lens. Prog Retin Eye Res. 1995;14(7):2780–2784.


