Poly(ADP-Ribose) Polymerase Is Involved in the Development of Diabetic Retinopathy via Regulation of Nuclear Factor-κB

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The current study investigated the role of poly(ADP-ribose) polymerase (PARP) in the development of diabetic retinopathy. Activity of PARP was increased in whole retina and in endothelial cells and pericytes of diabetic rats. Administration of PJ-34 (a potent PARP inhibitor) for 9 months to diabetic rats significantly inhibited the diabetes-induced death of retinal microvascular cells and the development of early lesions of diabetic retinopathy, including acellular capillaries and pericyte ghosts. To further investigate how PARP activation leads to cell death in diabetes, we investigated the possibility that PARP acts as a coactivator of nuclear factor-κB (NF-κB) in the retinal cells. In bovine retinal endothelial cells (BRECs), PARP interacted directly with both subunits of NF-κB (p50 and p65). More PARP was complexed to the p50 subunit in elevated glucose concentration (25 mmol/l) than at 5 mmol/l glucose. PJ-34 blocked the hyperglycemia-induced increase in NF-κB activation in BRECs. PJ-34 also inhibited diabetes-induced increase expression of intercellular adhesion molecule-1, a product of NF-κB–dependent transcription in retina, and subsequent leukostasis. Inhibition of PARP or NF-κB inhibited the hyperglycemia (25 mmol/l glucose)-induced cell death in retinal endothelial cells. Thus, PARP activation plays an important role in the diabetes-induced death of retinal capillary cells, at least in part via its regulation of NF-κB. *Diabetes* 53:2960–2967, 2004

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that is involved in the cellular response to DNA injury (1). DNA breaks are believed to be obligatory triggers for the activation of PARP. These DNA breaks are induced by a variety of environmental stimuli, such as oxidative and nitrosative stress. Upon encountering DNA strand breaks, PARP catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose, and then uses the latter to synthesize polymers of ADP-ribose, covalently attached to nuclear proteins, including PARP itself. When DNA damage is mild, poly(ADP-ribosyl)ation facilitates cell survival. When DNA damage is severe, PARP activation can induce cellular energetic disturbances, leading to cell dysfunction or death (2,3). Genetic disruption of PARP or pharmacologic inhibition of this enzyme has beneficial effects on inflammation, shock, stroke, myocardial ischemia/reperfusion, and prevents the onset of autoimmune diabetes (2–7).

PARP is now recognized to play a role also in the regulation of gene transcription. Several transcription factors, including nuclear factor-κB (NF-κB) (8,9), p53 (10, 11), and AP-1 (12), interact with PARP and are regulated by it. By using PARP inhibitors or knocking out PARP gene in cells or mice, both NF-κB activation and transcription of NF-κB–dependent genes, such as inducible nitric oxide synthase or intracellular adhesion molecule (ICAM)-1, can be reduced (9,13–15), suggesting that inhibition of poly(ADP-ribosyl)ation might prevent the consequences of inflammation or stress by modification of NF-κB–dependent pathways.

Evidence suggests that oxidative and nitrosative stress are greater than normal in retinas from diabetic animals (16–18), thus potentially activating PARP and contributing to the pathogenesis of diabetic retinopathy. In this study, we demonstrate that PARP activity in retina is increased in diabetes and that inhibition of PARP inhibits the development of early lesions of diabetic retinopathy. These beneficial effects of PARP inhibition are mediated at least in part via its regulation of NF-κB.

RESEARCH DESIGN AND METHODS

Type 1 diabetes was induced in fasted male rats (Lewis) with streptozotocin (60 mg/kg body wt), and animals were housed in ventilated microisolator cages. All experiments followed the guidelines set forth by the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research. Insulin was given as needed to maintain body weight and allow a slow increase in body weight while allowing hyperglycemia, polyuria, and hyperphagia (0–2 units every 2–3 days). Hyperglycemia was estimated every 2–3 months by assay of GHb using a Variant kit (Bio-Rad, Hercules, CA) and by assay of blood glucose concentration. One week after the injection of streptozotocin, diabetic rats were randomized to receive the PARP inhibitor PJ-34 (the hydrochloride salt of N-[-oxo-5,6-dihydro-phenanthridin-2-yl]-N, N-dimethylacetamide; 20 mg/kg body wt daily in food) or to remain as diabetic controls. PJ-34 is based on a modified phenanthridinone structure and is ~10,000 times more potent than the prototypical PARP inhibitors nicotinamide and 3-aminobenzamide (19). Diabetic rats and age-matched nondiabetic controls were killed at 12 and 36 weeks of treatment.

Cultured bovine retinal endothelial cells. Primary cultures of bovine retinal endothelial cells (BRECs) were established after isolating the cells

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Received for publication 3 February 2004 and accepted in revised form 12 August 2004.

C.S. holds stock in Inotek Pharmaceuticals.

BREC, bovine retinal endothelial cell; EMSA, electrophoretic mobility shift assay; ICAM, intercellular adhesion molecule; NF-κB, nuclear factor-κB; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling.

from fresh bovine eyes by homogenization and a series of filtration steps as described previously (20). A cell sorter was used to further purify the primary BRECs by using Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) uptake, and cell purity was confirmed by staining with factor VIII (Dako, Carpinteria, CA). BRECs were subsequently cultured in 5 mmol/l glucose with endothelial cell basal medium (Clonetics, Walkersville, MD) supplemented with 10% plasma-derived horse serum, 50 mg/l heparin (Sigma, St. Louis, MO), bovine brain extract (Clonetics), and 50 μg/ml endothelial cell growth factor (Clonetics). Only passage 3–6 BRECs were used in experiments. When cell populations reached 60–70% confluence, the concentration of horse serum was decreased to 2% to reduce the growth rate of the cells. Then, the cultures were incubated in 5 or 25 mmol/l D-glucose. Cells in 25 mmol/l glucose were treated with or without different doses of PJ-34 or SN-50 (Calbiochem, San Diego, CA). Cells were cultured at 37°C in 5% CO_2 and 95% air, and the media were changed every other day.

Isolation of retinal blood vessels. The retinal vasculature was isolated by two different methods because of different sensitivities of antibodies used for immunohistochemistry to fixation.

Osmotic shock method. Freshly isolated retinas from rats were incubated in distilled water (high-performance liquid chromatography grade) for 1 h, followed by brief exposure (2 min) to DNase 1 (2 mg/ml). Retinal vasculature was isolated under microscopy by repetitive inspiration and ejection through Pasteur pipettes with sequentially narrower tips. Retinal blood vessels isolated by this method showed a normal complement of nuclei and were devoid of nonvascular materials (21). The retinal vasculatures were laid out on glass slides and air dried for immunohistochemistry of PARP activity.

Trypsin digest method. Rat retinal vasculatures were isolated as described by us previously (22,23). Briefly, freshly isolated eyes were fixed with 10% neural buffered formalin. Retinas were isolated, washed in water overnight, and then incubated with 3% Difco crude trypsin (BD Biosciences, Sparks, MD) at 37°C for 1 h. Nonvascular cells were gently bushed away from the vasculature, and the isolated vasculatures were used for p50 immunostaining, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay, and assessment of pathology.

Immunohistochemistry on paraffin sections and isolated retinal microvasculature

Poly(ADP-ribose) groups. Paraffin sections (5 μm) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol, followed by rinsing with PBS (pH 7.4). These rehydrated sections or slides containing the retinal vasculature (isolated by the osmotic shock method) were treated with 3% hydrogen peroxide for 5 min. Nonspecific binding was blocked by incubating the slides for 1 h in PBS containing 2% horse serum. A monoclonal anti–poly(ADP-ribose) antibody (Alexis, San Diego, CA) was applied in a dilution of 1:400 for 1 h at room temperature. Immunoreactivity was detected with a biotinylated anti-mouse secondary antibody and the avidin-biotin-peroxidase complex, both supplied in the Vector Elite kit (Vector Laboratories, Burlingame, CA). Color was developed using the peroxidase substrate SG kit (Vector Laboratories) for 10 min. Sections were then counterstained with Nuclear Fast Red.

p50 subunit of NF-κB. Rabbit polyclonal antibody against rat p50 (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect NF-κB activation in rat retinal blood vessels. Retinal vessels isolated by the trypsin digest method were immunostained as above, except that p50 antibody was applied in a dilution of 1:50 overnight at 4°C. Approximately 350–500 capillary cells (endothelial cells and pericytes) of each sample were evaluated in a masked manner. The capillary endothelial cells (elongated nuclei shape) having p50 expression in nuclei were quantitated.

TUNEL assay in vivo. The retinal vasculature isolated by the trypsin digest method was washed extensively in PBS, and the TUNEL reaction performed (In Situ Cell Death Detection kit: fluorescein; Roche, Mannheim, Germany). For each assay, one sample of isolated retinal vessels was treated with DNase (50 units/100 μl) for 10 min to fragment DNA as a positive control. The number of TUNEL-positive nuclei was counted in all capillaries of the entire retinal vasculature. The number of TUNEL-positive cells in diabetic groups is reported relative to that in nondiabetic controls. The TUNEL assay was performed on three different occasions, and each yielded similar conclusions.

Quantitation of acellular capillaries and pericyte ghosts. After quantitation of TUNEL-positive cells, the coverslips were gently soaked from the slides. Sections were then stained with hematoxylin and periodic acid-Schiff, dehydrated, and coverslipped. Acellular capillaries were quantitated in four to seven field areas in the mid-retina (200× magnification) in a masked manner. Acellular capillaries were identified as capillary-sized vessel tubes having no nuclei anywhere along their length and were reported per square millimeter of retinal area. Tubes with a diameter <20% of the diameter of adjacent capillaries were identified as strands and not counted as acellular capillaries. Pericyte ghosts were estimated from the prevalence of spaces in the capillary basement membranes from which pericytes had disappeared. At least 1,000 capillary cells in five field areas in the mid-retina (400× magnification) were evaluated in a masked manner, and the number of pericyte ghosts was reported per 1,000 capillary cells. Ghosts on any acellular vessel were excluded.

Coimmunoprecipitation. The nuclear fraction of BRECs was isolated by the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Nuclear extracts (200 μg) were treated with agarose-conjugated control IgG for 1 h to minimize nonspecific binding. After centrifugation (3,500*g* for 5 min), the supernatant was incubated with agarose-conjugated antibodies (anti-p65 or anti-p50; Santa Cruz Biotechnology) in immunoprecipitation buffer (50 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 0.5% NP-40, and 0.5% sodium deoxycholate) overnight at 4°C. Beads were extensively washed sequentially with IP buffer, high-salt buffer (50 mmol/l Tris-HCl, pH 7.5, 500 mmol/l NaCl, 0.1% NP-40, and 0.05% sodium deoxycholate), and then low-salt buffer (50 mmol/l Tris-HCl, pH 7.5, 0.1% NP-40, and 0.05% sodium deoxycholate) for 10 min. Bound proteins were resolved by SDS-PAGE and subsequently detected by Western blot analysis for p50 (1:500 dilution), p65 (1:200 dilution), and PARP (1:2,000 dilution, Alexis).

Western blot analysis. Rat retinas were sonicated in RIPA buffer (25 mmol/l Tris, pH 7.4, 1 mmol/l EDTA, 150 mmol/l NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Proteins were fractionated by SDS-PAGE, and antibodies for ICAM-1 (1:200 dilution; Santa Cruz Biotechnology) or poly(ADP-ribose) groups (1:1,000 dilution; Alexis) were applied. After extensive washing, protein bands detected by the antibodies were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology) and evaluated by densitometry (Bio-Rad). Membranes then were stripped and reprobed with antibody against β-actin (Sigma) to confirm equal protein loading.

Electrophoretic mobility shift assay. Nuclear proteins (5 μg) were incubated with a labeled double-strand NF-κB consensus sequence (Promega, Madison, WI), and the electrophoretic mobility shift assay (EMSA) was performed according instructions provided by the manufacturer. DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel, and the bands were examined by autoradiography. Supershift assays were performed using antibodies against p50 and p65 to determine the composition of the NF-κB.

Cell death in vitro

Cell death in vitro was assessed by three different methods.

Trypan blue exclusion assay. BRECs were incubated for 5 days in 5 or 25 mmol/l glucose, with or without different concentrations of PJ-34 or SN-50. Cell death was determined by the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (vol/vol) with 0.1% trypan blue (Sigma), and the cells were counted with a hemocytometer. Cell death was defined as the percentage of blue-stained cells (dead cells) versus the total number of cells. Approximately 200–400 cells were counted in each sample, and each treatment was done in triplicate. The experiment was repeated three times with similar results.

Annexin V and propidium iodide staining. BRECs were incubated with 5 or 25 mmol/l glucose with or without PJ-34 for 3 days and then subcultured on chambered cover glasses (Fisher, Pittsburgh, PA) for 2 more days. Annexin V and PI staining was using to determine the cell death using a commercial available kit (Annexin-V-FLOUS staining kit; Roche).

Cell death detection enzyme-linked immunosorbent assay. After 4 days in 5 or 25 mmol/l glucose, with or without PJ-34, BRECs were collected. Apoptotic cell death was determined by using a commercial available kit (Cell death detection ELISA kit; Roche) reported to be selective for apoptosis.

Quantitative measurement of leukostasis. Blood was removed from the vasculature of anesthetized animals (100 mg/ml Ketaset:100 mg/ml Xylazine = 5:1) by complete perfusion with PBS via a heart catheter. Animals then were perfused with fluorescein-coupled concanavalin A lectin (20 μg/ml in PBS) (Vector Laboratories) as previously described (24,25). Flat-mounted retinas were imaged via fluorescence microscopy. The number of leukocytes adherent to the vascular wall was differentiated by location in the vasculature. The large vessels emanating from the optic nerve and their primary and secondary branches were defined as either arterioles or venular, and the remainder of vessels was called microvascular. The number of adherent leukocytes in the different locations was determined at 12 weeks of diabetes.

Statistical analysis. All results are expressed as means ± SD. The data were analyzed by the nonparametric Kruskal-Wallis followed by the Mann-Whitney test. Differences were considered statistically significant when the P values were <0.05.

RESULTS

GHb values and blood glucose in diabetic rats treated with the PARP inhibitor PJ-34 were not significantly different from those of diabetic control rats, but both diabetic

TABLE 1
Glycemia and body weights in the experimental groups

Group	*n*	Duration (weeks)	Body weight (g)	Nonfasting blood glucose (mg/dl)	GHb (%)
Nondiabetic	10	12	405 ± 27	62 ± 9	4.9 ± 1.0
Diabetic	10	12	263 ± 19	327 ± 32	10.7 ± 2.8
Diabetic + PJ-34	7	12	268 ± 20	288 ± 43	12.5 ± 1.1
Nondiabetic	28	36	575 ± 51	88 ± 26	4.0 ± 0.7
Diabetic	19	36	314 ± 45	340 ± 81	10.2 ± 1.4
Diabetic + PJ-34	19	36	268 ± 31	372 ± 78	9.9 ± 1.0

Data are means ± SD.

groups had significantly higher values than the corresponding ones in the nondiabetic control rats (Table 1). Diabetic rats were treated with insulin so that they did not lose weight, but failed to gain weight compared with nondiabetic rats. Body weights of both groups of diabetic rats remained significantly lower than those of nondiabetic control rats. Long-term administration of PJ-34 did not adversely affect the health or lifespan of diabetic rats.

Diabetes induces PARP activation in retina and retinal capillary cells. PARP activity was demonstrated using a monoclonal antibody to detect poly(ADP-ribosyl)ated proteins, the product of the enzyme. As shown in Fig. 1*A*, there was marked increase in poly(ADP-ribosyl)ation of proteins from the retinal extract of 12-week diabetic rats compared with nondiabetic controls, and this was significantly inhibited by PJ-34. Immunostaining to detect sites of PARP activity revealed that PARP activity was increased slightly in nuclei of the ganglion cell layer, inner nuclear layer, and outer nuclear layer of diabetic rats (Fig. 1*B*). PJ-34 inhibited PARP activation in each of these sites. Because diabetic retinopathy is a vascular disease, special effort was directed to assessing PARP activity within the retinal vasculature (Fig. 1*C*). Little or no PARP activity was detected in freshly isolated retinal vasculature from nondiabetic animals, whereas PARP activity was demonstrated in about one-half of all capillary endothelial cells and pericytes in the freshly isolated retinal vasculature from diabetic animals. Thus, PARP was activated in nonvascular as well as microvascular cells of the retina in diabetes.

Inhibition of PARP prevents the diabetes-induced death of retinal microvascular cells and early lesions of diabetic retinopathy. Accelerated death of capillary cells is believed to be the major cause of acellular capillary formation (26). We used the TUNEL assay to assess DNA damage and apoptosis of cells in trypsin-digested retinal vasculature preparations (Fig. 2*A*). There was a threefold increase in the number of TUNEL-positive capillary cells (endothelial cells and pericytes) in the retinas of 36-week diabetic animals compared with nondiabetic controls ($P < 0.0001$), and administration of PJ-34 prevented this increase in cell death ($P < 0.0001$ compared with diabetic controls).

The number of acellular capillaries was significantly increased in retinas from rats diabetic for 36 weeks compared with age-matched control rats (1.8-fold of control, $P < 0.0001$), and this increase was significantly inhibited by PJ-34 ($P < 0.0001$) (Fig. 2*B*). The number of pericyte ghosts was also significantly increased in retinal vessels from diabetic rats compared with age-matched control rats (2.4-fold of control, $P < 0.005$), and this increase also was significantly inhibited by PJ-34 ($P < 0.02$) (Fig. 2*C*).

The ability of PJ-34 to inhibit retinal endothelial cell death was also studied in vitro. By three different methods to assess cell death (trypan blue exclusion, annexin V staining, and cell death detection ELISA), 25 mmol/l glucose significantly increased BREC death compared with 5 mmol/l glucose (Fig. 3*A*–*C*). All of the methods showed that PJ-34 significantly inhibited the hyperglycemia-induced increase in endothelial death.

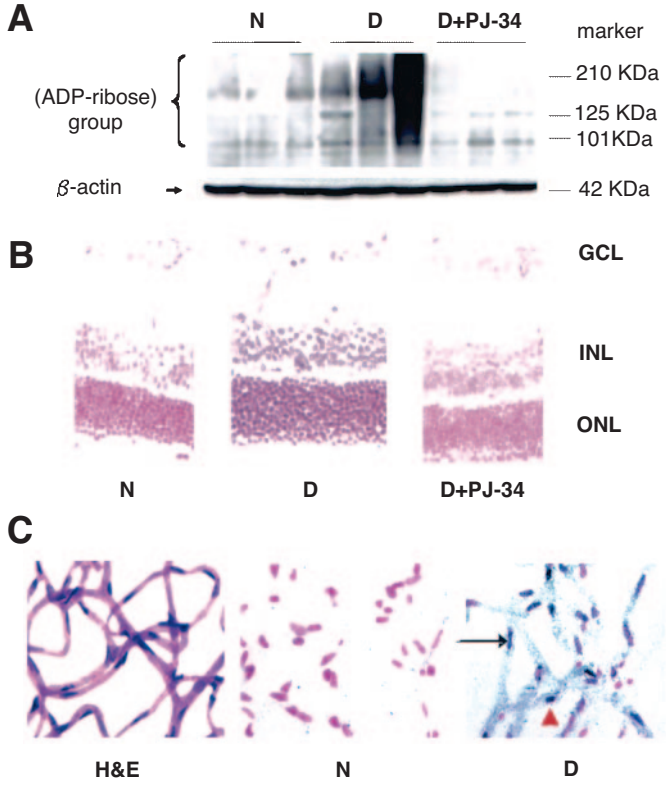

FIG. 1. PARP activation in diabetic retinas. *A*: Poly(ADP-ribosyl)ation was increased in retinas from rats diabetic for 12 weeks, and PJ-34 blocked this protein modification. N, nondiabetic rats; D, diabetic rats; D+PJ-34, diabetic rats treated with PJ-34. β-Actin is shown as a protein-loading control. *B*: PJ-34 blocked increased PARP activation in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) of diabetic rats (D). Poly(ADP-ribosy)lated proteins stained gray and nuclei stained red. N, nondiabetic rats; D+PJ-34, diabetic rats treated with PJ-34. *C*: PARP activation was evaluated in retinal blood vessels isolated from nondiabetic (N) and diabetic (D) rats. Conventionally stained blood vessels are shown on the left. An immunostained pericyte is illustrated by the red arrowhead, and a representative stained endothelial cell is indicated by the black arrow. H&E, hematoxylin and eosin.

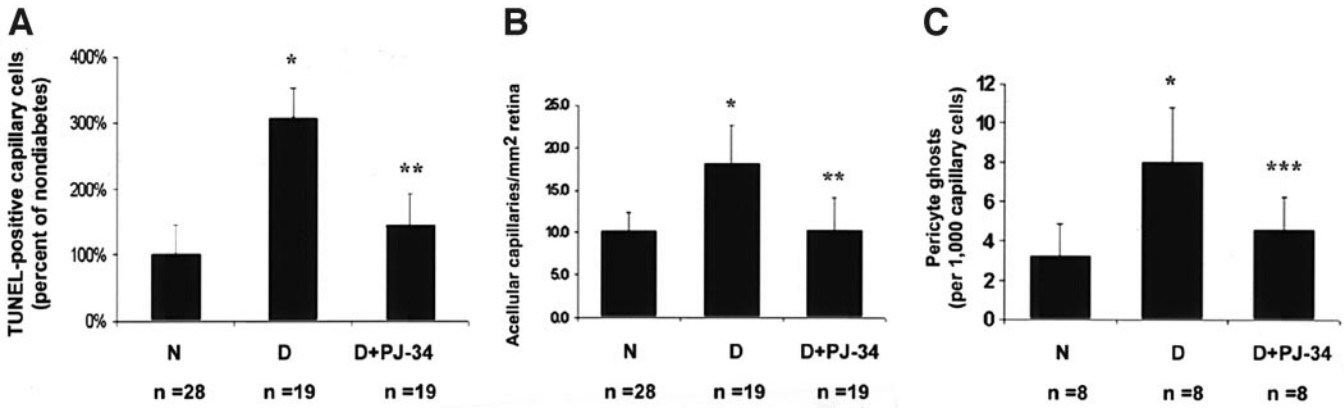

FIG. 2. PARP inhibitor inhibits retinal capillary cell death and development of lesions of diabetic retinopathy. TUNEL-positive cells (*A*), acellular capillaries (*B*), and pericyte ghosts (*C*) were counted on the isolated retinal vasculatures of rats diabetic for 9 months. TUNEL-positive cells were normalized to the average number of nondiabetic animals (5.5 ± 3.4 cells per retina), set as 100%. **P* < 0.005 vs. nondiabetic control; ***P* < 0.0001 vs. diabetic control; ****P* < 0.02 vs. diabetic control. N, nondiabetic rats; D, diabetic rats; D+PJ-34, diabetic rats treated with PJ-34.

PARP directly binds to NF-κB in cultured retinal cells. To further examine how PARP influences death of retinal capillary cells in elevated glucose, we initially focused on its ability to regulate the transcription factor, NF-κB. To test whether *PARP* was able to physically associate with NF-κB, we performed coimmunoprecipitation followed by Western blot analysis. Nuclear extracts of BRECs were immunoprecipitated with either an anti-p50 (Fig. 4*A*) or anti-p65 (Fig. 4*B*) antibody in 5 and 25 mmol/l glucose, and bound proteins were subsequently probed by Western blot using specific antibody against *PARP*. *PARP* was detected when immunoprecipitated with antibody against either p65 or p50, indicating that endogenous *PARP* and NF-κB form a complex in nuclei. More PARP was complexed to p50 at 25 mmol/l glucose than at 5 mmol/l glucose (Fig. 4*A*). In contrast, high glucose may have slightly decreased the association of PARP with the p65 subunit (Fig. 4*B*).

Inhibition of PARP inhibits the hyperglycemia-induced activation of NF-κB in retinal endothelial cells. Because PARP binds to NF-κB, we explored whether the enzyme activity of PARP was required for regulating NF-κB binding activity in retinal endothelial cells. Nuclear DNA binding activity of NF-κB in BRECs was examined by EMSA. Supershift assay demonstrated a classical p50/p65 heterodimer in this cell type (data not shown). There was increased DNA binding activity of NF-κB in cells incubated with 25 mmol/l glucose compared with that in 5 mmol/l glucose (Fig. 4*C*), and PJ-34 significantly inhibited this activation. The data suggest that poly(ADP-ribosyl)ation influences the interaction between NF-κB and DNA.

Inhibition of glucose-induced death in vitro by inhibition of NF-κB. Because PARP binds to NF-κB and regulates its activation, we evaluated whether PARP activation regulates the glucose-induced cell death via NF-κB activation. SN-50 is a cell-permeable peptide that binds to the nuclear translocation signal sequence of the p50 subunit of NF-κB, thus preventing NF-κB translocation to the nucleus. After 5 days' incubation in 5 or 25 mmol/l glucose with or without 6 μmol/l SN-50, the trypan blue exclusion assay was used to quantitate endothelial cell death. Death of BRECs incubated in 25 mmol/l glucose was significantly greater than that in 5 mmol/l glucose ($P < 0.05$), and this hyperglycemia-induced increase in cell death was significantly inhibited by SN-50 (Fig. 5) ($P < 0.05$), suggesting that endothelial cell death in elevated glucose is at least partially mediated via NF-κB activation.

NF-κB is activated in retinal endothelial cells from diabetic animals. To address whether p50 is increased in nuclei of retina endothelial cells in diabetes, we did p50 immunostaining on the trypsin-digested retinal vasculature in diabetic and nondiabetic animals. There was posi-

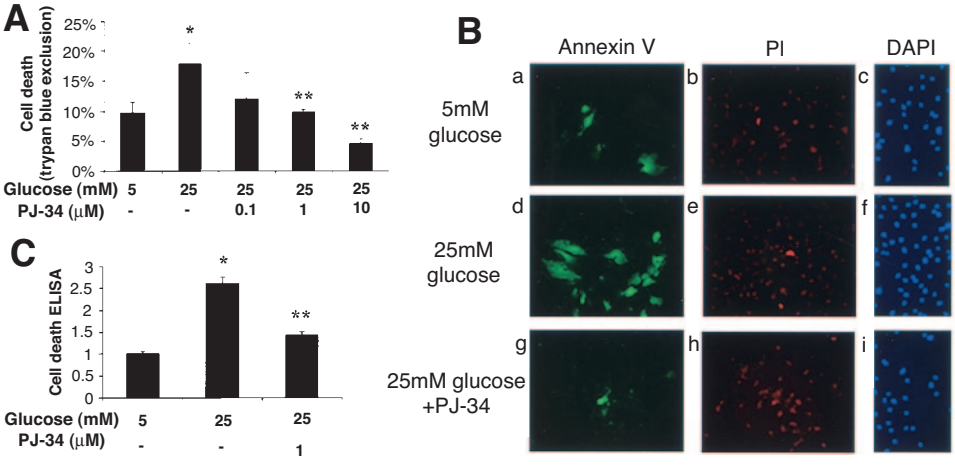

FIG. 3. PARP inhibitor inhibits high-glucose–induced endothelial cell death in vitro. *A*: Cell death detected by trypan blue exclusion assay. Cell death is reported as the percentage of cells failing to exclude trypan blue dye. Results shown are representative of three experiments. **P* < 0.05 vs. 5 mmol/l glucose; ***P* < 0.05 vs. 25 mmol/l glucose. *B*: Cell death detected by annexin V and PI staining. *a–c*: BRECs incubated with 5 mmol/l glucose. *d–f*: BRECs incubated with 25 mmol/l glucose. *g–i*: BRECs incubated with 25 mmol/l glucose treated with 1 μmol/l PJ-34. *a*, *d*, and *g*: BRECs stained with annexin V (green). *b*, *e*, and *h*: the same area stained with PI (red). *c*, *f*, and *i*: DAPI-stained nuclei (blue) demonstrate cell density. *C*: Apoptosis detected by ELISA. Cell death is reported as the fold difference relative to 5 mmol/l glucose media (i.e., cell death in 5 mmol/l glucose media set as 1). **P* < 0.05 vs. 5 mmol/l glucose; ***P* < 0.05 vs. 25 mmol/l glucose.

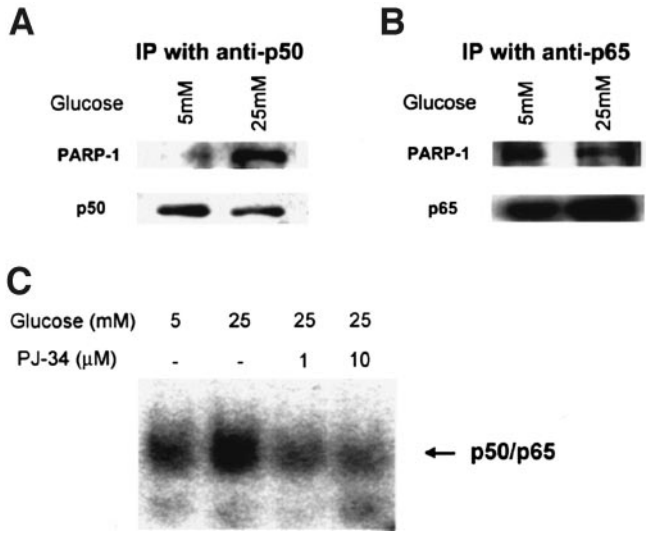

FIG. 4. PARP binds to NF-κB and regulates NF-κB activation. Nuclear proteins from BRECs incubated in 5 or 25 mmol/l glucose were incubated with agarose-conjugated anti-p50 (*A*) or anti-p65 (*B*). Coimmunoprecipitated proteins were analyzed by SDS-PAGE, followed by immunoblotting with antibody against PARP, and then with ones against p50 (*A*) or p65 (*B*). Nuclear proteins from BRECs treated with 5 mmol/l glucose, 25 mmol/l glucose, or 25 mmol/l glucose plus 1 or 10 μmol/l PJ-34 were incubated with NF-κB consensus sequence, and NF-κB DNA binding activity was monitored by EMSA (*C*).

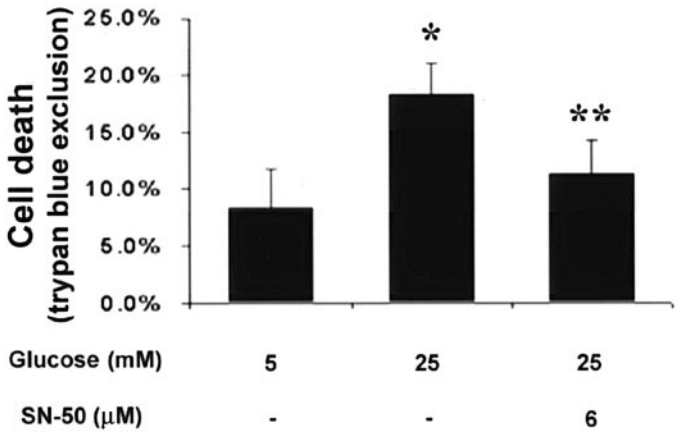

FIG. 5. NF-κB activation contributes to the death of retinal endothelial cells in high glucose. Cell death was detected by trypan blue exclusion assay **P* < 0.05 vs. 5 mmol/l glucose; ***P* < 0.05 vs. 25 mmol/l glucose.

tive p50 immunostaining on nuclei of endothelial cells and also on pericytes (Fig. 6*A*, left). Preincubation of the antibody with blocking peptide eliminated immunostaining, demonstrating that the immunostain was specific for p50 (Fig. 6*A*, right). Results are quantitated in Fig. 6*B*. Diabetes caused a 1.6-fold increase in the numbers of endothelial nuclei immunostained by p50 antibody compared with that in nondiabetic controls ($P < 0.02$).

Diabetes-induced induction of ICAM-1 and leukostasis are mediated via PARP activation. ICAM-1 expression is known to be regulated by NF-κB and plays a critical role in the diabetes-induced adherence of leukocytes to the vessel wall (27). Diabetes of 12 weeks' duration increased ICAM-1 expression 2.5-fold in the retinal extract of diabetic rats compared with nondiabetic controls (Fig. 7*A*) ($P < 0.05$). Chronic inhibition of PARP by PJ-34 significantly inhibited the upregulation of ICAM-1 in diabetic retina ($P < 0.05$), suggesting that PARP regulates ICAM-1 expression via regulation of NF-κB.

Leukostasis in the retinal microcirculation was significantly greater than normal in the arteriolar, venular, and microvascular portions of the retinal vasculature of 12-week diabetic rats ($P < 0.005$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 7*B*). Treatment of the diabetic animals with PJ-34 significantly inhibited the diabetes-induced increase in leukostasis in the retinal vasculature compared with control diabetic rats ($P < 0.005$, $P < 0.05$, and $P < 0.005$, respectively).

DISCUSSION

An early and important lesion in the development of diabetic retinopathy is death of capillary cells (26), resulting in an increased number of acellular capillaries and pericyte ghosts. Acellular capillaries are of interest because they are not perfused (28) and thus are causally related to the development of retinal ischemia and subsequent neovascularization. In the present study, we found that PARP activity regulated the diabetes-induced death of retinal capillary cells. PARP inhibition prevented both the hyperglycemia-induced death of retinal endothelial cells and the diabetes-induced increase in the number of acellular capillaries and pericyte ghosts in retina. Methods used for the in vivo (TUNEL) and in vitro (annexin V staining and ELISA) studies suggest that many of the cells dying in an elevated glucose concentration are doing so by an apoptosis-like process.

PARP inhibition or PARP deficiency is known to downregulate various mechanisms of cell death, including mitochondrial permeability transition, mitochondrial oxidant generation, and the release of the cell death mediator apoptosis-inducing factor (29,30). Recently, it was reported that PARP regulated several genes of apoptotic regulators, including caspase-1 and -3 (31). Which apoptotic pathways are activated in diabetic retinopathy and how PARP regulates them needs to be further investigated.

The mechanism by which *PARP* causes endothelial cell dysfunction could be via transcriptional regulation. In other cells (8,32,33) and now in retinal endothelial cells, PARP has been found to directly bind to subunits of NF-κB and to regulate its transcriptional activity. In vitro, we showed that diabetic-like concentrations of glucose resulted in more binding of PARP to the p50 subunit of NF-κB compared with that in normal glucose levels. We also showed that inhibition of PARP activity significantly inhibited the hyperglycemia-induced activation of NF-κB by EMSA. Our data suggest that increased DNA binding affinity of NF-κB in retinal endothelial cells exposed to elevated glucose is mediated via PARP activity, which is consistent with a prior report that the binding of p50 to DNA is dependent to poly(ADP-ribosyl)ation (33). Interestingly, others (34) recently found that *Drosophila* mutants lacking normal PARP levels display immune defects similar to those in mice lacking the p50 subunit. Increased interaction between p50 and *PARP* in elevated glucose may help assemble other coactivators of NF-κB, such as histone acetyltransferase p300 (35,36), to form a transcriptional complex and regulate NF-κB–dependent gene expression.

Retinal NF-κB is activated early in diabetes and remains activated for up to 14 months (24,37). NF-κB is apparently

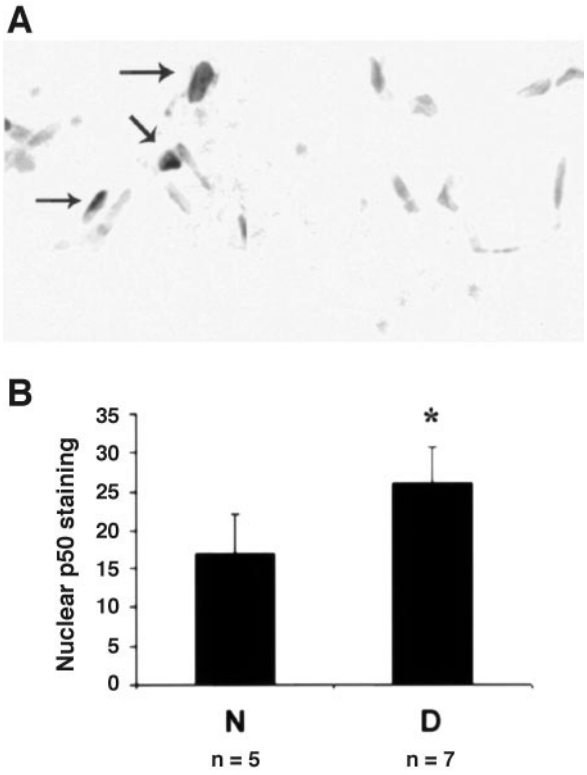

FIG. 6. Increased p50 expression in nuclei of endothelial cells. *A*: p50 immunostaining in nuclei from the retinal vasculature of a 12-week diabetic rat is shown on the left, and the lack of positive immunostaining following preincubation of the p50 antibody with p50 blocking peptide is shown on the right. Positive p50 immunostaining appears dark gray, and the counterstained nuclei appear light gray. *B*: p50-positive stained nuclei of endothelial cells were counted as described in the RESEARCH DESIGN AND METHODS and reported as stained endothelial nuclei per 100 capillary cells. *P < 0.02 vs. nondiabetic control. D, diabetic rat; N, nondiabetic rat.

activated also in the retinal vasculature in diabetes, inasmuch as there is increased accumulation of the p50 subunit of NF-κB in nuclei of retinal endothelial cells of diabetic animals. In contrast to findings with the p50 subunit, a prior report (20) demonstrated that retinal microvascular cells from diabetic humans and rats had increased p65 expression in nuclei of pericytes but not endothelial cells. More study will be necessary to understand the significance of this diabetes-induced difference in the binding of NF-κB subunits to DNA. Our in vitro studies demonstrated that hyperglycemia caused an increase in DNA binding activity of NF-κB in retinal endothelial cells, in contrast to an absence of increasing DNA binding activity of the same cell type in the prior study. The basis of this difference is not known, but data from this study and publications by Adamis et al. (27) showed that ICAM-1 (an endothelial protein regulated by NF-κB) is increased in retinas of diabetic animals, consistent with our data that NF-κB activity is increased in retinal endothelial cells in hyperglycemia.

The relative contribution of NF-κB transcriptional activity to either prosurvival or proapoptotic pathways depends on multiple factors, including intensity of the activating stimulus, cell type, and activation of other transcription factors (38–40). We report here that inhibition of NF-κB translocation to the nucleus with SN-50 inhibited death of retinal endothelial cells induced by an elevated glucose concentration in vitro, suggesting that NF-κB activation has at least some adverse effects in the retina in diabetes. These findings further support developing evidence (22,24,25,27,41–45) that inflammatory processes strongly contribute to the pathogenesis of diabetic retinopathy. Many effects of NF-κB activation are mediated via its regulation of inflammatory processes (40,46), and *PARP* also has an important role in inflammatory disorders as a coactivator of NF-κB (9).

PARP-induced activation of NF-κB likely results in at least two mechanisms that might contribute to capillary cell death in diabetes. In vivo, increased NF-κB–mediated transcription leads to increased expression of ICAM-1 on endothelial cells, resulting in excessive leukostasis in retinal vessels and possibly vaso-occlusion. Additionally, our finding that hyperglycemia-induced cell death was also regulated in vitro by PARP and NF-κB demonstrates that there is also another NF-κB–mediated route to cell death that does not require leukostasis or vaso-occlusion. PARP activation might also cause cell death independent of NF-κB activation. The best recognized of these alternate mechanisms is the depletion of NAD^+ and ATP by PARP overactivation (3). Recently, PARP has also been shown (47) to

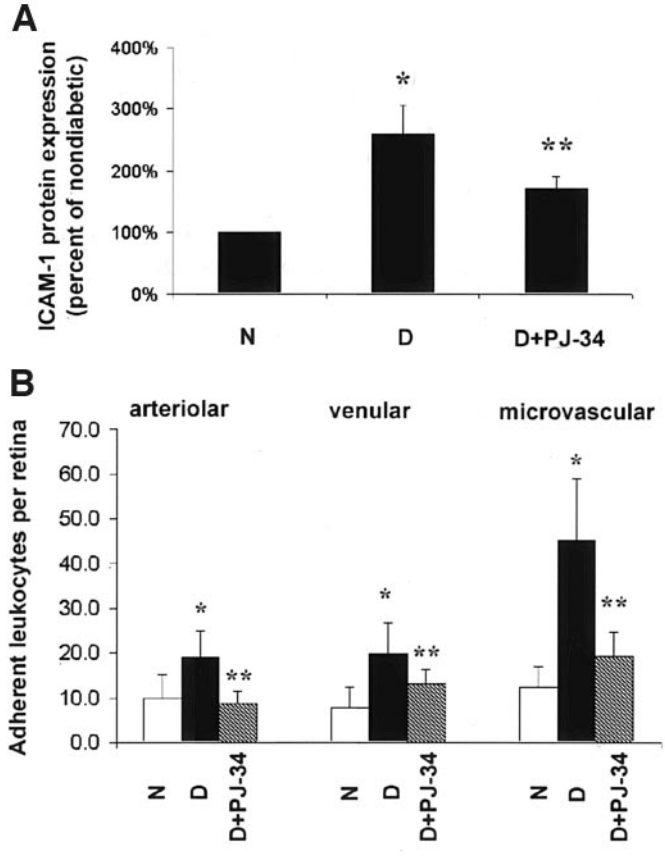

FIG. 7. PARP inhibitor prevents hyperglycemia-induced ICAM-1 expression and subsequent leukostasis. *A*: ICAM-1 protein expression was measured in retinal homogenates of nondiabetic rats (N; n = 3), diabetic rats (D; n = 3), and diabetic rats treated with PJ-34 (D+PJ-34; n = 3). Expression is normalized to nondiabetic control, set as 100%. *P < 0.05 vs. nondiabetic control; **P < 0.05 vs. diabetic control rats. *B*: Inhibition of the diabetes-induced increase in leukostasis in animals fed the PARP inhibitor for 12 weeks. Leukocyte adhesion was quantified in arteriolar, venular, and microvascular portions of the retinal vasculature. N, nondiabetic rats (n = 10); D, diabetic rats (n = 10); D+PJ-34, diabetic rats treated with PJ-34 (n = 7). *P < 0.005 vs. nondiabetic control; **P < 0.05 vs. diabetic rats.

mediate a partial inhibition of the glycolytic pathway in elevated concentrations of glucose, thus causing several metabolic abnormalities (including activation of NF-κB) that have been postulated to contribute to the development of diabetes complications.

Recent work has demonstrated the importance of PARP activation in diabetes-induced alterations in the function of macrovascular endothelium, cardiomyocytes, and neurons (48–50). Here we demonstrate that PARP activation also mediates the hyperglycemia-induced death of retinal capillary endothelial cells and development of at least the early stages of diabetic retinopathy. Our study suggests the special importance of the PARP-mediated activation of NF-κB in the pathogenesis of the retinopathy and thus offers several novel targets at which development of diabetic retinopathy might be inhibited.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Service of the Department of Veteran Affairs and the National Institutes of Health (R01EY000300, P01DK057733, and R01HL/DK71246).

We thank Dawn G. Smith for BREC isolation, Denise A. Hatala for histologic assistance, and Casey Miller, PhD, and Darlisha F. James for technical assistance. Cell culture and histology service were provided by the Case Western Reserve University Visual Science Research Center Core Facilities (P30EY11373).

REFERENCES

1. Szabo C, Ohshima H: DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide* 1:373–385, 1997
2. Pieper AA, Brat DJ, Krug DK, Watkins CC, Gupta A, Blackshaw S, Verma A, Wang ZQ, Snyder SH: Poly(ADP-ribose) polymerase-deficient mice are protected from streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* 96:3059–3064, 1999
3. Szabo C, Dawson VL: Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol Sci* 19:287–298, 1998
4. Yang Z, Zingarelli B, Szabo C: Effect of genetic disruption of poly (ADP-ribose) synthetase on delayed production of inflammatory mediators and delayed necrosis during myocardial ischemia-reperfusion injury. *Shock* 13:60–66, 2000
5. Szabo C, Cuzzocrea S, Zingarelli B, O'Connor M, Salzman AL: Endothelial dysfunction in a rat model of endotoxic shock: importance of the activation of poly (ADP-ribose) synthetase by peroxynitrite. *J Clin Invest* 100: 723–735, 1997
6. Takahashi K, Pieper AA, Croul SE, Zhang J, Snyder SH, Greenberg JH: Post-treatment with an inhibitor of poly(ADP-ribose) polymerase attenuates cerebral damage in focal ischemia. *Brain Res* 829:46–54, 1999
7. Virag L, Szabo C: The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. *Pharmacol Rev* 54:375–429, 2002
8. Kameoka M, Ota K, Tetsuka T, Tanaka Y, Itaya A, Okamoto T, Yoshihara K: Evidence for regulation of NF-kappaB by poly(ADP-ribose) polymerase. *Biochem J* 346:641–649, 2000
9. Hassa PO, Hottiger MO: The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-kappaB in inflammatory disorders. *Cell Mol Life Sci* 59:1534–1553, 2002
10. Mendoza-Alvarez H, Alvarez-Gonzalez R: Regulation of p53 sequence-specific DNA-binding by covalent poly(ADP-ribosyl)ation. *J Biol Chem* 276:36425–36430, 2001
11. Wesierska-Gadek J, Schmid G: Poly(ADP-ribose) polymerase-1 regulates the stability of the wild-type p53 protein. *Cell Mol Biol Lett* 6:117–140, 2001
12. Andreone TL, O'Connor M, Denenberg A, Hake PW, Zingarelli B: Poly-(ADP-ribose) polymerase-1 regulates activation of activator protein-1 in murine fibroblasts. *J Immunol* 170:2113–2120, 2003
13. Chiarugi A, Moskowitz MA: Poly(ADP-ribose) polymerase-1 activity promotes NF-kappaB-driven transcription and microglial activation: implication for neurodegenerative disorders. *J Neurochem* 85:306–317, 2003
14. Le Page C, Sanceau J, Drapier JC, Wietzerbin J: Inhibitors of ADP-ribosylation impair inducible nitric oxide synthase gene transcription through inhibition of NF kappa B activation. *Biochem Biophys Res Commun* 243:451–457, 1998
15. Oliver FJ, Menissier-de Murcia J, Nacci C, Decker P, Andriantsitohaina R, Muller S, de la Rubia G, Stoclet JC, de Murcia G: Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly (ADP-ribose) polymerase-1 deficient mice. *EMBO J* 18:4446–4454, 1999
16. van Reyk DM, Gillies MC, Davies MJ: The retina: oxidative stress and diabetes. *Redox Rep* 8:187–192, 2003
17. Kowluru RA, Engerman RL, Case GL, Kern TS: Retinal glutamate in diabetes and effect of antioxidants. *Neurochem Int* 38:385–390, 2001
18. Du Y, Smith MA, Miller CM, Kern TS: Diabetes-induced nitrative stress in the retina, and correction by aminoguanidine. *J Neurochem* 80:771–779, 2002
19. Jagtap P, Soriano FG, Virag L, Liaudet L, Mabley J, Szabo E, Hasko G, Marton A, Lorigados CB, Gallyas F Jr, Sumegi B, Hoyt DG, Baloglu E, VanDuzer J, Salzman AL, Southan GJ, Szabo C: Novel phenanthridinone inhibitors of poly (adenosine 5′-diphosphate-ribose) synthetase: potent cytoprotective and antishock agents. *Crit Care Med* 30:1071–1082, 2002
20. Romeo G, Liu WH, Asnaghi V, Kern TS, Lorenzi M: Activation of Nuclear factor-κB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. *Diabetes* 51:2241–2248, 2002
21. Badr GA, Tang J, Ismail-Beigi F, Kern TS: Diabetes downregulates Glut1 expression in retina and its microvessels, but not in cerebral cortex or its microvessels. *Diabetes* 49:1016–1021, 2000
22. Kern TS, Engerman RL: Pharmacologic inhibition of diabetic retinopathy: aminoguanidine and aspirin. *Diabetes* 50:1636–1642, 2001
23. Kern TS, Tang J, Mizutani M, Kowluru RA, Nagaraj RH, Romeo G, Podesta F, Lorenzi M: Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. *Invest Ophthalmol Vis Sci* 41:3972–3978, 2001
24. Joussen AM, Poulaki V, Mitsiades N, Kirchhof B, Koizumi K, Dohmen S, Adamis AP: Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. *FASEB J* 16:438–440, 2002
25. Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP: Leukocyte-mediated endothelial cell injury and death in the diabetic retina. *Am J Pathol* 158:147–152, 2001
26. Mizutani M, Kern TS, Lorenzi M: Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest* 97:2883–2890, 1996
27. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP: Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. *Proc Natl Acad Sci U S A* 96:10836–10841, 1999
28. Bresnick G, Engerman R, Davis MD, de Venecia G, Myers FL: Patterns of ischemia in diabetic retinopathy. *Trans Am Acad Ophthalmol Otolaryngol* 81:694–709, 1976
29. Virag L, Salzman AL, Szabo C: Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidant-induced cell death. *J Immunol* 161:3753–3759, 1998
30. Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL: Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* 297:259–263, 2002
31. Zingarelli B, Hake PW, O'Connor M, Denenberg A, Kong S, Aronow BJ: Absence of poly(ADP-ribose)polymerase-1 alters nuclear factor-kappa B activation and gene expression of apoptosis regulators after reperfusion injury. *Mol Med* 9:143–153, 2003
32. Hassa PO, Covic M, Hasan S, Imhof R, Hottiger MO: The enzymatic and DNA binding activity of PARP are not required for NF-kappa B coactivator function. *J Biol Chem* 276:45588–45597, 2001
33. Chang WJ, Alvarez-Gonzalez R: The sequence-specific DNA binding of NF-kappa B is reversibly regulated by the automodification reaction of poly (ADP-ribose) polymerase 1. *J Biol Chem* 276:47664–47670, 2001
34. Tulin A, Spradling A: Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at Drosophila puff loci. *Science* 299:560–562, 2003
35. Ota K, Kameoka M, Tanaka Y, Itaya A, Yoshihara K: Expression of histone acetyltransferases was down-regulated in poly(ADP-ribose) polymerase-1-deficient murine cells. *Biochem Biophys Res Commun* 310:312–317, 2003
36. Hassa PO, Buerki C, Lombardi C, Imhof R, Hottiger MO: Transcriptional coactivation of nuclear factor-kappaB-dependent gene expression by p300 is regulated by poly(ADP)-ribose polymerase-1. *J Biol Chem* 278:45145–45153, 2003
37. Kowluru RA, Koppolu P, Chakrabarti S, Chen S: Diabetes-induced activa-

tion of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. *Free Radic Res* 37:1169–1180, 2003
38. Barkett M, Gilmore TD: Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* 18:6910–6924, 1999
39. Mattson MP, Camandola S: NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Invest* 107:247–254, 2001
40. Perkins ND: The Rel/NF-kappa B family: friend and foe. *Trends Biochem Sci* 25:434–440, 2000
41. Zhang J, Gerhardinger C, Lorenzi M: Early complement activation and decreased levels of glycosylphosphatidylinositol-anchored complement inhibitors in human and experimental diabetic retinopathy. *Diabetes* 51: 3499–3504, 2002
42. Yamashiro K, Tsujikawa A, Ishida S, Usui T, Kaji Y, Honda Y, Ogura Y, Adamis AP: Platelets accumulate in the diabetic retinal vasculature following endothelial death and suppress blood-retinal barrier breakdown. *Am J Pathol* 163:253–259, 2003
43. Adamis AP: Is diabetic retinopathy an inflammatory disease? *Br J Ophthalmol* 86:363–365, 2002
44. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP: A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J* 18:1450–1452, 2004
45. Mohr S, Tang J, Kern TS: Caspase activation in retinas of diabetic and galactosemic mice and diabetic patients. *Diabetes* 51:1172–1179, 2002
46. Baldwin AS Jr: Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 107:3–6, 2001
47. Du X, Matsumura T, Edelstein D, Rossetti L, Zsengeller Z, Szabo C, Brownlee M: Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 112:1049–1057, 2003
48. Garcia Soriano F, Virag L, Jagtap P, Szabo E, Mabley JG, Liaudet L, Marton A, Hoyt DG, Murthy KG, Salzman AL, Southan GJ, Szabo C: Diabetic endothelial dysfunction: the role of poly(ADP-ribose) polymerase activation. *Nat Med* 7:108–113, 2001
49. Pacher P, Liaudet L, Soriano FG, Mabley JG, Szabo E, Szabo C: The role of poly(ADP-ribose) polymerase activation in the development of myocardial and endothelial dysfunction in diabetes. *Diabetes* 51:514–521, 2002
50. Obrosova IG, Li F, Abatan OI, Forsell MA, Komjati K, Pacher P, Szabo C, Stevens MJ: Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy. *Diabetes* 53:711–720, 2004