Activation of Nuclear Factor-κB Induced by Diabetes and High Glucose Regulates a Proapoptotic Program in Retinal Pericytes

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To reconstruct the events that may contribute to the accelerated death of retinal vascular cells in diabetes, we investigated in situ and in vitro the activation of nuclear factor-κB (NF-κB), which is triggered by cellular stress and controls several programs of gene expression. The retinal capillaries of diabetic eye donors showed an increased number of pericyte nuclei positive for NF-κB, when compared with nondiabetic donors, whereas endothelial cells were negative. Microvascular cell apoptosis and acellular capillaries were increased only in the diabetic donors with numerous NF-κB–positive pericytes. Likewise, high glucose in vitro activated NF-κB in retinal pericytes but not in endothelial cells, and increased apoptosis only in pericytes. Studies with NF-κB inhibitors suggested that in pericytes, basal NF-κB has prosurvival functions, whereas NF-κB activation induced by high glucose is proapoptotic. Pericytes exposed to high glucose showed increased expression of Bax and of tumor necrosis factor-α, which were prevented by the NF-κB inhibitors and mimicked by transfection with the p65 subunit of NF-κB, and failed to increase the levels of the NF-κB–dependent inhibitors of apoptosis. Colocalization of activated NF-κB and Bax overexpression was observed in the retinal pericytes of diabetic donors. A proapoptotic program triggered by NF-κB selectively in retinal pericytes in response to hyperglycemia is a possible mechanism for the early demise of pericytes in diabetic retinopathy. Diabetes 51:2241–2248, 2002

Activation of the transcription factor nuclear factor-κB (NF-κB) has been proposed to play a role in the vascular complications of diabetes. NF-κB is activated by oxidative stress (1), which can be caused in diabetes by high glucose (2) and advanced glycation end products (AGEs) (3). In turn, NF-κB is a pivotal mediator of inflammatory responses (4), which play a role in pathologies, such as atherosclerosis, that are highly prevalent in diabetes. To date, the studies testing NF-κB in vascular cells in relation to diabetic angiopathy all have been performed in cultured cells, mostly endothelial or smooth muscle cells derived from large vessels and exposed to high glucose (2,5–8) or AGEs (9,10) in vitro. In these models, NF-κB activation has been noted to induce proinflammatory responses (5), upregulation of endothelin (10), and apoptosis (7). It is becoming apparent that NF-κB activation may promote or inhibit apoptosis depending on the cell type and the nature of the inducing stimulus (11). Proapoptotic effects of NF-κB in diabetes would be relevant to the molecular pathogenesis of retinopathy, a vascular complication of diabetes in which both types of capillary cells—pericytes and endothelial cells—undergo accelerated apoptosis (12).

We performed this study to determine whether NF-κB activation occurs in diabetic retinal vessels. The activation of NF-κB is exquisitely posttranslational (1,4). In unstimulated cells, NF-κB dimers (most frequently composed of the p50 and p65 subunits) are sequestered in the cytosol via noncovalent interactions with inhibitory proteins called IκBs. Signals that induce NF-κB activity cause the phosphorylation and subsequent degradation of IκB proteins, allowing NF-κB dimers to enter the nucleus and induce gene expression. Hence, NF-κB activation in excised tissues can be detected by its nuclear localization (13,14), and we used this approach to examine the status of NF-κB in retinal vessels from human diabetic donors and experimentally diabetic rats. We targeted the study to the p65 subunit (RelA in the Rel/NF-κB family of polypeptides) because it is a potent activator of gene expression (1). Having observed nuclear localization of NF-κB in the pericytes but not in endothelial cells of diabetic retinal vessels, we investigated its association with microangiopathy and sought to identify mechanisms and consequences of the cell-selective NF-κB activation.

RESEARCH DESIGN AND METHODS

Human eyes. Human eyes were provided by certified Eye Banks and obtained through the National Disease Research Interchange (Philadelphia, PA). The donors remained anonymous. Criteria for selection were age <75 years, duration of diabetes <15 years to address early nonproliferative retinopathy (15), the fewest possible chronic pathologies other than diabetes, absence of retinal or hematologic diseases and uremia, and absent administration of chemotherapy or life-support measures. The eyes were fixed in 10% buffered formalin by the Eye Banks as soon as possible after the donor’s death. The characteristics of the donors are reported in Table 1; the diabetic donors likely had type 2 diabetes. In the diabetic group, the time elapsed from death to enucleation and fixation of the eyes was 3 ± 1 h and 13 ± 6 h, respectively; in
the control group, it was 4 ± 2 h and 12 ± 6 h, respectively. The eyes were processed after at least 48 h but no more than 15 days of formalin fixation. Each eyecup was cut into six sectors centered at the optic disc so that six retinal samples of similar size were obtained from each eye. Isolation of the retinal vasculature by trypsin digestion was performed according to the method of Kuwabara and Cogan (16) with some modifications (17). The digestion buffer contained 0.1 mol/l sodium fluoride to inhibit the DNase activity of the crude trypsin preparation.

**Immunohistochemistry.** Localization of the p65 subunit of NF-κB was tested in retinal trypsin digests with a rabbit polyclonal antibody raised against the aminoterminal domain of p65 (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) and with a mouse monoclonal antibody (clone 12H11; Boehringer Mannheim Biochemicals, Indianapolis, IN) raised against a peptide containing the nuclear localization sequence of p65. This antibody selectively recognizes the activated, IκB-released form of NF-κB localized to the nucleus (13) and was used at a concentration of 5 μg/ml. Negative controls received the same concentration of nonimmune rabbit IgG and mouse IgG3 (Pharmingen, San Diego, CA), respectively. Peroxidase immunohistochemistry was performed as described (18). NF-κB-κB nuclear DNA-protein complexes were resolved on a 4% nondenaturing gel and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL). DNA was stained with 4′,6-diamidino-2-phenylindole (2 μg/ml; Boehringer Mannheim, Germany). NF-κB activation was detected with rabbit anti-human IκB, raised against residues 50–206 of the human 105-kDa protein (Santa Cruz Biotechnology, Santa Cruz, CA) and with a mouse monoclonal antibody (clone 12H11; Boehringer Mannheim Biochemicals, Indianapolis, IN) raised against a peptide containing the binding sequence for transcription factor AP-1 (Promega).

**Apoptosis in cultured cells.** Annexin V binding, which we had previously used to measure BRP apoptosis in flow cytometry (18), proved unreliable for BRP, yielding up to 50% positive cells in control cultures. The outcome was attributable to a greater sensitivity to trypsinization of BREC than BRP plasma membrane, because annexin V binding in situ to BREC monolayers showed only occasional positive cells. The Cell Death Detection ELISA PLUS (Roche), which does not require trypsinization, thus was used to measure apoptosis quantitatively in BRP and BREC. This photometric enzyme-immunoassay measures mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. After separation of the cytoplasmic fraction to be used for the assay, the pellet containing cell nuclei was resuspended in high-salt buffer (2 mol/l NaCl, 0.05 mol/l NaHPO₄ [pH 7.5]), sonicated, and used for DNA measurement (21). DNA was an accurate indicator of cell density because the DNA content per cell in BRP as well as BREC cultures was not altered by high

**Data are means ± SD. CA, cancer (gastrointestinal); CAD, coronary artery disease; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident; MI, myocardial infarction.**
glucose or mannitol. Apoptosis thus was measured as absorbance units of cytoplasmic nucleosomal DNA/μg DNA.

**Transfection.** Biosynthetic consequences of NF-κB activation were studied in BRP transiently overexpressing p65. The p65 expression plasmid was produced by PCR amplification of the p65 coding region (22) and subcloning of the PCR product by blunt-end ligation into the pcDNAI/amp expression vector (Invitrogen, Carlsbad, CA). The insert was confirmed by double-stranded sequencing. The pcDNAI/amp parental vector served as the “empty vector.” Reporter plasmids pCH110 Eukaryotic Assay Vector (Amersham Pharmacia Biotech, Piscataway, NJ) and pGL2-Promoter DNA Vector (Promega) were used to monitor transfection efficiency. Cells at 50–60% confluence in 35-mm culture dishes were transfected in serum-free, antibiotic-free medium for 6 h with 7.5 μg of the p65 plasmid or the empty vector previously mixed with the TransFast Transfection Reagent (Promega) in a 1:1 ratio. At the end of the transfection period, cells were returned to complete culture medium and harvested after 24–36 h for total RNA or protein extraction. Cultures transfected with the reporter plasmids were assayed in parallel to document efficient plasmid delivery. β-Galactosidase activity (23) was visualized in cells fixed in 0.5% glutaraldehyde for 10 min at room temperature; luciferase activity was determined in lysates of transfected cells using the Luciferase Assay Substrate (Promega) in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

**RT-PCR.** Total RNA was extracted from BRP by centrifugation on cesium chloride followed by ethanol precipitation. The RT reaction was performed as described previously (24), using 1 μg of total RNA. The PCR reaction contained 10 mmol/l Tris-HCl, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.625 units of Taq DNA polymerase (Boehringer Mannheim), 0.5 mmol/l sense and antisense primer, and 5 μl of the RT reaction in a 25-μl volume. The primers for Bax, tumor necrosis factor-α (TNF-α), and cyclophilin are reported in Table 2. Cycles were 26 for Bax, 33 for TNF-α, and 23 for cyclophilin. Ten microliters of each PCR reaction was electrophoresed on 1% agarose gel, and signals were quantified with a BioRad Molecular Imager FX (Bio-Rad, Hercules, CA). The Bax and TNF-α signals were normalized for cyclophilin expression.

**Immunoblotting.** BRP protein extraction and immunoblotting were as described (18). The primary antibodies were antisera 1712 to human Bax (1:1,000) and polyclonal antibodies to the inhibitors of apoptosis cIAP-1 (1 μg/ml) and cIAP-2 (1.5 μg/ml) from R & D Systems, Minneapolis, MN.

**Statistical analysis.** Data are summarized with the mean ± SD, except for the results obtained in the studies of retinal microvessels, which are summarized with the median and range because the data were not normally distributed. These data were analyzed with nonparametric statistics. Comparisons between two groups were performed with the Mann-Whitney rank test; when three groups were compared, the preliminary Kruskal-Wallis test was followed by multiple comparisons with the Mann-Whitney test (closed testing procedure). Correlations were tested with the Spearman rank correlation coefficient. Analyses yielded identical results when performed using parametric statistics. The data from cultured cells were analyzed with the paired t test.

**RESULTS**

**NF-κB in diabetic retinal vessels and association with microangiopathy.** Human retinal vessels tested with the antibodies that detect the p65 subunit of NF-κB irrespective of its activation and localization showed diffuse staining, indicating the presence of the p65 subunit in the cytosol of retinal vascular cells. We thus studied the retinal trypsin digests of diabetic and nondiabetic donors with monoclonal antibody 12H11 to detect NF-κB activation. The brown immunoreaction was present almost exclusively in the round or oval nuclei of pericytes, characteristically protruding from the outline of the vessels (Fig. 1). The NF-κB+ nuclei of pericytes were scattered throughout the vascular network and were more frequent in the diabetic (eight per one-sixth of retina, range 0–42) than in the nondiabetic vessels (0.7 per one-sixth of retina, range 0–8; P = 0.009). The counts performed in two different trypsin digests of five diabetic and two nondiabetic donors were highly reproducible (r = 0.9). The majority of both diabetic and nondiabetic trypsin

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**TABLE 2**

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**FIG. 1.** NF-κB activation in retinal pericytes of diabetic donors. A: A trypsin digest prepared from the retina of a 69-year-old donor with a 2-year history of diabetes was tested with monoclonal antibody 12H11, and the immunoreaction was revealed by peroxidase histochemistry; no counterstain was applied. A prominent brownish reaction (arrow) identifies the characteristic eccentric nucleus of a pericyte in the absence of nonspecific background staining. A pericyte nucleus negative for NF-κB is indicated by the arrowhead. B: The same area presented in A after counterstaining with periodic acid-Schiff and hematoxylin to reveal vascular and nuclear morphology. The vessel with the NF-κB+ pericyte nucleus (arrow) as well as the other vessels appear morphologically normal. In the immediate vicinity of the NF-κB+ pericyte, the elongated endothelial cell nuclei (small arrow) are clearly negative for NF-κB immunostaining. Bar, 30 μm.
digests showed no NF-κB + endothelial cells; in both groups, the median was 0 and the range was 0–3 per one-sixth of retina.

Noticing that a group of diabetic donors had as few NF-κB + pericytes (two or fewer per one sixth of retina) as the nondiabetic donors (Fig. 2A), we examined the relationship of NF-κB activation with the severity of microangiopathy. Only the group of diabetic donors with numerous NF-κB + pericytes (Fig. 2) showed in their trypsin digests a number of TUNEL + cells—inclusive of pericytes, endothelial cells, and fragmented chromatin of uncertain cellular attribution—(15 per one-sixth of retina, range 3–38), and acellular capillaries (7.5 per mm² of retina, range 0–80) greater than the control group (P < 0.04 for TUNEL + cells and P < 0.02 for acellular capillaries). Also TUNEL + pericytes as well as pericyte ghosts were more numerous in this group (P = 0.04). We did not observe nuclei positive for both NF-κB and TUNEL. The number of TUNEL + cells correlated with that of acellular vessels (P < 0.03). The group of diabetic donors with the large number of NF-κB + pericytes and more severe microangiopathy tended to have a longer diabetes duration (10 ± 5 years) than the donors without microangiopathy (6 ± 4 years), but the difference was not significant. Other donor characteristics as well as the characteristics of the specimens were similar in the two groups.

Observations made in experimentally diabetic rats supported a relationship of NF-κB activation in pericytes with microangiopathy. In six rats with 4-month diabetes duration, time at which retinal microangiopathy is not yet detectable (12), NF-κB + pericytes were three per whole retina (range 0–3) versus 0 (range 0–1) in the six nondiabetic controls (P = 0.02). More NF-κB + pericytes were counted in the four diabetic rats with 18-month diabetes duration (9, range 4–10 vs. 1.5, range 1–2, in the nondiabetic controls; P = 0.02). As in the human specimens, endothelial cell nuclei were negative in both diabetic and nondiabetic rat retinas.

**NF-κB and apoptosis in retinal microvascular cells exposed to high glucose in vitro.** We tested whether exposure to high glucose in vitro (Fig. 3) reproduced the discordant effect of diabetes on NF-κB activation in retinal pericytes and endothelial cells. Nuclear extracts from BRP as well as BREC cultured in normal glucose showed a small degree of binding to the radiolabeled NF-κB sequence, consistent with reports of NF-κB activation by serum in cultured cells (25). NF-κB binding was substantially greater to nuclear extracts from BRP but not BREC cultured in high glucose. NF-κB activation in BRP was detected as early as 2 h after exposure to high glucose (data not shown). In BRP, only one of the complexes was supershifted by antibodies to the p65 subunit, whereas in BREC both complexes were supershifted. The p50 antibodies did not induce supershift even though they effectively immunoprecipitated a p50-p65 complex from BRP whole cell lysates (data not shown). Either the binding of bovine p50 to the antibodies does not survive the EMSA conditions or the NF-κB translocated to the nucleus in BRP and BREC does not contain the p50 subunit. Quantification of the bands containing the p65 subunit showed a threefold increase in binding to nuclear extracts of BRP exposed to high glucose for 1 week (333 ± 130% of normal glucose control, n = 5, P = 0.01) or 3 weeks (328 ± 134%, n = 4, P = 0.04) but no changes in nuclear extracts of BREC exposed to high glucose for 2 h (116 ± 25% of control), 8 h (121 ± 30%), 24 h (123 ± 33%), 48 h (142 ±

FIG. 2. Association of NF-κB activation in pericytes with retinal microvascular cell apoptosis and capillary acellularity. Trypsin digests from diabetic donors with minimal or substantial NF-κB activation, as well as digests from nondiabetic donors, were tested with the TUNEL reaction for detection of microvascular cell apoptosis and examined for the presence of acellular capillaries. All counts were performed in a masked manner. The count of TUNEL + nuclei includes nuclei of pericytes, endothelial cells, and the occasional TUNEL + chromatin with undetermined cellular attribution. Only the group of diabetic subjects with a substantial number of NF-κB + pericytes (A) showed a number of TUNEL + cells (B) and acellular vessels (C) significantly greater than the nondiabetic subjects (P = 0.006 and 0.0009, respectively).
FIG. 3. Electrophoretic mobility shift assays for NF-κB in BRP (A) and BREC (B). Confluent cultures were exposed to normal (N) or high (H, 30 mmol/l) glucose in their culture medium containing 10% serum for the indicated periods of time (h, hours; w, weeks). The left panels show that binding of nuclear proteins to the labeled NF-κB nucleotide was increased in BRP but not BREC cultured in high glucose. Binding was competed by 25-fold excess of cold NF-κB oligonucleotide but not by irrelevant AP1, and antibodies to p65 but not p50 retarded the electrophoretic migration of the NF-κB complex (arrowheads indicate the supershift). The p65-containing bands (arrows) were quantified by densitometry, and bars in the right panels show the means ± SD of the results obtained in at least three independent isolates at each time point. *P < 0.05 versus normal glucose.

56%), or 1 week (100 ± 17%) (n = 3 at all time points). Also under conditions of reduced serum (3%) and no brain extracts, BREC failed to activate NF-κB in response to high glucose. Exposure to high concentrations of galactose or mannitol did not induce NF-κB activation in BRP or BREC (data not shown).

To test the relationship of apoptosis and NF-κB activation, we compared apoptosis in BRP and BREC exposed to high glucose, and we treated BRP with NF-κB inhibitors (Fig. 4). The measurement of cytoplasmic nucleosomal DNA detected a small but significant increase in apoptosis in BRP cultured in high glucose for 1 week (134 ± 10% of normal glucose control; P = 0.02), whereas no changes were observed in BREC exposed to high glucose for 1 week (101 ± 17% of control). The NF-κB inhibitor MG262 used at a concentration (0.05 μmol/l) that did not affect the morphology of the cultures doubled the rate of apoptosis of BRP cultured in normal glucose (215 ± 75% of untreated cultures; P = 0.01) but did not further increase apoptosis in BRP exposed to high glucose (175 ± 59% of untreated cultures in normal glucose; P = 0.2 versus untreated cultures in high glucose). Similar results were obtained using a different NF-κB inhibitor, Bay 11-7082. These observations indicated that in the basal state NF-κB has prosurvival influences on BRP, but that in BRP exposed to high glucose the situation is more complex. The apparent lack of effect of NF-κB inhibitors on the apoptosis of BRP exposed to high glucose could be interpreted to indicate either that in these cells NF-κB activation does not modulate apoptosis, or that enhanced NF-κB activation by high glucose adds proapoptotic to the basal prosurvival signals and that NF-κB inhibitors reduce both sets of signals. Because the first interpretation could not be reconciled with the observations made in cultures grown in normal glucose, we tested the second possibility.

**NF-κB and regulators of apoptosis in cultured BRP.** Previous studies had shown that high glucose can increase the expression of Bax (18,26), a proapoptotic member of the Bcl-2 family, and of TNF-α (27), a proinflammatory cytokine that under certain conditions can induce apoptosis (28). We examined the Bax and TNF-α mRNA levels in BRP exposed to high glucose and whether NF-κB activa-
the expression of Bcl-XS (proapoptotic) or Bcl-X L (antiapop-
the twofold c-IAP2 increase in BRP transfected with the p65
A and B: The FITC-conjugated secondary antibodies detect localization of NF-κB to the round, pro-
that NF-κB indeed transactivates Bax and TNF-α in BRP
That NF-κB is not prominent in other retinal vasculopathies (32). The
Bax indeed transactivates Bax and TNF-α in BRP exposed to high glucose for 1 or 3 weeks as measured by
A and B: The FITC-conjugated secondary antibodies detect localization of NF-κB to the round, pro-
To probe further the effect of high glucose and NF-κB on
A and B: The FITC-conjugated secondary antibodies detect localization of NF-κB to the round, pro-
pericyte nuclei (18), the
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to capture a possible colocalization of activated NF-κB
the absence or presence of MG262 (MG) for 48 h, as
by RT-PCR. High glucose but not equimolar mannitol causes
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Bax overexpression in human retinal vessels. In the
to the round, protruding nuclei of some pericytes, and the Cy3-conjugated antibodies
Bax staining was often localized in correspondence of NF-κB+ pericyte nuclei (Fig. 6). Because we
we had previously observed that in the retinal vessels of
diabetic patients intense Bax immunoreactivity sur-
capillaries are patent (30). In reality,
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endothelial cells are also lost from diabetic retinal vessels, as documented by their early apoptosis (12) and the development of acellular capillaries (30). Even if the loss of pericytes is only apparently selective and probably explained by the fact that these cells do not replicate in the adult retina (33), it remains possible that the modalities with which pericytes sense, respond, and eventually succumb to diabetes differ from those of endothelial cells. This study has uncovered that differences do in fact exist, and it begins to chart the events accounting for the loss of pericytes in diabetic retinopathy.

Only retinal pericytes, not endothelial cells, showed NF-kB activation in the presence of high glucose in vivo as well as in vitro. The observations in retinal vessels could potentially reflect a longer-lasting activation of NF-kB in pericytes, facilitating detection. However, the studies in vitro failed to detect early transient activation in endothelial cells. None of the previous studies showing NF-kB activation by high glucose or AGEs in cultured endothelial cells had been performed in endothelial cells from retinal vessels. Endothelial cells in general are known to be endowed with a multiplicity of mechanisms to limit NF-kB activation and its proinflammatory and proapoptotic consequences (34), and in retinal endothelial cells such mechanisms may be especially active as means of protecting the barrier function of vessels that serve neural tissue. Pericytes join instead the smooth muscle cells of larger vessels in responding to high glucose in vitro with prolonged NF-kB activation, although the subunit composition of the NF-kB complexes formed in aortic smooth muscle cells (8) may not be identical to that observed in pericytes. A role for oxidative stress was proposed for the effects of high glucose on smooth muscle cells (8) and will need to be investigated in pericytes. Of note, high concentrations of galactose in vitro did not induce NF-kB activation in BRP. Our previous in vivo studies using aminoxydianine had suggested that galactose may affect retinal microvascular cells through mechanisms different from those of high glucose (19).

The association of NF-kB+ pericytes with microvascular cell death and acellular capillaries in the retina of diabetic donors suggested that NF-kB activation induced by diabetes could have proapoptotic consequences. Although the association might alternatively reflect the presence of NF-kB–inducing stimuli in retinas with microangiopathy, the NF-kB+ pericytes found in the retinal vessels of rats with duration of diabetes too short to have caused vascular histopathology (12) were supportive of NF-kB with duration of diabetes too short to have caused vascular histopathology preceding microangiopathy. A more direct association between NF-kB activation and apoptosis was established by the studies in BRP and BREC cultured in high glucose. A causal relationship between the two events was tested using NF-kB inhibitors, but the interpretation of results had to take into account a substantial enhancement of apoptosis in control BRP treated with the inhibitors. MG262 inhibits the proteasome and thus may cause apoptosis by reducing the degradation of p53 (35). However, p53 accumulating under such conditions seems to be sequestered in the nucleolus, with reduced potential for transactivation of its target genes (36). In fact, the levels of Bax—one of the mediators of p53-induced apoptosis (37)—were suppressed rather than increased by MG262 in our cultures. Furthermore, the effects of MG262 were mimicked by Bay 11-7082, which does not inhibit the proteasome. It thus is reasonable to attribute the enhanced apoptosis of control BRP treated with NF-kB inhibitors to withdrawal of NF-kB–dependent survival signals. Several classes of NF-kB target genes are antiapoptotic (11), and future studies will address which among them are most important for the survival of retinal pericytes. On the basis of the increased apoptosis in control BRP, the unchanged rates of apoptosis when the NF-kB inhibitors were tested on BRP cultured in high glucose could not be taken to indicate that in these cells NF-kB was irrelevant in the regulation of apoptosis. The possibility that high glucose had already blunted the very survival signals targeted by the inhibitors was not favored because the levels of molecules known to be involved in NF-kB–mediated antiapoptosis—IAps and Bcl-XL (11)—were not decreased in BRP exposed to high glucose (this work and ref. 18). We thus entertained a third possibility, that whereas basal NF-kB activity in BRP is prosurvival, the activation occurring in the presence of high glucose is proapoptotic.

Indeed, BRP cultured in high glucose showed increased expression of Bax, which was prevented by NF-kB inhibitors, and failed instead to upregulate the expression of IAPs, prototypical antiapoptotic targets of NF-kB. A role for NF-kB in Bax upregulation was further confirmed in BRP overexpressing the p65 subunit. The Bax promoter contains an imperfect NF-kB consensus sequence (38), and our observations indicate that Bax should now be added to the known proapoptotic effectors of NF-kB activation in selected contexts (11). Also TNF-α expression was increased in BRP by high glucose and p65 transfection. TNF-α can generate both proapoptotic (28) and proinflammatory (39) signals, and because it is a secreted cytokine, it could affect cells beyond the cells of origin. For example, if in diabetic vessels pericytes with activated NF-kB produce excess TNF-α, then the cytokine could reach neighboring endothelial cells and change their phenotype to proinflammatory and procoagulant (39), contributing to capillary occlusion and endothelial cell apoptosis. This construct is speculative, and the leukostasis (40) and microthrombosis (41) that occur in diabetic retinal vessels and are associated with endothelial apoptosis (41) may be triggered by abnormalities originating in the endothelial cells themselves and/or in circulating leukocytes and platelets.

A firm conclusion from our work is that retinal pericytes undergo changes in diabetes that do not occur in endothelial cells and are proapoptotic. The NF-kB activation, increased Bax expression, and apoptosis found in the pericytes of diabetic retinal vessels and recapitulated in pericytes exposed to high glucose in vitro identify a pathway whereby hyperglycemia results in the pericyte ghosts of diabetic retinopathy.

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REFERENCES