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High glucose evokes an intrinsic proapoptotic signaling pathway in mesangial cells

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High glucose evokes an intrinsic proapoptotic signaling pathway in mesangial cells.

Background. In response to chronic hyperglycemia, microvascular cells undergo stress and injury, which can lead to cell death. We characterized a proapoptotic signaling pathway whereby high glucose evokes an intrinsic, caspase-9–dependent mechanism of cell death in human mesangial cells.

Methods. Biochemical (caspase activity, cytochrome-c release, etc.) and morphologic (chromatin condensation and nuclear segmentation) features of apoptotic cell death were assessed in cultured human mesangial cells exposed to high glucose, a risk factor for mesangial cell injury and diabetic glomerulosclerosis. Proapoptotic signaling was also analyzed in the *db/db* murine model of kidney injury in diabetes.

Results. Incubation in high glucose caused cytotoxicity and apoptosis in mesangial cells. High glucose stimulated mitochondrial release of cytochrome-c, cleavage of procaspase-9, and caspase-9 enzyme activity, suggesting an intrinsic pathway of proapoptotic signaling. In contrast, caspase-8 was unaffected by high glucose. A cell-permeable, caspase-9-selective inhibitor blocked caspase-3 activation and prevented chromatin condensation and nuclear segmentation in cells treated with high glucose. To determine whether an intrinsic signaling pathway occurs in the diabetic kidney in vivo, apoptosis was investigated in diabetic 8- and 16-week *db/db* murine kidneys. Effector caspases-3 and -7 were activated in diabetic *db/db* kidneys but not in age-matched nondiabetic *db/m* controls. At 16 weeks, apoptotic cells in *db/db* glomeruli were identified on the basis of nuclear segmentation and DNA fragmentation. Apoptosis of glomerular cells correlated with expansion of the mesangial matrix and with worsening of albuminuria. Consistent with an intrinsic signaling pathway, caspase-9 cleavage was elevated only in *db/db* kidneys, whereas activation of caspase-8 and caspase-12 was undetectable.

Conclusion. These findings support the hypothesis that hyperglycemia evokes an intrinsic pathway of proapoptotic signaling in mesangial cells. In addition, these results point to an important role for the intrinsic pathway in microvascular injury in the diabetic kidney in vivo.

Key words: hyperglycemia, mesangial cells, diabetic glomerulosclerosis.

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Microvascular injury occurs in all forms of diabetes and is thought to be caused principally by hyperglycemia (reviewed in [1–4]). Diabetes-specific microvascular injury in the kidney occurs in the glomerulus, which is an important step in developing diabetic nephropathy. In the renal glomerulus, mesangial cells are prone to hyperglycemia-induced cell stress and injury [5–8]. Several mechanisms have been proposed to explain mesangial injury by hyperglycemia, including hyperfiltration, abnormal vascular permeability, production of inflammatory cytokines and growth factors, formation of advanced glycation end products (AGEs), and increased oxidative stress. More recently, chronic hyperglycemia has been proposed to cause mesangial injury by evoking pathways leading to apoptotic cell death. Indeed, hyperglycemia-induced apoptosis might be an important pathogenic factor in other forms of microvascular injury in diabetes [1, 2, 4].

Although the ability of hyperglycemia to induce apoptosis in mesangial cells has not been widely studied, Kang et al [9] reported that high glucose stimulates caspase-3 cleavage and DNA fragmentation in cultured human mesangial cells. Apoptosis of cultured mesangial cells is induced by AGEs [10], which have been implicated in hyperglycemia-induced diabetic microvascular complications. Mesangial cell apoptosis in these studies was attenuated by the antioxidant N-acetylcysteine, implicating oxidative stress induced by the AGEs [10]. Two additional hyperglycemia-related diabetic stressors, methylglyoxal [11] and transforming growth factor-β (TGF-β) [12], have also been shown to induce apoptosis in cultured mesangial cells.

Clinical studies in patients with type 2 diabetic nephropathy report a loss of glomerular mesangial cells and podocytes that correlates with progression to diabetic glomerulosclerosis [13–16]. Depletion of mesangial cells has been noted in segments of the glomerular tuft where expansion of the mesangial matrix has occurred [8, 15, 17–19]. In one study, the loss of mesangial cells even correlates with the development of incipient nephropathy and with significant structural changes resulting in

the loss of single nephron K_f [15]. These clinical studies did not address the mechanisms of glomerular cell death, but other studies of microvascular injury in diabetes have associated hyperglycemia with apoptotic death of microvascular cells in diabetes [1, 2, 4]. So it seems possible that hyperglycemia-induced apoptosis might contribute to mesangial cell injury, but the pathways of apoptotic signaling evoked by hyperglycemia are unclear.

In this study, we investigated the pathways of proapoptotic signaling activated in mesangial cells exposed to high glucose. We report here that high glucose causes apoptosis in human mesangial cells and activates a caspase-9–dependent intrinsic pathway of proapoptotic signaling. In addition, we observed an intrinsic pathway of apoptotic signaling in *db/db* diabetic mouse kidneys in vivo and apoptosis of cells in the glomerular microvasculature. Taken together, these results suggest an important role for hyperglycemia-induced proapoptotic signaling in microvascular cell injury in diabetes.

METHODS

Reagents

Antibodies used in these studies for Western blotting were from Cell Signaling (Beverly, MA, USA) except where indicated: caspase-3 active fragment (9661), caspase-3 total (9668), caspase-7 active fragment (9491), murine-specific pro- and active fragment caspase-9 (9504), human-specific pro- and active fragment of caspase-9 (9502), murine-specific pro- and cleaved caspase-12 (2202), Bax (2772), human cytochrome-c (4272), murine and human pro- and active fragment caspase-8 (55932) (BD Biosciences San Diego, CA, USA), and β-actin (A5316) (Sigma, St. Louis, MO, USA). The cell-permeable caspase-9 selective inhibitor, Z-LEHD-FMK, was from R&D Systems (Minneapolis, MN, USA).

Human mesangial cell culture and viability assays

Human mesangial cells, purchased from Cambrex Bioscience, Inc. (Walkersville, MD, USA), were maintained in Dulbecco's modified essential medium (DMEM) (11885) (Gibco-BRL, Gaithersburg, MD, USA), supplemented with 17% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 5 ng/mL selenite, and 5 μg/mL each of insulin and transferrin. Characterization was performed by phase-contrast microscopy and by immunostaining for intermediate filaments and surface antigens as described previously [20, 21]. Briefly, cells were positive for desmin, vimentin, and myosin, but did not stain for factor VIII, keratin, or common leukocyte antigen. For glucose activation, cells were plated in 60 mm plates in the DMEM containing 17% FBS. After 24 hours, the medium was changed to DMEM/5% FBS with 5.5 mmol/L glucose, 30 mmol/L glucose, or 30 mmol/L mannitol and cells were incubated for the time indicated. If necessary, the medium was changed every 48 hours to maintain the appropriate concentration of glucose. For cell viability assays, cells in 24-well plates were exposed to normal (5.5 mmol/L) or high glucose (30 mmol/L) exactly as described above, and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reduction was measured for 30 minutes at 37°C using a protocol from the manufacturer (G3580) (Promega, Inc., Madison WI, USA) and recording the absorbance at 490 nm in 96-well plates using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale CA, USA). Wells with media alone served as the blank. Release of lactate dehydrogenase (LDH) into the media was measured enzymatically (Promega).

Assessment of apoptosis in cultured human mesangial cells

Western blotting of active caspase fragments in human mesangial cells was performed as previously reported by us with minor modification [22]. Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped in CHAPS extraction buffer [50 mmol/L Pipes/HCI, pH 6.5, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% CHAPS, 20 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL aprotinin, 5 mmol/L dithiothreitol (DTT), 2 mmol/L sodium pyrophosphate, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF]. An aliquot was saved for protein determination using the DC assay (Bio-Rad, Hercules, CA, USA). An equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer was immediately added to the extraction buffer, and Western blotting was carried out using antibodies for human caspase-9, caspase-8, and the active fragments of human caspase-3. As previously described [23], the Western blots were analyzed by densitometry in NIH Image by normalizing values for the relevant caspase fragment to the highest value within each experiments (maximum level = 1). To measure caspase-9 enzyme activity, human mesangial cells were lysed (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 1.0% Triton X-100) and equivalent amounts of total human mesangial cells protein were added to a buffer containing the LEHD caspase-9 peptide substrate linked to a cleavable luciferase substrate, aminoluciferin (Promega). The amount of light produced in a coupled reaction with luciferase was measured in a Berthold Luminometer (Berthold, Bad Wilbad, Germany). To quantify the number of pyknotic nuclei, a cardinal feature of apoptotic cells, human mesangial cells on coverslips were washed once with PBS and fixed for 20 minutes with freshly prepared 3.7% formaldehyde/20% sucrose in PBS. After washing twice with PBS

the human mesangial cells were stained with 5 μg/mL Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and mounted in Slow Fade Light (Molecular Probes). The number of pyknotic nuclei were expressed as a percentage of the total number of nuclei counted (N = >300 nuclei per condition). To analyze cytochrome-c redistribution, human mesangial cells were fractionated into cytosol and membrane fractions using 0.05% digitonin in an isotonic sucrose buffer exactly as described by Dong et al [24]. Because cytochrome-c release occurs mostly from mitochondria, Western blot analysis of cytosol and membrane fractions is expected to reflect cytochrome-c translocation from mitochondria to the cytoplasm.

Studies in *db/db* mice: Diabetes and renal structure/function measurements

This study protocol was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University according to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Male *db/db* (BKS.Cg-*m*+/+*Lepr*^{db}) and *db/m* mice were used for all studies (Jackson Laboratories, Bar Harbor, ME, USA). Mice were housed in a sterile environment with 12-hour/12-hour light/dark cycles and had free access to food and water. Blood glucose was monitored using an Accu-Chek meter (Roche Diagnostics, Indianapolis, IN, USA). To measure 24-hour albumin excretion, mice were placed in individual mouse diuresis cages (Nalgene, Rochester, NY, USA) with access to water but no food for 24 hours. Following 24-hour ad libitum access to food and water, the mice were anesthetized and serum was collected and frozen at −40°C. Kidney tissue was rapidly processed for histology or Western blotting as described below. Serum creatinine was measured using a kinetic micro-Jaffé reaction (10 μL sample) with care taken to measure only the initial reaction rate to minimize interference by glucose [25]. However, this assay is biased by the presence of unidentified noncreatinine chromagens in mice [25]. Urinary albumin concentration was measured with an enzyme-linked immunosorbent assay (ELISA) specific for mouse albumin (Exocell, Philadelphia, PA, USA). At the time of sacrifice, sagittal sections of kidney were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm. Morphometric analysis of renal structure was conducted using previously established techniques [8, 26]. The mesangial matrix fraction was analyzed in coded sections stained with periodic acid-Schiff (PAS) reagent. Twenty glomeruli from each of three sections per mouse were imaged. Digital images for analysis and for photomicroscopy were acquired with a SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA).

In vivo measurements of apoptosis in the glomeruli of *db/db* and *db/m* mice

Several well-established techniques were used to identify apoptosis in tissue sections [27–29]. First, light microscopy was used to identify changes characteristic of apoptotic cells in standard hematoxylin-eosin sections. These changes include condensation of nuclear chromatin to sharply delineated granular masses along the perimeter of the nuclear envelope (also known as crescent formation), convolution of the cellular and nuclear outlines with general cell shrinkage, and segmentation of the nucleus. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) (R&D Systems) was used to identify nuclei with DNA fragmentation characteristic of apoptotic cells. The manufacturer's instructions were followed except that 20 μg/mL of proteinase K (161519) (Roche Diagnostic) for 30 minutes at 37°C was used to achieve adequate permeability of reagents into the nuclei of glomeruli. All measurements of TUNEL staining in *db/db* kidneys were conducted concurrently with identically processed *db/m* controls. We also performed unlabeled experimental controls [i.e., omission of terminal deoxynucleotidyl transferase (TdT) enzyme] and positive controls with nuclease-treated sections in each assay. The number of TUNEL-positive glomerular cells were counted blindly in 20 glomeruli from each of three separate sections from each mouse. The number of TUNEL-positive specific glomerular cells was averaged from the individual glomerular counts (i.e., $N = 60$ glomeruli per mouse).

Western blotting for active caspase fragments in *db/db* kidney extracts

Western blotting for proteins in *db/db* kidneys was performed essentially as previously described [23]. Kidney homogenates (15% wt/vol) were prepared in CHAPS extraction buffer and centrifuged at 2000 × *g* for 10 minutes at 4°C. Protein content in the supernatant was assayed with the DC protein assay. An aliquot of the lysate (25 μg protein) was boiled in SDS sample buffer, resolved on a 4% to 12% SDS-polyacrylamide gel electrophoresis (PAGE) gradient gel, and transferred to a 0.2 μm nitrocellulose membrane. After blocking in 5% nonfat dried milk in TBS-T (20 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, and 0.05% Tween 20) for 1 hour, the membrane was washed 3 times with TBS-T for 5 minutes each and incubated overnight at 4°C with primary antibody (1:1,000) in 3% bovine serum albumin (BSA) in TBS-T. After incubating with suitable horseradish peroxidase-labeled secondary antibody (1:2,000) and extensive washing, the proteins were detected by chemiluminescence with an average exposure ranging from 10 to 30 seconds. Analysis

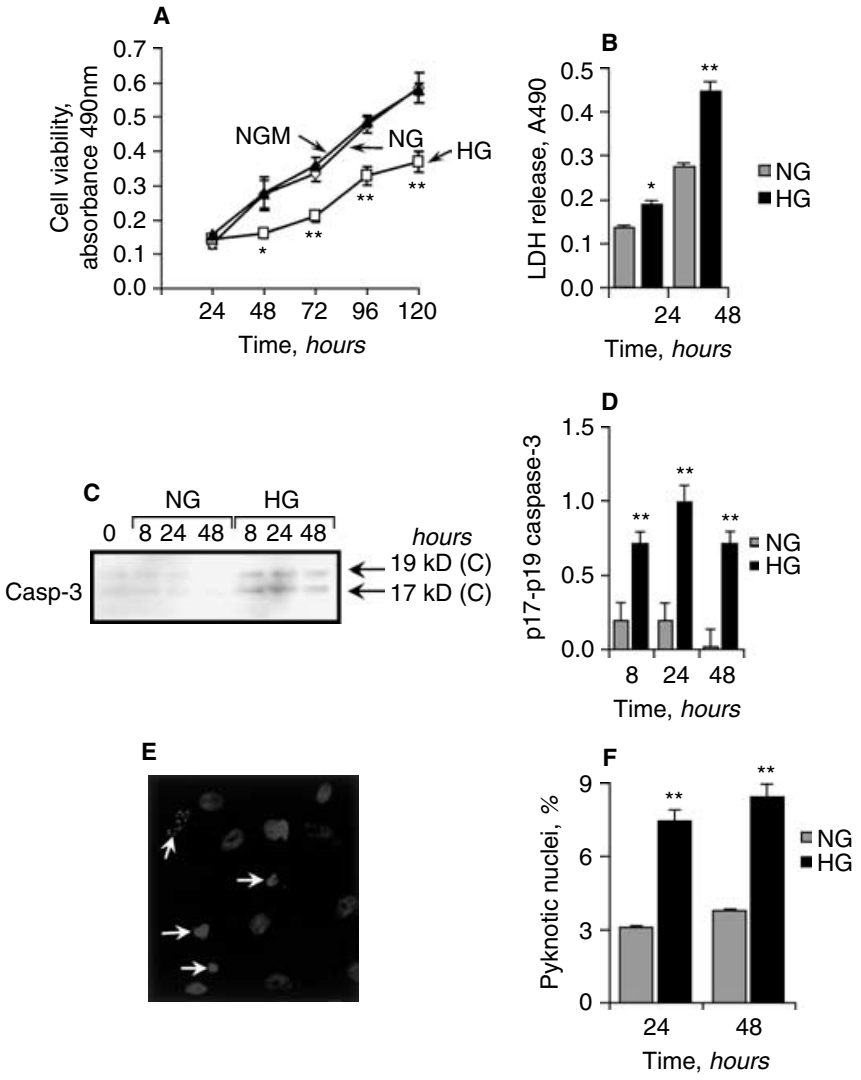

Fig. 1. Cytotoxicity and apoptosis in human mesangial cells treated with high glucose. (*A*) Human mesangial cells were plated in Dulbecco's modified essential medium (DMEM) containing 17% fetal bovine serum (FBS) for 24 hours, after which the medium was changed to 5% FBS and normal glucose (NG) (5.5 mmol/L), high glucose (HG) (30 mmol/L), or normal glucose plus 30 mmol/L mannitol (NGM) were added. The media was changed every 2 days to maintain the appropriate concentrations of glucose or mannitol. At the times indicated, cell viability was measured in an MTS assay. (*B*) Lactate dehydrogenase (LDH) release into the media by human mesangial cells treated with normal glucose or high glucose. (*C*) Western blots for the active fragments of human caspase-3 (p17 and p19 kD) in human mesangial cells incubated with normal glucose or high glucose. (*D*) Densitometric analysis of the combined p17 and p19 caspase-3 levels from three experiments. (*E*) Photomicrograph illustrating morphology of pyknotic nuclei (arrows) (i.e., nuclear condensation and segmentation) revealed by Hoechst 33342 staining in human mesangial cells treated with high glucose for 48 hours (magnification ×600). (*F*) Percentage of pyknotic nuclei identified in human mesangial cells incubated with normal glucose or high glucose. (A, B, and D) are mean ± SEM from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ by *t* test vs. normal glucose at same time point. (F) Three experiments. $^{**}P < 0.01$ by χ^2 test vs. normal glucose.

of the results by densitometry using NIH Image was as described [23].

RESULTS

High glucose stimulates apoptosis in human mesangial cells

Hyperglycemia is a major risk factor for mesangial cell injury in vivo, so we determined if hyperglycemia stimulates apoptosis of cultured human mesangial cells. As shown in Figure 1A, the proliferative capacity of human mesangial cells was significantly impaired in media containing high glucose (30 mmol/L) to mimic hyperglycemia compared to cells cultured in normal glucose (5.5 mmol/L). After 48 hours of 30 mmol/L glucose, human mesangial cell proliferation was significantly depressed, an effect that was observed up to 120 hours of treatment with 30 mmol/L glucose (Fig. 1A). Treatment of human mesangial cells with mannitol, to control for possible nonspecific effects of changes in osmolarity on human mesangial cell viability, demonstrated that the decrease in proliferation by high glucose was not due to the change in osmolarity of the culture medium (Fig. 1A). Release of LDH into the media, a marker of cytotoxicity, was significantly increased by high glucose compared to normal glucose (Fig. 1B). The conclusion from these studies is that high glucose reduces the proliferative capacity and viability of cultured human mesangial cells.

We next asked whether high glucose causes apoptosis in cultured human mesangial cells. The caspase family of cysteine acid proteases are key regulators of apoptosis. When activated by apoptotic stimuli, the initiator caspases (8, 9, 10, and 12) cleave and activate downstream effector caspases (including 3, 6, and 7). Using an antibody that selectively detects the p17 kD active fragment of caspase-3 in Western blots, we found that high glucose activated caspase-3 at 8, 24, and 48 hours compared to normal glucose (Fig. 1C and D). In addition, high glucose increased the formation of pyknotic nuclei after 24 and 48 hours compared to incubation in normal glucose

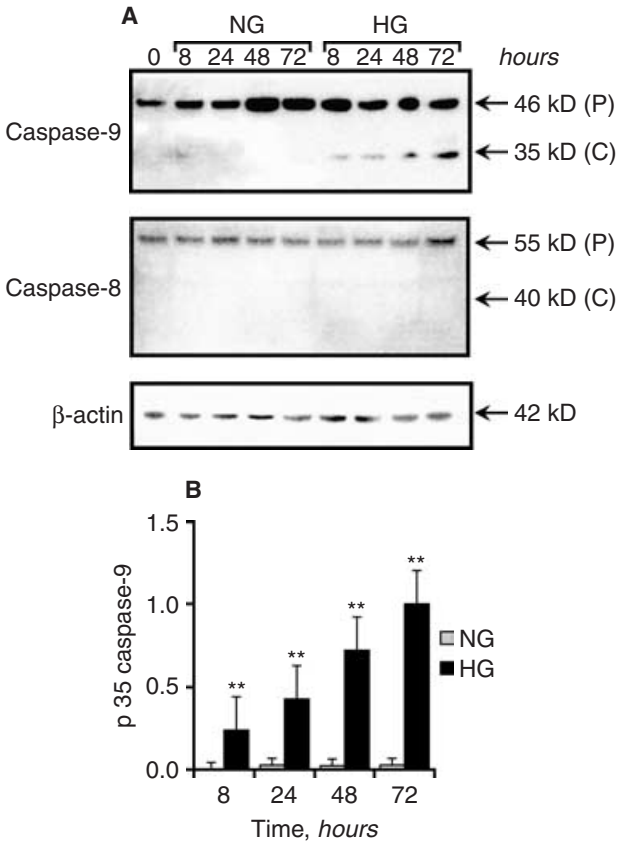

Fig. 2. High glucose stimulates caspase-9 cleavage in cultured human mesangial cells. (*A*) Human mesangial cells treated with normal glucose (NG) or high glucose (HG) were harvested, and equivalent amounts of cell protein (P) were analyzed by Western blotting for the cleaved (C) fragments of human caspase-9 (p35 kD) and caspase-8 (p40 kD). (*B*) Densitometric analysis of active caspase-9 fragment levels in Western blots from three independent experiments. $^{**}P < 0.01$ by *t* test vs. normal glucose at same time point.

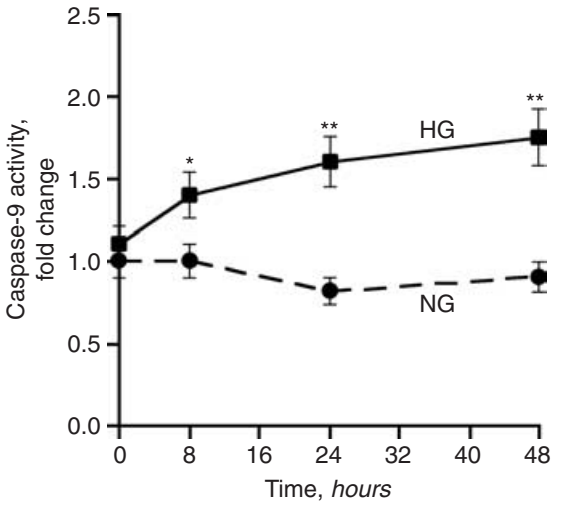

Fig. 3. High glucose increases caspase-9 enzyme activity in human mesangial cells. Cells incubated in high glucose (HG) for the times indicated were lysed, and caspase-9 enzyme activity (i.e., LEHDase activity) was measured using equivalent amounts of human mesangial cell protein. Enzyme activity measured as relative light units was normalized relative to caspase-9 activity at 0 time in normal glucose. $^{*}P < 0.05$; $^{**}P < 0.01$ by one-way analysis of variance (ANOVA) with Bonferroni post hoc test vs. control (three independent experiments in duplicate).

(Fig. 1E and F). Thus, using two established criteria for apoptotic cell death [27–29], we conclude that high glucose stimulates apoptosis in cultured human mesangial cells.

Proapoptotic signaling in human mesangial cells treated with high glucose

Diverse pathways of apoptosis regulation have been proposed, but two canonical pathways are the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway [30–32]. To identify the apoptotic signaling pathways activated by high glucose in human mesangial cells, Western blotting for the active fragment of caspase-9 and caspase-8 was performed. High glucose stimulated caspase-9 cleavage and release of the p35 kD active fragment in cultured human mesangial cells (Fig. 2). The p35 kD caspase-9 active fragment was detectable at 8 hours and increased progressively up to 72 hours (Fig. 2B). Cells cultured in normal glucose had no detectable p35 kD active fragment. The increase in caspase-9 by high glucose paralleled the decline in human mesangial cell viability observed in Figure 1. In contrast, high glucose did not detectably increase levels of the p40 kD active fragment of caspase-8, the major initiator caspase of the death receptor pathway of apoptotic signaling (Fig. 2A). Because caspase-9 is an important initiator caspase in the intrinsic pathway, these results suggest that high glucose stimulates the intrinsic pathway of proapoptotic signaling in human mesangial cells. This conclusion was further supported by enzymatic analysis of caspase-9 activity in extracts from human mesangial cells treated with high glucose (Fig. 3), which showed elevation of caspase-9 activity (i.e., LEHDase activity) as early as 8 hours after addition of high glucose. Caspase-9 activity in cells treated with high glucose was also elevated at 24 and 48 hours (Fig. 3).

The intrinsic pathway of apoptotic signaling involves release of multiple polypeptides from mitochondria and formation of the apoptosome containing activated caspase-9. Release of cytochrome-c from mitochondria is a key event in the initiation and regulation of apoptosis through the caspase-9–dependent intrinsic pathway. Incubation of human mesangial cells with normal glucose for up to 24 hours caused no detectable redistribution of cytochrome-c from membrane-bound to cytosolic fractions (Fig. 4). High glucose rapidly stimulated release of cytochrome-c into the cytosol (Fig. 4). Cytosolic levels of cytochrome-c remained elevated for up to 24 hours in cells treated with high glucose. These results are consistent with activation of an intrinsic pathway of apoptosis signaling by high glucose.

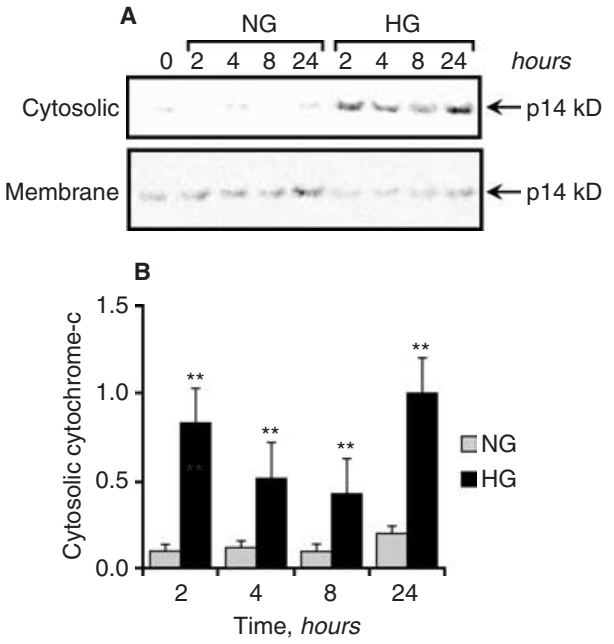

Fig. 4. High glucose elevates cytosolic levels of cytochrome-c in human mesangial cells. (*A*) Translocation of p14 kD cytochrome-c to the cytosol was measured by Western blotting of cells fractionated into cytosol (top panel) and membrane-bound (bottom panel) preparations by digitonin. Equivalent amounts of either the cytosol or membrane-bound fractions from cells treated with normal glucose (NG) or high glucose (HG) were loaded in each lane. (*B*) Densitometric analysis of p14 cytochrome-c levels in cytosolic fractions from three independent experiments. $^{**}P < 0.01$ by *t* test vs. normal glucose at same time.

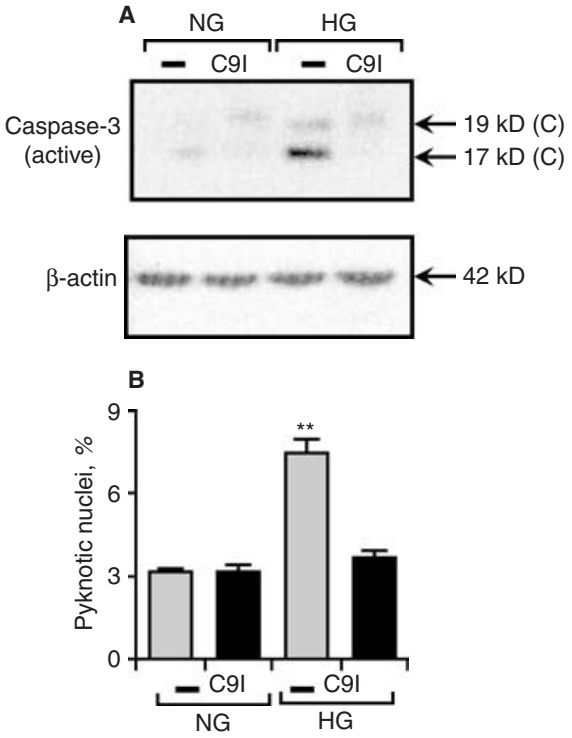

Fig. 5. Inhibition of caspase-9 blocks apoptosis induced by high glucose in cultured human mesangial cells. (*A*) Cells were treated for 24 hours with normal glucose (NG) or high glucose (HG) in the absence or presence of 40 μmol/L Z-LEHD-FMK to inhibit caspase-9 (C9I). The levels of p17 and p19 kD cleaved caspase-3 (upper panel) were measured by Western blotting. Inhibition of caspase-3 cleavage by Z-LEHD-FMK was observed in two independent experiments. (*B*) Human mesangial cells treated with high glucose as in (A) were fixed, stained with Hoechst 33342, and the percentage of pyknotic nuclei were counted. $^{**}P < 0.01$ by χ^2 test.

To determine the functional role of caspase-9 in high glucose–mediated apoptosis in human mesangial cells, cells were treated with a cell-permeable caspase-9 selective inhibitor, Z-LEHD-FMK. As shown in Figure 5A, treatment with high glucose for 24 hours increased the level of p17-p19 active caspase-3; normal glucose had no effect. Coincubation with Z-LEHD-FMK prevented the increase in active caspase-3 by high glucose (Fig. 5A). Z-LEHD-FMK also blocked the increase in pyknotic nuclei observed in cells treated with high glucose (Fig. 5B). Collectively, these results support a role for caspase-9 and the intrinsic signaling pathway in the proapoptotic action of high glucose in cultured human mesangial cells.

Apoptosis in *db/db* diabetic kidneys

To further investigate the possible relevance of apoptotic signaling in glomerular injury in vivo, we studied apoptosis in the *db/db* murine model of kidney injury in type 2 diabetes [25]. Table 1 presents the metabolic and renal parameters of the 8- and 16-week *db/db* mice used in these studies. Hyperglycemia was evident in 8- and 16-week *db/db* but not *db/m* mice. Albuminuria was higher in 8-week *db/db* compared to *db/m* mice, but the mesangial matrix fraction was normal compared to the 8-week *db/m* controls (Table 1). At 16 weeks, the hyperglycemic *db/db* mice manifested evidence of renal injury including increments in albuminuria, mesangial matrix fraction, and serum creatinine (Table 1). These results confirm that 8-week *db/db* mice have relatively normal renal histology despite hyperglycemia and albuminuria and that 16-week *db/db* mice elaborate significant glomerular damage.

As a first step in determining whether an apoptotic signaling cascade had been initiated in *db/db* kidneys, we assessed caspase-3 cleavage in extracts of 8- and 16-week *db/m* and *db/db* kidneys. As shown in Figure 6, the p19 kD cleaved active fragment of murine caspase-3 was elevated in 16-week *db/db* kidneys. Longer exposures of the Western blots revealed a modest increase in p19 in the 8-week *db/db* kidneys but no detectable p19 in the 8- or 16-week *db/m* kidneys (data not shown). However, the amount of the active fragment of caspase-3 was always much higher in the 16-week *db/db* kidneys. These results demonstrate that caspase-3 is specifically activated in the diabetic kidney. Activation of caspase-3 in the 16-week *db/db* kidney was associated with the appearance of mesangial matrix expansion (Table 1).

Table 1. Metabolic and renal parameters in 8- and 16-week *db/m* and *db/db* mice

	8-week *db/m*	8-week *db/db*	16-week *db/m*	16-week *db/db*
Body weight *g*	25.9 ± 3.3	36.4 ± 2.6[a]	28.9 ± 0.9	42.8 ± 1.3[a,b]
Blood glucose *mg/dL*	116 ± 5	389 ± 32[a]	168 ± 9	486 ± 23[a,b]
Kidney weight *mg*	168 ± 12	212 ± 15[a]	173 ± 9	232 ± 17[a]
Albuminuria μg*/24 hours*	0.9 ± 0.4	12.8 ± 3.8[a]	1.8 ± 1.6	35.1 ± 6.9[a,b]
Serum creatinine *mg/dL*	0.38 ± 0.17	0.41 ± 0.2	0.35 ± 0.7	0.53 ± 0.7[a,b]
Mesangial matrix fraction%	3.9 ± 1.4	4.9 ± 0.9	5.7 ± 3.2	12.8 ± 3.1[a,b]

Data are mean ± SD for five *db/m* and five *db/db* mice at each time. Statistical significance calculated using one-way analysis of variance (ANOVA) and Bonferroni post hoc test.

[a] $P < 0.05$ vs. corresponding *db/m.*

[b] $P < 0.05$ vs. 8-week *db/db.*

Additional support for apoptosis in the diabetic kidneys comes from our studies of the effector caspase-7, which is an important substrate for active caspase-3. We used an antibody that specifically recognizes the p20 kD active fragment of caspase-7 to evaluate caspase-7 activity in *db/db* kidneys (Fig. 6). The 20 kD cleaved fragment of caspase-7 was present only in 8- and 16-week *db/db* kidney extracts but not in extracts from the *db/m* nondiabetic kidneys. Long exposures failed to detect the p20 kD cleavage product in *db/m* extracts (data not shown). The presence of active caspase-3 and -7 in *db/db* but not *db/m* kidneys provides evidence that an apoptotic signaling cascade has been initiated in the diabetic kidneys.

The intrinsic pathway of apoptotic signaling in *db/db* kidneys

We next analyzed the pathways of apoptotic signaling in *db/db* mouse kidneys. As shown in Figure 7, caspase-9 is activated in *db/db* mice as revealed by processing of murine procaspase-9 (p47 kD) to the p39 and p37 kD active fragments. The p39 and p37 kD fragments were observed exclusively in *db/db* kidneys and never in the nondiabetic *db/m* controls (Fig. 7). We also noted that the steady-state level of procaspase-9 was higher in the *db/db* mice, which is consistent with the 2.6-fold increase in caspase-9 mRNA observed in our previous microarray studies [23]. These findings suggest that diabetes causes an increase in kidney expression of the procaspase-9 gene. Immunoblotting for β-actin confirmed that equal amounts of kidney protein were present in each lane, which further supports that notion that steady-state caspase-9 protein is elevated in the *db/db* kidneys (Fig. 7).

Caspase-8 is the major initiator caspase of death receptor-mediated pathway but the p40 and p23 kD active caspase-8 fragments were undetectable in the *db/db* kidney extracts (Fig. 7). In separate experiments, we confirmed that the caspase-8 antisera correctly detected the p40 and p23 kD active fragment in Jurkat cells stimulated by etoposide to induce apoptosis (not shown). We also asked whether diabetes activates an apoptotic regulatory pathway initiated by stress in the endoplasmic reticulum stress. A pathway involving activation of procaspase-12 in the endoplasmic reticulum, presumably in response to endoplasmic reticulum stress, was recently described in murine cells [33]. Using an antibody that recognizes the full-length and cleaved form of murine caspase-12, we did not observe a difference in caspase-12 activation in *db/db* kidneys; however, a small amount of the 42 kD cleavage fragment was observed in all the lysates and might reflect nonspecific proteolysis in the extraction procedure (Fig. 7). Alternatively, a small amount of constitutive caspase-12 activity might be present in both *db/db* and *db/m* kidneys. Taken together, our preliminary results provide strong support for the hypothesis that diabetes activates apoptosis in the *db/db* kidneys by evoking the intrinsic pathway of apoptotic signaling.

To further test the hypothesis that diabetes initiates the intrinsic pathway of apoptosis in diabetes, we asked whether Bax levels were higher in *db/db* kidneys. Bax is a critical regulator of apoptosis induced by mitochondrial stress through the intrinsic pathway. Bax increases permeability of the mitochondrial membrane leading to release of cytochrome c, formation of the apoptosome, and activation of caspase-9 [30]. Immunoblotting demonstrated that although some p20 kD Bax protein was detected in *db/m* kidney extracts, Bax protein was consistently elevated in *db/db* vs. *db/m* mice (Fig. 8) These results provide further support for apoptotic signaling through the intrinsic pathway in *db/db* diabetic mouse kidney.

Apoptosis in glomerular cells of the *db/db* kidney

Because the glomerular microvasculature is a major site of injury in the diabetic kidney, our next goal was to determine whether apoptosis was occurring in glomeruli of *db/db* mice. Nuclear segmentation and DNA fragmentation were assessed to identify apoptotic cells. Nuclear morphology in glomeruli of hematoxylin-eosin–stained sections from 16-week *db/m* controls were normal (Fig. 9A and C). In contrast, numerous apoptotic changes were observed in the nuclei of glomerular cells of 16-week *db/db* glomeruli (Fig. 9B and D). Many apoptotic nuclei that displayed prototypical signs of apoptosis, including segmentation and condensation

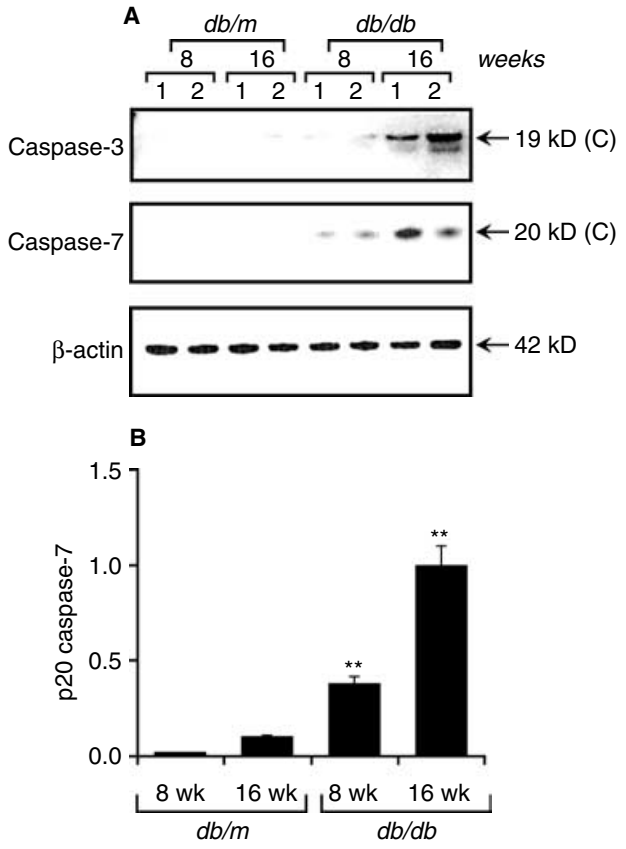

Fig. 6. Caspases-3 and -7 are activated specifically in *db/db* kidneys. (*A*) Kidney extracts containing equal amounts of protein were analyzed by Western blotting for caspase-3 activation using an antibody that recognizes a "neoepitope" in the cleaved (C) 19 kD murine casapse-3 active fragment. To determine if caspase-7 was activated in *db/db* kidneys, we performed Western blots with an anticleaved caspase-7 (Asp198) antibody that detects endogenous levels of the large active fragment (p 20 kD) of caspase-7 resulting from cleavage at aspartic acid 198. This antibody does not crossreact with full-length caspase-7 or with other caspases. The blot was reprobed with an antibody that recognizes β-actin to confirm equal protein loading (bottom panel). Two different mouse kidney extracts have been presented at each time point (lanes 1 and 2). (*B*) Densitometric analysis of Western blots for the active caspase-7 fragment in extracts prepared from five mice per condition. $^{**}P < 0.01$ by one-way analysis of variance (ANOVA) with Bonferroni post hoc test vs. *db/m*.

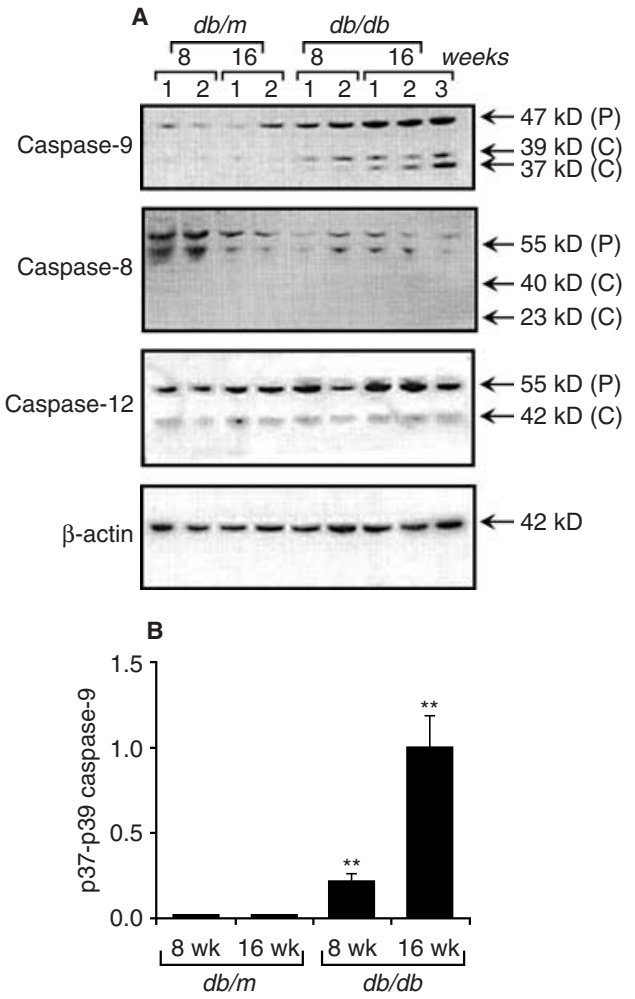

Fig. 7. Caspase-9 is activated in *db/db* diabetic kidneys. (*A*) Kidneys from *db/db* and *db/m* kidneys were homogenized, and equivalent amounts of tissue protein were analyzed by Western blotting. Specific antibodies were used that recognize the procaspase (P) and cleaved (C) forms of murine caspase-9, murine caspase-8, and murine caspase-12. β-actin was used as a control for protein loading. The position and molecular weight of the procasapse and cleaved caspase are indicated. Each lane represents a different mouse kidney extract from each group (i.e., lanes 1 and 2). (*B*) Densitometric analysis of the active caspase-9 fragment by Western blots performed on five mice per condition. $^{**}P < 0.01$ by one-way analysis of variance (ANOVA) with Bonferroni post hoc test vs. *db/m*.

(Fig. 9D, arrows) appeared to be in the mesangial/endocapillary regions, but a definitive assignment of cell type can not be made from these studies. Importantly, apoptotic nuclear changes were only rarely observed in the glomeruli of 8-week *db/db* kidneys and were not present in the nondiabetic *db/m* mice at any time.

Another indicator of apoptosis (DNA fragmentation) was assessed by TUNEL staining in paraffin-embedded sections. TUNEL-positive nuclei were observed in glomeruli of 16-week *db/db* kidneys (Fig. 9E and F, arrows) (8-week *db/m*, 0.2 ± 0.1; 8-week *db/db*, 0.6 ± 0.3; 16-week *db/m*, 0.4 ± 0.3; 16-week *db/db*, 2.7 ± 0.6; data are mean ± SEM, $P < 0.01$ by Student *t* test versus 16-week *db/m*). Controls in the TUNEL assay included nuclease-treated sections to ensure the adequacy of proteinase-k digestion (Fig. 9G) and omission of the TdT enzyme (Fig. 9H). Collectively, the results demonstrate that apoptosis is occurring in glomerular cells of 16-week *db/db* mice. In addition, apoptosis of glomerular cells correlates with mesangial matrix expansion and worsening of albuminuria in 16-week *db/db* mice (Table 1).

DISCUSSION

Chronic hyperglycemia causes cell stress and apoptosis in the microvasculature, but the proapoptotic

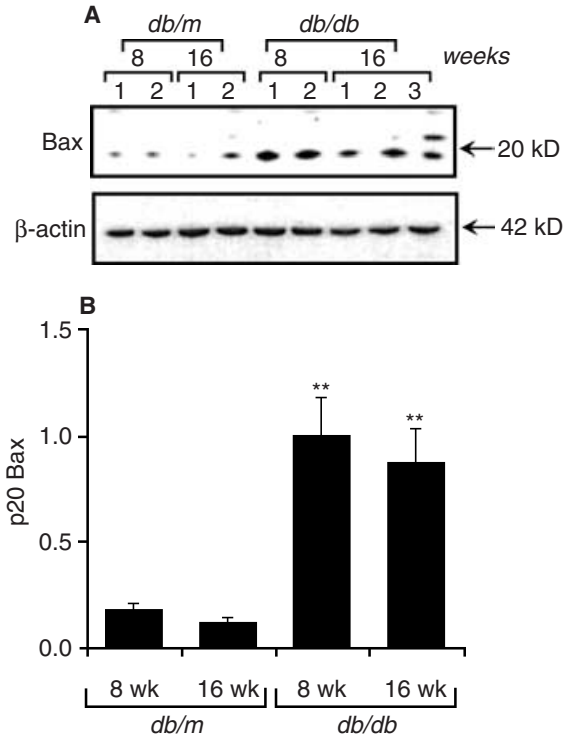

Fig. 8. Bax protein is specifically elevated in the diabetic compared to the nondiabetic kidneys. (*A*) Equivalent amounts of kidney protein were analyzed for Bax by Western blotting. The antibody specifically recognizes the full-length p20 kD protein, which was elevated in the diabetic kidneys. Modest levels of p20 were observed in the 8- and 16-week *db/m* kidneys but were always lower than levels in the corresponding *db/db* mice. Lanes 1 and 2 represent two different mice in each condition. (*B*) Densitometric analysis of p20 Bax levels from a total of five mice in each group. $^{**}P < 0.01$ by one-way analysis of variance (ANOVA) with Bonferroni post hoc test vs. *db/m*.

signal transduction pathways evoked by high glucose are unclear. Our results support the hypothesis that hyperglycemia evokes an intrinsic mitochondrial pathway of proapoptotic signal transduction in cultured human mesangial cells. In addition, an intrinsic pathway of apoptotic signaling is activated in diabetic murine kidneys and is associated with apoptosis of glomerular cells in vivo.

Proapoptotic signaling pathways in mesangial cells exposed to high glucose and in diabetic kidney injury in vivo

Consistent with results from a previous study [9], we found that exposure to high glucose increased apoptosis and reduced mesangial cell viability. In support of a role for the intrinsic pathway in hyperglycemia-induced mesangial cell apoptosis, the cleaved fragment of procaspase-9, which reflects activation of caspase-9 in the intrinsic pathway, was present only in the cells exposed to high glucose and was never observed in the cells exposed to normal glucose. Caspase-9 enzyme activity was also activated specifically by high glucose. In human mesangial cells, high glucose failed to activate caspase-8, a key initiator caspase in the death receptor pathway. In addition, mitochondrial release of cytochrome-c, which helps form the apoptosome to activate caspase-9 in the intrinsic pathway, was observed only in human mesangial cells exposed to high glucose. Most important, a selective caspase-9 inhibitor blocked both caspase-3 activity and formation of pyknotic nuclei in cells exposed to high glucose. Collectively, these results demonstrate that high glucose stimulates apoptosis in human mesangial cells by activating an intrinsic pathway of proapoptotic signaling.

Several lines of evidence suggested that an intrinsic pathway of apoptotic signaling was also activated in the kidney of *db/db* mice, a model for glomerular injury in type 2 diabetes. Caspase-9 but not caspase-8 was elevated exclusively in the diabetic kidneys. Although endoplasmic reticulum stress, particularly in response to protein unfolding, seems to be a possible pathway for cell injury in diabetes, we did not observe an increase of caspase-12 processing in the *db/db* kidneys. However, the paucity of information regarding a procaspase-12 homolog in human cells makes the importance of this pathway difficult to evaluate. Two downstream effector caspases of caspase-9, caspase-3 and caspase-7, were also stimulated in *db/db* kidneys. Bax protein was increased in the *db/db* kidneys, consistent with an intrinsic pathway of regulation. Ortiz, Ziyadeh, and Neilson [34] have reported elevated Bax mRNA in the renal cortex of *db/db* mice, and Murata et al [35] have previously demonstrated increased Bax in the kidneys of the Otsuka Long-Evans Tokushima Fatty rats, another experimental model of type 2 diabetes. Although our studies of caspase activation by Western blotting were performed on whole kidney extracts, the results are presumably relevant to apoptosis in the glomerular cells because the vast majority of apoptosis in the 16-week *db/db* kidneys was detected in the glomerulus, not in the renal tubules. However, considerable work remains to be done to identify the relevant stimuli of the intrinsic pathway in the diabetic kidney in vivo. Hyperglycemia in *db/db* mice is prominent, but we can not rule out activation by other known apoptotic stimuli, including AGEs, which have been widely implicated in diabetic microvascular complications. In addition, TGF-β causes apoptosis in specific cell types, and this cytokine is elevated in *db/db* kidneys [36]. Administration of TGF-β–neutralizing antisera retards the progression of glomerular injury in these mice [37, 38], so it would be important to determine whether TGF-β could initiate the intrinsic pathway of apoptotic signaling in glomeruli in vivo.

Apoptosis in glomerular cells of the *db/db* kidney

An important implication of our study is that the intrinsic pathway of apoptotic signaling could lead to glomerular injury in the kidney in diabetes. A previous study in

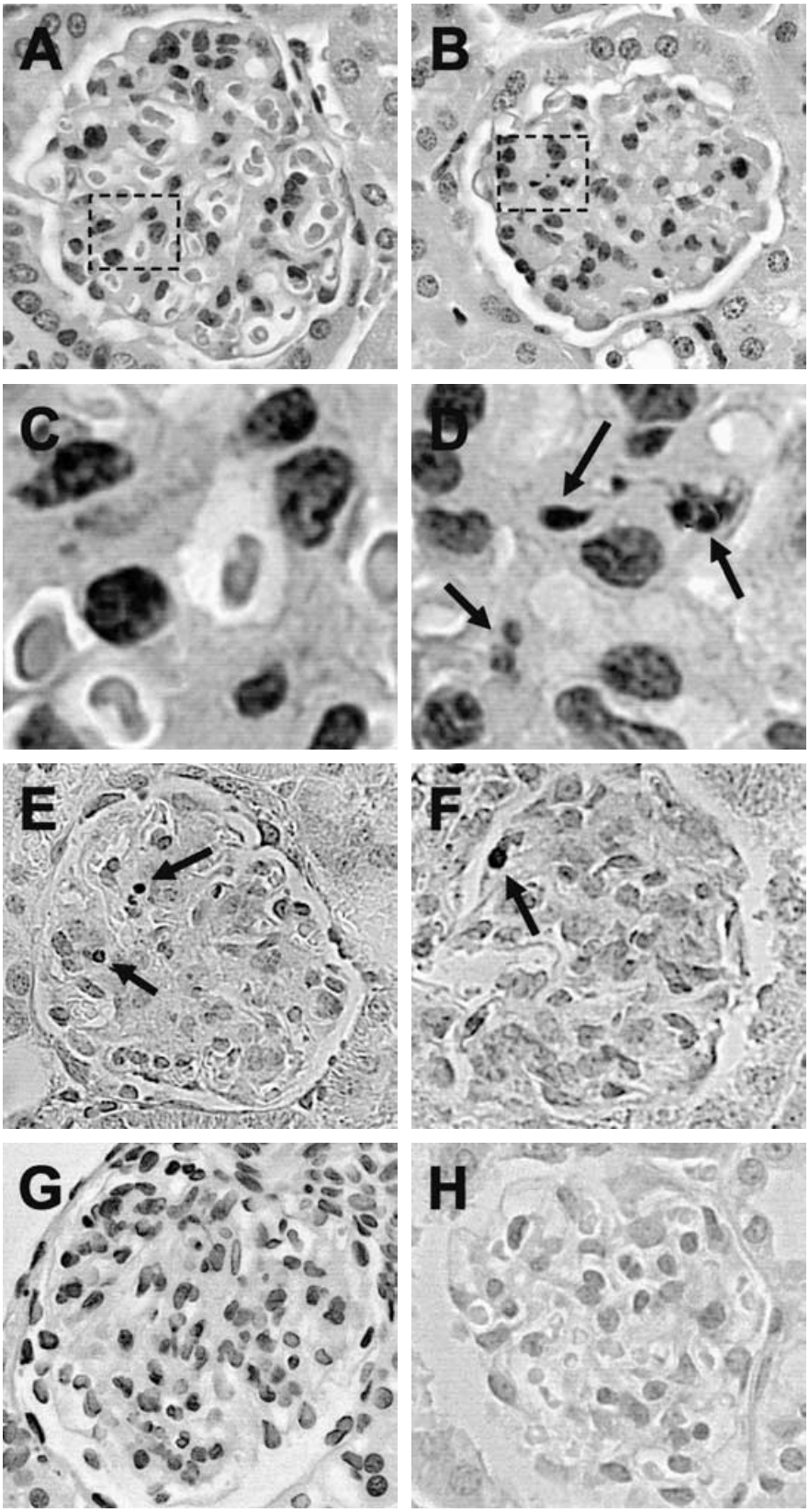

Fig. 9. Localization of apoptotic cells to the glomeruli of 16-week *db/db* mice. Representative hematoxylin-eosin staining of (*A*) 16-week *db/m* nondiabetic control kidney and (*B*) 16-week *db/db* diabetic kidneys. The magnified insets from (A) and (B) are shown in (*C*) and (*D*), respectively. Histologic hallmarks of apoptosis, including condensation of nuclear chromatin and segmentation of the nuclei (D, arrows), were evident in glomeruli of *db/db* kidneys but not in the *db/m* controls. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) identifies DNA fragmentation, an important characteristic of apoptotic cells, and is evident in glomeruli of 16-week *db/db* glomeruli (*E* and *F*, arrows). The TUNEL-stained sections were counterstained with methyl green. (*G*) TACS-nuclease-treated controls were performed in each assay to confirm sufficient permeabilization as were (F) unlabeled experimental controls [i.e., omission of terminal deoxynucleotidyl transferase (TdT)]. Both (G and *H*) are representative glomeruli from 16-week *db/db* mice. All measurements of TUNEL staining in *db/db* kidneys were conducted concurrently with identically processed *db/m* controls (magnification $\times 400$).

db/db mice [34] showed that Bcl-2 mRNA was decreased while Bax mRNA was elevated in the renal cortex of *db/db* mice, which is consistent with a pro-apoptotic phenotype. In addition, a study by Bamri-Ezzine et al [39] showed caspase-3 activation in the kidneys of rats injected with streptozotocin to cause diabetes. High glucose causes atrophy and apoptosis in the metanephric mesenchyme [40], which also suggests that hyperglycemia induces apoptosis in the kidney. Murata et al [35] found glomerular cell apoptosis in a rat model of type 2 diabetes, and Pesce et al [41] observed TUNEL-positive glomerular cells in long-term (6 months) streptozotocin-injected rats. Thus, our present results are consistent with previous reports that diabetes results in apoptosis of glomerular cells in vivo.

Although our present results and the existing literature do not prove a pathogenic role for apoptosis in glomerular injury, clinical studies of patients with type 2 diabetes are consistent with this hypothesis. Evidence to support this idea comes from several studies by Pagtalunan et al [13], Meyer, Bennet, and Nelson [14], and Lemley et al [15], in which a reduction in the number of podocytes correlates with proteinuria and glomerular structural damage in humans with diabetic nephropathy. In fact, the loss of glomerular podocytes is an excellent predictor of renal disease progression [14]. Another recent study by Dalla Vestra et al [16] shows loss of podoyctes in type 2 diabetics and that the density of podocytes per glomerulus was inversely correlated with the rate of albumin excretion. Loss of podocytes has also been reported in patients with diabetic nephropathy secondary to type 1 diabetes [42, 43]. Also in humans with type 2 diabetes, loss of mesangial cells correlates with the development of incipient nephropathy and with significant structural changes resulting in the loss of single nephron K_f [15]. It is important to note that these clinical studies did not investigate the mechanism of mesangial and epithelial cell death, but it seems possible that apoptosis is in part responsible for this loss of podoctyes and mesangial cells in the diabetic kidney. Apoptotic death of podocytes and mesangial cells might be especially deleterious given the low replicative capacity of these glomerular cells in vivo.

CONCLUSION

Our results support the hypothesis that hyperglycemia activates an intrinsic pathway of proapoptotic signaling in mesangial cells and that the intrinsic pathway is activated in *db/db* kidneys in vivo. Studies are now needed to confirm these results in other experimental models of diabetic kidney injury, to determine whether antidiabetic therapies might act in part by attenuating glomerular cell apoptosis, and particularly to see if specific inhibition of the intrinsic pathway of apoptosis reduces the progression of renal injury in experimental models of diabetes.

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