Insulin-Like Growth Factor-Binding Protein-3 Mediates High Glucose-Induced Apoptosis by Increasing Oxidative Stress in Proximal Tubular Epithelial Cells

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IGF-binding protein-3 (IGFBP-3) is the major circulating carrier protein for IGF, and also acts as a potent antiproliferative agent in various cell types. Recently, IGFBP-3 was reported to mediate high glucose-induced apoptosis in mesangial cells and podocytes. In this study, we investigated the role of IGFBP-3 in high glucose-induced apoptosis in proximal tubular epithelial cells (PTEC). Expression of IGFBP-3 protein and mRNA in a porcine PTEC line (LLC-PK1 cells) was measured after exposure to either standard (5.5 mM) or high-glucose (30 mM) medium. We quantified apoptosis after treatment with small interfering RNA against IGFBP-3 (siRNA:IGFBP-3) in high-glucose medium or in cells that overexpressed IGFBP-3. Oxidative stress was measured in high-glucose medium, in the presence of siRNA:IGFBP-3, or in IGFBP-3-overexpressing cells. IGFBP-3 protein and mRNA expression in LLC-PK1 cells was higher in high-glucose medium than in standard-glucose medium. Exposure to high-glucose medium increased apoptosis, and high-glucose-induced apoptosis was abolished by siRNA:IGFBP-3. IGFBP-3 overexpression induced apoptosis in LLC-PK1 cells. Both high-glucose medium and IGFBP-3 overexpression increased reactive oxygen species, and siRNA:IGFBP-3 reduced this increase. Antioxidant treatment decreased IGFBP-3 expression and apoptosis, whereas oxidative stress from hydrogen peroxide increased IGFBP-3 expression, suggesting that oxidative stress increases IGFBP-3 expression. Our results suggest that increased IGFBP-3 expression by high glucose mediates high-glucose-induced apoptosis in PTEC. Increased oxidative stress from high glucose enhances IGFBP-3 expression, inducing apoptosis. Increased expression of IGFBP-3 by high glucose induces additional oxidative stress, which may result in amplification of hyperglycemic damage. (Endocrinology 152: 3135–3142, 2011)
IGFBP-3 may contribute to the pathogenesis of diabetic nephropathy by modulating the bioactivity of IGF via an IGF-dependent mechanism and by inducing apoptosis in kidney cells via both IGF-independent and IGF-dependent mechanisms (5). IGFBP-3 is reported to increase mesangial cell apoptosis under high ambient or standard levels of glucose, and an antisense IGFBP-3 oligodeoxynucleotide inhibits apoptosis induced by high glucose or by TNF-α (18). Recent human biopsy studies have proposed evidence that podocytes are functionally and structurally injured very early in the natural history of diabetic nephropathy (19). Exogenous IGFBP-3 induces IGF-independent podocyte apoptosis (5).

Both the glomerular and tubular compartments may be involved in the development of diabetic nephropathy. The proximal tubule plays a crucial role in the pathogenesis of diabetic kidney disease and is susceptible to a variety of metabolic and hemodynamic factors associated with diabetes, especially hyperglycemia. Glucose entry into proximal tubular epithelial cells (PTEC) is insulin independent, which makes PTEC particularly sensitive to the deleterious effects of chronic hyperglycemia, and high glucose also induces programmed mesangial cell death in vitro by apoptosis (17).

IGFBP-3 may contribute to the pathogenesis of diabetic nephropathy by modulating their bioactivity (5, 6). IGFBP-3 has a role as a potent IGF-IGF receptor-independent antiproliferative agent in various cell types, and acts by blocking the cell cycle and inducing apoptosis (7–9). In addition, IGFBP-3 may contribute to insulin resistance in adipocytes (10, 11) and inhibit adipocyte differentiation (12).

Apoptosis is rarely observed in the normal kidney but is reported in renal diseases including renal ischemia-reperfusion injury (13) and chronic renal failure (14). In diabetic rats, apoptosis increases in renal tubular and interstitial cells, and this is reversed by insulin therapy (15). Apoptosis also occurs in the human diabetic kidney, especially in the tubular epithelial cells (16). Although high ambient glucose induces mesangial IGF signaling and cellular hypertrophy, high glucose also induces programmed mesangial cell death in vitro by apoptosis (17).

Overexpression of GGG-IGFBP-3, a mutant IGFBP-3 that cannot bind to IGF, was also performed to verify the IGF-independent effect of IGFBP-3 on apoptosis in LLC-PK1 cells. The GGG-IGFBP-3 mutant cDNA was generated by site-directed mutagenesis at residues Ile 56, Leu 80, and Leu 81 to Gly 56, Gly 80, and Gly 81, as described previously (23). Binding studies, including Biacore analysis, showed that the GGG-IGFBP-3 mutant protein, generated in Escherichia coli and baculovirus expression systems, lacks affinity for IGF (23).

Small interfering RNA transfection
Small interfering RNA against IGFBP-3 (siRNA:IGFBP-3, siGENOME SMARTpool M-004777-01-0005), and mismatch control (ON-TARGETplus siCONTROL, D-001810-01-05) were from Dharmacon Research (Lafayette, CO), and 12.5 nM siRNA:IGFBP-3 was transfected as above. Cells were incubated in growth medium with 10% fetal bovine serum for 24 h.

Reagents and antibodies
Antihuman IGFBP-3 antibody was from AbCam (Cambridge, MA). Horseradish peroxidase-labeled IgG (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA) was used for secondary antibody. Primer pairs for real-time PCR were from Applied Biosystems (HS-00181211-m1 for IGFBP-3, Hs99999903 for β-actin).

Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits were from R&D Systems (Minneapolis, MN), and the cellular DNA fragmentation ELISA kit was from Roche Diagnostics (Mannheim, Germany). The cell-permeable fluorogenic probe dichlorodihydrofluorescein diacetate (DCF-DA), and the antioxidants, N-acetyl-L-cysteine (NAC) and glutathione (GSH) were from Sigma-Aldrich (St. Louis, MO).
Western blot analysis

LLC-PK1 cells were analyzed after 72 h exposure to standard-glucose (5.5 mM) or high-glucose (30 mM) medium. For Western blots, 20 μl medium from LLC-PK1 cells was electrophoresed on 11% acrylamide gels in the presence of sodium dodecyl sulfate and electroblotted onto polyvinylidene fluoride membranes. After blocking 1 h with 5% wt/vol skim milk, membranes were incubated overnight at 4 C with anti-human IGFBP-3 antibody. Secondary antibody was horseradish peroxidase-labeled IgG (1:3000), and detection was by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

RT-PCR and real-time quantitative PCR

RT-PCR and real-time quantitative PCR were performed using whole-cell lysates from LLC-PK1 cells after 48 h exposure to standard- or high-glucose medium. In brief, total RNA was isolated using Trizol reagent (Invitrogen) and 2 μg total RNA subject to reverse transcription using a Superscript III first-strand synthesis system (Invitrogen) with primers for RT-PCR (IGFBP-3 up): aatgtaga caacgcga, IGFBP-3 (down): tacttatca cgacacca), according to the manufacturer’s instructions.

Amplification was in duplicate, using the ABI 7300 system (Applied Biosystems, Foster City, CA) with the following profile: 94 C for 5 min, 35 cycles of 95 C for 30 sec, 50 C for 30 sec, and 72 C for 30 sec, followed by 72 C for 10 min.

Real-time quantitative PCR was with 20 μl PCR amplification reaction mixture containing DNA, TaqMan primer pairs, and TaqMan universal PCR master mix (Applied Biosystems). Amplification was in duplicate as above with the following profile: 50 C for 2 min, 95 C for 10 min, and 40 cycles of 95 C for 15 sec with 60 C for 1 min. Gene expression for each sample was expressed as cycle threshold (Ct) normalized to β-actin (Ct). Ct values were compared between samples from high-glucose-treated cells and control cells in serum-free medium, for Ct calculations. The final comparison of transcript ratios between samples is given as 2^−ΔΔCt.

Quantification of apoptosis by annexin V-FITC staining

Subconfluent monolayers of LLC-PK1 cells were exposed to medium containing either standard or high glucose for 72 h. Detection of annexin V on the cell surface was done using a flow cytometer (Bekman Coulter Corp., Fullerton, CA). System II software was used for acquisition and analysis. Annexin V-FITC conjugate (R&D Systems) was used to stain cells. In brief, cells and supernatants were harvested, washed, and incubated for 15 min with annexin V-FITC and propidium iodide to differentiate between early apoptotic cells (annexin V-positive), late apoptotic and/or necrotic cells (annexin V and propidium iodide positive), and viable cells (unstained). The sum of early apoptotic and late apoptotic/necrotic cells were considered as total apoptotic cells and was used for statistical analysis.

Quantification of apoptosis by DNA fragmentation

Cells were prelabeled by incubation with 10 μM bromodeoxyuridine (BrdU) for 12 h at 37 C. Labeled cells were incubated in 5.5 or 30 mM glucose or 30 mM mannitol for an osmotic control. After 48 h, cells were centrifuged, and the supernatant was analyzed for apoptosis. After adsorbing anti-DNA antibody onto the wells of a microplate, sample was added to the microplate, allowing BrdU-labeled DNA fragments to bind to the immobilized antibody. Immunocomplexed BrdU-labeled DNA-
fragments were denatured by microwave irradiation, and anti-BrdU antibody peroxidase conjugate was added to form an immunocomplex. Bound anti-BrdU-peroxidase was quantified using an ELISA plate reader.

### Oxidative stress measurement

The cell-permeable fluorogenic probe DCF-DA diffuses across cell membranes and is hydrolyzed by nonspecific cellular esterases to the nonfluorescent compound dichlorofluorescin (DCFH), which is predominantly trapped within the cell. In the presence of reactive oxygen species (ROS), DCFH rapidly undergoes one-electron oxidation to the highly fluorescent compound dichlorofluorescein (DCF). Our assay was a modification of the Bestwick and Milne (24) procedure. Glucose-exposed cell monolayers were incubated with 10 μM DCF-DA in PBS (pH 7.4) for 15 min at 37°C. Cells were washed once with PBS, and cell debris was cleared by centrifugation at 3000 rpm for 5 min. Cells were treated with trypsin and washed once with PBS, and cell debris was cleared by centrifugation at 1000 rpm for 5 min. Fluorescence was monitored using a flow cytometer as above, using 480-nm excitation and 530-nm emission wavelengths. fluorescent levels are expressed as the percent increase over the control.

### Data expression and statistical analysis

Results of three or more independent experiments are expressed as mean ± SD. Statistical comparison among groups were performed using one-way ANOVA followed by Tukey’s post hoc analysis. *P* < 0.05 was considered significant.

### Results

**High glucose increases IGFBP-3 expression in PTEC**

We examined whether high glucose increases IGFBP-3 expression in LLC-PK1 cells. Western blots revealed that IGFBP-3 protein is increased in LLC-PK1 cells exposed to high-glucose medium, compared with cells exposed to standard-glucose medium (Fig. 1A). IGFBP-3 expression was decreased in LLC-PK1 cells in high-glucose medium after cotreatment with siRNA:IGFBP-3 (Fig. 1A). IGFBP-3 mRNA expression was also increased in cells in high glucose, as measured by RT-PCR (Fig. 1B) or real-time quantitative PCR (Fig. 1C).

To confirm the effect of high-glucose medium on IGFBP-3 expression, we examined the expression of IGFBP-3 at different glucose concentrations and time courses. IGFBP-3 expressions in 30 and 45 mM glucose media were significantly increased compared with those in 5.5 mM glucose medium (Supplemental Fig. 1, A and B, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). IGFBP-3 protein expression was significantly increased by 24 h exposure to high-glucose medium, which was inhibited by siRNA:IGFBP-3 (Supplemental Fig. 1, A and B). IGFBP-3 mRNA expression was also increased in cells in high glucose, as measured by RT-PCR (Fig. 1B) or real-time quantitative PCR (Fig. 1C).

**IGFBP-3 mediates high-glucose-induced apoptosis in PTEC**

We investigated whether high glucose increases apoptosis in LLC-PK1 cells. Annexin V-FITC staining revealed that the percentage of apoptotic cells in high-glucose medium was higher than in standard-glucose medium (18.3 ± 1.2 vs. 5.8 ± 1.7%, *P* < 0.05), indicating that high glucose increases
apoptosis in LLC-PK1 cells (Fig. 1D). The apoptosis-inducing effect of high glucose was abolished by cotreatment with siRNA:IGFBP-3, suggesting that IGFBP-3 mediates high-glucose-induced apoptosis (Fig. 1D). Moreover, measurement of apoptosis by DNA fragmentation demonstrated the same pattern in LLC-PK1 cells in high glucose, with or without siRNA:IGFBP-3 (Fig. 1E). Treatment with 30 mM mannitol did not increase apoptosis in LLC-PK1 cells (Fig. 1E), suggesting that the increased apoptosis in high glucose did not result from high osmolarity.

To confirm the effect of high-glucose medium on PTEC apoptosis, we performed annexin V-FITC staining under different glucose concentrations and exposure time. Apoptosis in LLC-PK1 cells were significantly increased after exposure to 15, 30, and 45 mM glucose media compared with 5.5 mM glucose medium. Because apoptosis was most prominent in 30 mM glucose medium, we labeled 30 mM glucose as a high-glucose condition throughout the experiment (Supplemental Fig. 2A). Apoptosis was significantly increased in LLC-PK1 cells after 24, 48, and 72 h exposure to high-glucose medium, which was most prominent at 72 h (Supplemental Fig. 2B). Preincubation with siRNA:IGFBP-3 significantly inhibited apoptosis after 12, 24, and 48 h exposure to high-glucose medium (Supplemental Fig. 2C).

IGFBP-3 overexpression increases apoptosis in PTEC

To confirm the effect of increased IGFBP-3 expression on apoptosis in LLC-PK1 cells, we established an IGFBP-3-overexpressing PTEC line by transfection with IGFBP-3 cDNA. Figure 2, A–C, shows the increase in IGFBP-3 protein and mRNA in the IGFBP-3-overexpressing PTEC line. Annexin V-FITC staining showed that IGFBP-3 overexpression increased apoptosis (empty vector control, 8.1 ± 0.7%, vs. IGFBP-3 overexpression, 21.8 ± 4.2%; P < 0.01) (Fig. 2D). GGG-IGFBP-3 overexpression also increased apoptosis (empty vector control, 8.1 ± 0.7%, vs. GGG-IGFBP-3 overexpression, 18.4 ± 3.6%; P < 0.01), suggesting that IGFBP-3 promotes PTEC apoptosis in an IGF-independent manner (Fig. 2D). Measurement of apoptosis by DNA fragmentation also demonstrated that GGG-IGFBP-3 overexpression, as well as IGFBP-3 overexpression, increased apoptosis (P < 0.05 compared with cells in standard glucose, Fig. 2E).

IGFBP-3 is involved in oxidative stress induction by high glucose in PTEC

We investigated the effect of high glucose and IGFBP-3 expression on oxidative stress in LLC-PK1 cells. Exposure to high-glucose medium increased oxidative stress, as measured by DCF-DA staining (standard glucose, 9.7 ± 0.4%, vs. high glucose, 20.8 ± 0.7%, P < 0.01; Fig. 3A). NAC and GSH, the antioxidants, prevented ROS increase by high-glucose medium (n = 4). ROS was significantly increased by high glucose at 6 h, and treatment with siRNA:IGFBP-3 reduced oxidative stress in cells exposed to high-glucose medium (n = 4). IGFBP-3 overexpression increased oxidative stress in LLC-PK1 cells (n = 4). *, P < 0.05.
Oxidative stress increases IGFBP-3 expression and mediates PTEC apoptosis

We further investigated the effect of oxidative stress on IGFBP-3 expression and high-glucose-induced apoptosis in LLC-PK1 cells. Preincubation with antioxidants NAC and GSH abolished increase in IGFBP-3 protein expression by high-glucose media, whereas elevating oxidative stress with hydrogen peroxide increased IGFBP-3 expression (Fig. 4A). IGFBP-3 mRNA expression was also reduced by NAC or GSH, whereas it was increased by hydrogen peroxide (Fig. 4, B and C). These findings suggest that increased oxidative stress by high glucose may stimulate IGFBP-3 expression.

We also investigated whether antioxidant prevented high-glucose-induced apoptosis in LLC-PK1 cells. Annexin V-FITC staining showed that NAC and GSH decreased apoptosis in high-glucose medium (NAC with high glucose, 10.9 ± 1.2%, and GSH with high glucose, 13.9 ± 1.9%, vs. high glucose only, 20.2 ± 4.0%, P < 0.01; Fig. 4D). Taken together, these results imply that increased oxidative stress from high glucose increased IGFBP-3 expression, resulting in increased apoptosis.

Discussion

Chronic hyperglycemia is the central initiating factor in diabetic nephropathy. Although all diabetic cells are exposed to elevated levels of plasma glucose, most cells are able to reduce glucose transport into the cell during hyperglycemia, so that internal glucose concentrations remain constant. In contrast, in cells damaged by hyperglycemia, the glucose transport rate does not decline rapidly during hyperglycemia (2). PTEC, like mesangial cells and podocytes, develop intracellular hyperglycemia, which can result in hyperglycemic damage (25). Samikkannu et al. (26) demonstrated that acute exposure of human PTEC to high glucose induces a time-dependent dual effect of early proliferation and late apoptosis.

Cellular responses to high glucose are numerous and varied but ultimately result in functional changes and often cell death. High glucose induces oxidative and nitrosative stress, causing activation of proteins involved in apoptotic cell death, which is important to the development of diabetic complications (27). In this study, high-glucose medium increased IGFBP-3 protein and mRNA levels in PTEC. IGFBP-3 mRNA expression is reported to be increased in the proximal tubules of diabetic rat kidney (28), but few studies have reported on IGFBP-3 expression in high-glucose medium in a PTEC line.

Initially, IGFBP-3 was considered to modulate only the actions of circulating IGF, but it also exhibits clear, distinct biological effects independent of the IGF/IGF-1 receptor (IGF-IR) axis. Apart from modulating IGF actions (IGF/IGF-IR-dependent actions), IGFBP-3 clearly exerts intrinsic bioactivity either in the absence of IGF or without triggering IGF-IR signaling (IGF/IGF-IR-independent actions) (6). IGFBP-3 inhibits growth and induces apoptosis by both IGF-dependent and IGF-independent mechanisms. GH and tumor suppressors such as p53 induce
IGFBP-3 expression. Physiological stimuli, including DNA damage by irradiation and hypoxia, are reported to induce IGFBP-3, suggesting that IGFBP-3 is involved in physiological protection against aberrant cell growth (29).

In this study, high glucose increased IGFBP-3 expression and apoptosis in PTEC, and high-glucose-induced apoptosis was abolished by cotreatment with siRNA: IGFBP-3, suggesting that IGFBP-3 mediates high-glucose-induced apoptosis in PTEC. In addition, IGFBP-3 overexpression increased PTEC apoptosis. Moreover, overexpression of GGG-IGFBP-3, a mutant IGFBP-3 that cannot bind to IGF, also increased apoptosis. These findings suggest that increased IGFBP-3 by high glucose induces PTEC apoptosis, at least partly by an IGF-independent mechanism. Although similar results have been reported in mesangial cells (18) and podocytes (5), to our knowledge, this is the first report on the independent role of IGFBP-3 in high-glucose-induced PTEC apoptosis.

Changes in cellular function by hyperglycemia that result in oxidative stress are crucial in the development and progression of diabetic nephropathy. A unifying hypothesis suggests that mitochondrial overproduction of ROS initiates hyperglycemia-induced damage in the diabetic kidney, leading to activation of four major biochemical pathways: AGE formation, protein kinase C isoforms, and flux through the polyol and hexosamine pathways. Each of these pathways can contribute to the perpetuation of, and in some cases initiate, cellular ROS generation (25).

Increased levels of ROS in hyperglycemia induce renal cell apoptosis and diabetic nephropathy (30). Verzola et al. (31) reported that hyperglycemia induces apoptotic changes in human tubular cells by increasing oxidative stress. In this study, high glucose or IGFBP-3 overexpression increased oxidative stress, and siRNA: IGFBP-3 abolished the increase in oxidative stress caused by high glucose or IGFBP-3 overexpression, suggesting that IGFBP-3 is involved in inducing oxidative stress by high glucose in PTEC. On the other hand, antioxidants decreased IGFBP-3 expression and high-glucose-induced apoptosis, and elevation of oxidative stress by hydrogen peroxide increased IGFBP-3 expression, suggesting that oxidative stress increases IGFBP-3 expression. In addition, ROS increase by high glucose or IGFBP-3 overexpression was significant at 6 h, whereas IGFBP-3 mRNA expression was significantly increased at 12–48 h, and apoptosis was most prominent at 72 h exposure to high-glucose medium. Taken together, these results suggest a positive loop between ROS and IGFBP-3, amplifying ROS production and resulting in amplification of hyperglycemic damage (Fig. 5).

In the present study, treatment of siRNA:IGFBP-3 under standard-glucose conditions also resulted in decreased apoptosis. PTEC itself normally secrete IGFBP-3 in some degree. It is possible that that inhibition of intrinsic IGFBP-3 secretion by siRNA:IGFBP-3 resulted in subnormal IGFBP-3 expression, resulting in decreased apoptosis.

Although it is well documented that IGFBP-3 promotes apoptosis, the underlying mechanism of action remains uncertain (7). Kim et al. (8) reported that IGFBP-3 induces apoptosis through the activation of caspases, via a death receptor-mediated pathway in breast cancer cells. It was also demonstrated that IGFBP-3 significantly enhances apoptosis by inhibiting nuclear factor-kB activation in colon carcinoma-derived cell lines (32). However, the actual mechanism of IGFBP-3 action on PTEC apoptosis is still unclear and needs to be elucidated.

In conclusion, this study found that high glucose increases IGFBP-3 expression and induces oxidative stress and apoptosis in PTEC. The results suggest that increased IGFBP-3 expression by high glucose mediates high-glucose-induced apoptosis in PTEC. We propose that elevated levels of oxidative stress from high glucose increase IGFBP-3 expression, thereby inducing apoptosis. In addition, increased expression of IGFBP-3 by high glucose induces additional oxidative stress, which may result in amplification of hyperglycemic damage in PTEC.

Acknowledgments

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