IGF-1 inhibits the mitochondrial apoptosis program in mesangial cells exposed to high glucose

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Kang, Barinder P. S., Arunas Urbonas, Andrew Baddoo, Stuart Baskin, Ashwani Malhotra, and Leonard G. Meggs. IGF-1 inhibits the mitochondrial apoptosis program in mesangial cells exposed to high glucose. Am J Physiol Renal Physiol 285: F1013–F1024, 2003. First published July 22, 2003; 10.1152/ajprenal.00209.2003.—The activated insulin-like growth factor-1 receptor (IGF-1R) protects cells from a wide range of apoptotic stimuli. Hyperglycemia promotes the intracellular generation of superoxide anion and hydrogen peroxide, both of which have been linked to the activation of the mitochondrial apoptosis program. Here, we report for the first time that ligand activation of the IGF-1R protects normal human mesangial cells and SV40 murine mesangial cells from the glycol-oxidant-induced apoptosis program. The IGF-1R antiapoptosis program was dependent on the recruitment of both Akt/PI3K and the ERK subfamily of mitogen-activated protein kinases. IGF-1 treatment also protected the redox potential of mesangial cells maintained at high ambient glucose concentration, by inhibiting the generation of reactive oxygen intermediates and preserving mitochondrial transmembrane potential. IGF-1R survival signals targeted the Bcl-2 family of proteins to protect against glucose-induced apoptosis and oxidative stress. IGF-1-treated cells exhibited a decrease in the Bax/Bcl-2 ratio; increased phosphorylation/inactivation of Bad at Ser112 and Ser136; inhibition of cytochrome c release; perturbations directionally opposed to the initiation of the apoptosis program. In addition, we demonstrate IGF-1R-activated ERK signaling modules phosphorylate Ser112 of the mitochondrial Bad protein, establishing a direct link between surface IGF-1R and the mitochondrial apoptosis program. Our findings indicate that in mesangial cells maintained at high ambient glucose concentration, IGF-1 activates a survival program that maintains the integrity of mitochondria and prevents the expression of the genetic program for apoptosis.

mitochondria; cytochrome c; reactive oxygen species; Bad; Bcl-2

STRATEGIES THAT INTERRUPT or suppress initiation of the apoptosis program offer a novel approach to preserving cell number and organ function. The activated insulin-like growth factor-1 receptor (IGF-1R) protects several cell lines from a wide range of apoptotic stimuli (8, 27, 29, 30, 35). Ligand binding to the IGF-1R results in the phosphorylation of specific tyrosine residues at the IGF-1R β-subunit, activating the signaling cascade. The phosphorylated IGF-1R transmits intracellular signals via tyrosyl phosphorylation of the insulin receptor substrate (IRS) family and Shc, adaptor proteins that interact with a myriad of signaling molecules (41). In this scheme, the recruitment of IRS-1 results in the activation of phosphatidylinositol 3-kinase (PI-3K), which in turn activates Akt/protein kinase B (Akt/PKB) (34). The latter signaling cascade is generally regarded as the main pathway used by the IGF-1R for protection against apoptotic cell death (22, 25, 41). Compelling evidence has also been provided for the existence of alternate survival pathways (26, 41, 47). In several cell lines (26, 40, 41, 53), the ERK subfamily of MAPKs have been reported to protect against apoptosis. The IGF-1R via its interaction with the adaptor protein Shc (41, 42) promotes activation of the ERK subfamily. It has been suggested that the remarkable efficacy of the IGF-1R against a variety of apoptotic stimuli may reflect the ability to recruit multiple survival pathways (41).

Cell death by apoptosis is a tightly orchestrated event, under the control of genetic programs that have been highly conserved during the evolutionary process. Mesangial cells possess the genetic program for apoptosis (21, 33, 43–46), and this mechanism of cell death has been reported during the resolution phase of inflammatory glomerular lesions (3, 33). Reactive oxygen species (ROS) are recognized as important mediators of biological responses, including cell death by apoptosis (1, 50). The mitochondria are the primary source of reactive oxygen intermediates and critical determinants of cell death and cell survival (1, 14). Hyperglycemia increases the generation of superoxide anion (O2−) by interfering with the flow of electrons along the mitochondrial electron transport chain (37). In a recent communication, our laboratory provided evidence for a cause and effect relationship between glucose-induced ROS generation and activation of the apoptosis program in normal human mesangial cells (NHMC) and SV40 murine mesangial cells (MMC) (21). The Bcl-2 family of proteins constitutes a critical checkpoint in both the susceptibility to apoptosis and mitochondrial...
dysfunction. IGF-1R-dependent signals inactivate the proapoptosis protein Bad by phosphorylating Ser112 and Ser136 (41). The nonphosphorylated Bad protein is capable of forming heterodimers with Bcl-XL and Bcl-2 at the level of mitochondria, neutralizing their antiapoptotic function and promoting cytochrome c release (2, 17, 19). After release from the mitochondrial intermembrane space, cytochrome c binds to Apaf-1 to form a macromolecular complex known as the apoptosome (36), which recruits and activates caspase-9, initiating the terminal apoptosis program.

In the present study, we test the hypothesis that ligand activation of the IGF-1R will protect NHMC and MMC from the apoptotic stimulus of high glucose concentration. To explore the mechanism(s) of IGF-1R cytoprotection, selective inhibitors of Akt/PKB and ERKs were employed to determine if either of these signaling pathways is necessary and sufficient for protection against the glucose-induced death signal. On the basis of previous work (21), implicating oxidative stress as the proximate event in the glucose-induced apoptosis program, the effect of IGF-1 on glucose-induced ROS generation, mitochondrial transmembrane potential (ΔΨm), and cytochrome c release was examined. Finally, we demonstrate that IGF-1R-dependent signals target the Bcl-2 family of proteins to protect against glycol-oxidative stress and activation of the apoptosis program.

METHODS

Reagents. IGF-1, wortmannin, and α-glucose were purchased from Sigma. LY294002 and U0126 were purchased from Cell Signaling. Calyculin A, leupeptin, PMSF, and protease inhibitor cocktail were purchased from Calbiochem-Novabiochem. All culture media were purchased from Gibco-BRL and BioWhittaker.

MMC culture. MMC were obtained from American Type Culture Collection. MMC exhibit the phenotypic characteristics of mesangial cells in primary culture (51). A limited number of studies were also performed with NHMC to ensure that results were not influenced by transformation. MMC cultures were maintained under conditions previously established in our laboratory (21). For experimental studies, 80% confluent MMC were plated in serum-free medium (SFM; 0.2% BSA) and incubated for 12 h and divided into different experimental groups, as described below.

To determine if IGF-1 rescues MMC from the apoptotic stimulus of high glucose concentration, MMC were maintained at 5 or 25 mM glucose for 16 h, in the presence and absence of 100 ng/ml IGF-1 (33). Protocols were performed by adding the following pharmacological agents to culture media, MEK inhibitor (15 μM U0126) and the PI-3K inhibitors (10 μM LY294002 and 100 nM wortmannin). The percentage of cells undergoing apoptosis was determined by ELISA cell death detection assay and confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).

NHMC culture. To determine if IGF-1 activates a survival program in NHMC maintained at high ambient glucose concentration, an identical protocol to that described immediately above was performed with NHMC obtained from BioWhittaker. Culture conditions were as follows: NHMC were maintained in mesangial cell basal medium (Bio-Whittaker), supplemented with 5% FBS, 30 mg/l gentamycin, and 15 μg/l amphotericin-B in a humidified incubator at 37°C and 5% CO₂-95% air (21). For experimental studies, 70% confluent primary NHMC cultures were incubated in SFM (0.2% BSA) for 12 h. The culture media were replaced with fresh SFM containing 5 or 25 mM glucose for 16 h, with or without IGF-1 (100 ng/ml). All experiments were performed using NHMC from passages 5-6.

Analysis of DNA fragmentation by ELISA. Histone-associated DNA fragments were quantified by the Cell Death Detection ELISA (Roche Diagnostic) as previously described (21).

In situ TUNEL assay. TUNEL assay was performed to study the double-strand cleavage of DNA in NHMC and MMC as previously described (21).

Akt/PKB kinase assay. MMC were harvested and Akt/PKB kinase activity was determined using a nonradioactive Akt/PKB kinase assay kit (Cell Signaling Technology). Cell extracts were prepared according to manufacturer’s instructions. Two-hundred microliters of samples containing 200 μg protein were selectively immunoprecipitated for active Akt/PKB protein, using immobilized Akt/PKB 1G1 monoclonal antibody. Immobilized immune complexes (immunoprecipitates) were washed and incubated with GSK-3 fusion protein (1 μg), for 30 min at 30°C, in the presence of 40 μl kinase buffer supplemented with 200 μM ATP. Kinase reaction was terminated with 25 μl of 3× SDS sample buffer. Akt-mediated phosphorylation of GSK-3 was measured by Western blotting using phospho-GSK-3α/β (Ser21/9) antibody. After chemiluminescent detection (Pierce), Akt/PKB kinase activity was quantitated using computerized image analysis software, Quantity One (Bio-Rad).

p44/42 Kinase assay. MMC were harvested and p44/p42 activity was determined in lysates using nonradioactive MAPK assay kit (Cell Signaling Technology). Cell extracts were prepared according to manufacturer’s instructions. Two-hundred microliters of samples containing 200 μg protein were selectively immunoprecipitated for active MAPK, using immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody. Immobilized immune complex (immunoprecipitates) was washed and incubated with Elk-1 fusion protein (2 μg), for 30 min at 30°C, in the presence of 50 μl kinase buffer supplemented with 200 μM ATP. Kinase reaction was terminated with 25 μl of 3× SDS sample buffer. p44/p42 Kinase-mediated phosphorylation of Elk-1 at Ser383 was measured by immunoblot analysis using phospho-Elk-1 (Ser383) antibody. p44/p42 Kinase activity was quantitated as described above.

Immunofluorescent detection of glycol-oxidative stress. Glucose-mediated oxidative stress in MMC and NHMC was studied by trafficking of 2,3,4,5,6-pentafluorobenzaldehyde-2′,3′,5′,6′-tetrahydroxynaphthalene (PF-H2TMRos or Redox Sensor Red CC-1; Molecular Probes) using fluorescence microscopy as previously described (21). Redox Sensor Red CC-1 is oxidized in the presence of O₂⁻ and H₂O₂. Culture slides were washed and mounted with PBS and visualized using Nikon fluorescence microscope (Nikon Eclipse E800) equipped with triple filter cube and charge-coupled device (CCD) camera (Nikon DMDX1200). The staining was performed in quadruplicate for each group, and 30 random fields (average 500 cells) were studied in each replicate. Images were captured using Nikon ACT-1 (Version 1.12) software and combined for publishing format using Adobe Photoshop 6.0 software.

Assessment of ΔΨm. ΔΨm was monitored with the dye, 5′,5′,6′,6′-tetrachloro-1′,1′,3′,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Molecular Probes) using fluorescence microscopy and flow cytometric analyses (1). NHMC and MMC were cultured on chambered culture slides at 5 mM glucose, 25 mM glucose, and 25 mM glucose + IGF-1 (100
ng/ml) for 16 h. At the end of the incubation, cells were loaded at 37°C for 10 min with JC-1 cationic dye (1 µg/ml) added to respective media. Culture slides were washed and mounted with PBS and visualized using Nikon fluorescence microscope (Nikon Eclipse E800) equipped with triple filter cube and CCD camera (Nikon DXM1200). JC-1 dye exhibits potential-dependent accumulation in mitochondria (J-aggregates; accumulate at high membrane potential), indicated by a fluorescence shift from green to red fluorescence. Green fluorescence reflects the monomeric form of JC-1, appearing in cytosol after mitochondrial membrane depolarization. The staining was performed in quadruplicates for each group and 30 random fields (average 500 cells) were studied in each replicate. Images were captured using Nikon ACT-1 (Version 1.12) software and combined for publishing format using Adobe Photoshop 6.0 software.

For flow cytometry, cells were harvested by trypsinization, loaded with JC-1 dye, and analyzed (FACSCalibur; Becton Dickinson; 10,000 cells/sample) (1). The excitation wavelength was 488 nm (argon ion laser), and the emission fluorescence for JC-1 was monitored at 530 nm (FL-1) and 585 nm (FL-2). The flow cytometry data were acquired and analyzed using Cell Quest and Cell Quest Pro software (Becton Dickinson). Data were plotted based on changes detected in mean red fluorescence (FL-2) intensity.

**Immunoblot analysis.** MMC were homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 0.5 mM PMSF, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 0.5 µg/ml leupeptin, and 0.5 µg/ml aprotinin. Protein samples were separated by 4–15% (w/vol) SDS-PAGE and were transferred onto nitrocellulose membranes using a Semi-Dry Transfer Cell apparatus (Bio-Rad). Primary rabbit polyclonal antibodies for Bax and Bcl-2 (1:1,000; Santa Cruz Biotechnology); and phosphorylated Bad Ser136, a dilution of 1:3,000 of secondary antibody (BD Biosciences) was used. The blots were developed by ECL-kit (Amersham-Pharmacia-Biotech). Brieﬂly, protein samples were separated by SDS-PAGE (4–15% gels) to ascertain the separation of cytosolic and mitochondrial fractions. Anti-COX IV antibody (1:1,000; Molecular Probes) was used to probe the protein COX-IV as a marker in mitochondria and its absence in cytosol by Western blot analysis. For detection, horseradish peroxidase-linked secondary antibody was used at a dilution of 1:3,000. Cytochrome c was detected and quantitated by probing cytosolic fractions with mouse monoclonal anticytochrome c antibody (BD Biosciences) as previously described (21).

**Mitochondrial Bad and ERK expression.** Mitochondrial subfractions of MMC were subjected to 4–15% gradient gel electrophoresis, transferred to nitrocellulose, and probed for Bad and ERK proteins. For Bax, phospho-Bad Ser112, and phospho-Bad Ser136 expression, conditions were identical to those described above. Primary antibodies for ERK (rabbit polyclonal; Santa Cruz Biotechnology) and phospho-ERK Tyr204 (mouse monoclonal; Santa Cruz Biotechnology) were used at a dilution of 1:1,000. The proteins were detected by HRP-linked secondary antibodies (Bio-Rad) using 1:7,500 dilution. Densitometric analyses were performed as described above.

**Statistical analysis.** Data are expressed as means ± SE. Comparison between two values was performed by unpaired Student’s t-test. For multiple comparisons among different groups of data, the significant differences were determined by the Bonferroni method. Significance was defined at *P* ≤ 0.05.

**RESULTS**

**Effect of IGF-1R on glucose-induced mesangial cell apoptosis.** To determine whether ligand activation of the IGF-1R inhibits glucose-induced mesangial cell apoptosis, NHMC and MMC were plated in SFM containing 5 or 25 mM glucose for 16 h, in the presence and absence of IGF-1 (100 ng/ml). As shown in Fig. 1, A and B, NHMC and MMC maintained in SFM + Fig. 1. Effect of insulin-like growth factor-I (IGF-1) on normal human mesangial cells (NHMC; A) and SV40 murine mesangial cells (MMC; B) apoptosis. Mesangial cells were maintained under one of the following conditions for 16 h: serum-free medium (SFM) + 5 mM glucose, SFM + 25 mM glucose, SFM + 25 mM glucose + IGF-1, and SFM + 5 mM glucose + IGF-1. A baseline level of apoptosis was detected in serum-starved NHMC and MMC under euglycemic conditions (C) by ELISA cell death assay. The histone-associated DNA fragments are presented as optical density at 405 nm relative to control value. For each assay, 20 µl of lysate (2.0 mg/ml) was used. Data are presented as means ± SE and represent 6–8 independent experiments. * or + *P* ≤ 0.05. • C vs. H; • H vs. Hi or CI. The following abbreviations are used throughout the figures: C, SFM + 5 mM glucose; H, SFM + 25 mM glucose; Hi, H + 100 ng/ml IGF-1; CI, C + IGF-1; Hi, H + 50 µM LY294002; Hw, H + 100 nM wortmannin; HU, H + 10 µM U0126; HIL, H + I + L; HiW, H + I + W.
5 mM glucose (control) exhibit a baseline level of apoptotic cell death. A 50% increase in apoptosis was detected in both cell lines when the glucose concentration in the media was increased to 25 mM. In an earlier communication from our laboratory (21), to control for the potential effect of osmolarity on MMC apoptosis, cells were maintained under osmolar equivalent conditions of 5 mM glucose/H1100120 mM mannitol and were demonstrated to exhibit levels of apoptosis similar to control values. The addition of IGF-1 to cultures of NHMC and MMC maintained at 25 mM glucose reduced apoptosis to baseline values. TUNEL staining confirmed the increased number of NHMC apoptotic nuclei at 25 mM glucose (Fig. 2b), which was attenuated in the presence of IGF-1 (Fig. 2c). The results indicate that the activated IGF-1R protects NHMC and MMC from the apoptotic stimulus of high glucose concentration.

Effect of PI-3K inhibitor on Akt/PKB activity and glucose-induced apoptosis. The cytoplasmic serine/threonine kinase Akt/PKB is a downstream effector of the PI-3K signaling pathway and has been reported to play a critical role in the IGF-1R survival pathway (8, 41). To assess Akt/PKB activity in IGF-1-treated cells and under basal conditions, immunoblot analysis was performed to detect Akt/PKB-mediated phosphorylation of GSK-3 fusion protein. This approach provides a semiquantitative analysis of Akt/PKB activity. As shown in Fig. 3A, basal Akt/PKB activity was decreased by nearly 50% in lysates from serum-starved MMC maintained at 25 mM glucose. The addition of IGF-1 to MMC maintained under identical conditions induced an increase in Akt/PKB activity, which was completely blocked in the presence of the PI-3K inhibitor LY294002. To determine whether Akt/PKB is necessary and sufficient for the IGF-1R survival program, the effect of LY294002 on the glucose-induced component of MMC apoptosis was evaluated. A threefold increase in apoptosis was detected in LY294002-treated MMC maintained at 25 mM glucose (Fig. 3B). Under identical conditions, the coadministration of IGF-1 with LY294002 reduced apoptosis to the level detected in MMC at 25 mM glucose. Taken together, the activated IGF-1R recruits Akt/PKB via PI-3K, which is necessary, but not sufficient, alone to protect against the glucose-induced apoptosis program.

Effect of MEK inhibitor on ERKs (p44/p42) activity and glucose-induced apoptosis. Activation of the ERK subfamily of MAPKs has also been implicated in the
IGF-1R survival program in mesangial cells

Activated IGF-1R stabilizes $\Delta \psi_m$. Apoptosis is characterized by the collapse of $\Delta \psi_m$ due to the opening of the mitochondrial permeability transition pore (14, 36). To determine whether ligand activation of the IGF-1R prevents collapse of $\Delta \psi_m$, this parameter was examined in NHMC and MMC, under control and experimental conditions. Cells were loaded with the fluorescent probe JC-1, which exhibits potential-dependent accumulation in mitochondria. As shown in Fig. 6, $a$ and $d$, under control conditions NHMC and MMC show punctate red staining due to the accumulation of $J$ aggregates in mitochondria, indicative of preserved $\Delta \psi_m$. NHMC and MMC maintained at 25 mM glucose (Fig. 6, $b$ and $e$) exhibit mitochondrial depolarization indicated by the reduction in $J$ aggregates (red fluorescence) and increased JC-1 monomers (green fluorescence). Figure 6, $c$ and $f$, show NHMC and MMC maintained at 25 mM glucose in the presence of IGF-1. Cells show intense punctate red staining, indicative of preserved $\Delta \psi_m$. Flow cytometric analysis (Fig. 6$g$) of $\Delta \psi_m$ under control and experimental conditions indicates a shift (FL-2 channel) of the distribution curve (HI) toward control (C), whereas the distribution curve for 25 mM glucose alone (H) shifts to the left. A quantitative analysis of mean red fluorescence intensity for control and experimental groups (Fig. 6$h$) indicates a 50% reduction for MMC at 25 mM glucose and 10% reduction for MMC at 25 mM glucose + IGF-1. Taken together, high ambient glucose concentration is a potent stimulus for the collapse of $\Delta \psi_m$ in NHMC and MMC. Ligand activation of the IGF-1R abrogates the glucose-induced loss of $\Delta \psi_m$, preserving the integrity of mitochondria and promoting the survival phenotype.

We next asked whether the activated IGF-1R preserves $\Delta \psi_m$ via the recruitment of Akt/PKB and/or ERKs. To determine whether the activated IGF-1R requires Akt/PKB to preserve $\Delta \psi_m$ at high glucose (H) concentration, studies were performed with LY294002. As shown in Fig. 7$a$, LY294002 + IGF-1 (HIL) shifted the distribution curve leftward compared with IGF-1 alone (HI). This result indicates a marked decrease in

cytoprotective properties of the IGF-1R (27, 41). To assess p44/p42 activity in IGF-1-treated cells and under basal conditions, immunoblot analysis was performed to detect phosphorylation of the Elk-1 fusion protein at Ser383. This approach provides a semiquantitative analysis of p44/p42 activity. Basal p44/p42 activity (Fig. 4A) was decreased by 40% in lysates from starved MMC maintained at 25 mM glucose. The addition of IGF-1 to MMC maintained under identical conditions induced an increase in p44/p42 activity, which was completely blocked by the MEK inhibitor U0126. To determine whether ERKs are necessary and sufficient for the IGF-1R survival program, the effect of U0126 on the glucose-induced component of apoptosis was evaluated. A 5.5-fold increase in apoptosis was detected in U0126-treated MMC maintained at 25 mM glucose (Fig. 4$B$). Under identical conditions, the coadministration of IGF-1 with U0126 resulted in a level of apoptosis that exceeded that detected in MMC at 25 mM glucose. Taken together, ERKs are necessary but not sufficient to activate the IGF-1R survival program in this system. The aggregate results indicate that both Akt/PKB and ERKs are required for protection against the glucose-induced component of apoptosis.

Activated IGF-1R prevents glucose-induced ROS generation. High ambient glucose concentration alters the redox status of cells through the overproduction of ROS by the mitochondrial electron transport chain (37). To document the presence of $O_2^*$ and $H_2O_2$, NHMC and MMC were loaded with Redox Sensor Red CC-1 and the mitochondria-specific dye mitotracker green FM as previously described (21). Figure 5, $a$ and $d$, show NHMC and MMC under control conditions. NHMC and MMC maintained at 25 mM glucose exhibit bright yellow/orange fluorescence in mitochondria (Fig. 5, $b$ and $e$) due to the colocalization of oxidized Red CC-1 dye (red fluorescence) and mitotracker green (green fluorescence). To determine if IGF-1R-dependent signals suppress glucose-induced $O_2^*$ and $H_2O_2$ generation, IGF-1 was added to cultures of NHMC and MMC. IGF-1 inhibited $O_2^*$ and $H_2O_2$ generation as illustrated by the marked reduction of bright yellow/orange fluorescence (Fig. 5, $c$ and $f$). Taken together, IGF-1R-dependent signals interrupt glucose-induced ROS generation, defending the redox status of NHMC and MMC maintained at high glucose concentration.

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**Fig. 4.** Effect of MEK inhibitor on p44/p42 activity and glucose-induced apoptosis. MMC were maintained under one of the following conditions for 16 h: C, H, HI, HU, or HIU. A: p44/p42 activity in lysates prepared from MMC. Data are presented as means $\pm$ SE and represent 6 independent experiments. B: effect of MEK inhibitor on MMC apoptosis. Apoptosis was detected by ELISA cell death assay as described above. Data are presented as means $\pm$ SE and represent 8 independent experiments. * vs. H, HI, HU, or HIU; **H vs. HI, HI, HU, or HIU; #HI vs. HU or HIU; $\#$H vs. HIU.
Fig. 5. Glucose-induced reactive oxygen species (ROS) generation. NHMC (a–c) and MMC (d–f) were maintained under one of the following conditions for 16 h: C, H, or HI. NHMC and MMC were loaded with the oxidant-sensitive dye Redox Sensor red CC-1 and the mitochondrial-specific dye MitoTracker green FM. b And e: bright yellow-orange fluorescence seen in mitochondria (arrows) due to the colocalization of oxidized red CC-1 (red fluorescence) and MitoTracker green FM (green fluorescence) compared with controls (a and d). c And f: inhibition of red CC-1 oxidation by IGF-1. Magnification ×20 (NHMC) and ×40 (MMC).

Fig. 6. Effect of activated IGF-1 receptor (IGF-1R) on mitochondrial transmembrane potential (∆ψm). NHMC (a–c) and MMC (d–f) were maintained under one of the following conditions for 16 h: C, H, or HI. Control cells (a and d) show predominantly punctate red staining with the fluorescent dye JC-1, indicative of preserved ∆ψm. Cells exposed to H (b and e) lose punctate red staining and increased monomeric dye (green fluorescence), indicative of collapse of ∆ψm. c And f: preservation of ∆ψm in cells exposed to HI. Magnification ×20 (NHMC) and ×40 (MMC). Overlays of flow cytometric histogram (g) of FL2 channel (red fluorescence) in C, H, and HI. h: Quantitative analysis depicting mean red fluorescence intensity. Results of flow cytometric analyses are representative of 4 independent experiments. * or + P < 0.05. *C vs. H; +H vs. HI.
IGF-1R preservation of $\Delta\psi_m$ with the addition of LY294002. Figure 7B depicts a quantitative analysis of mean red fluorescence intensity for both distribution curves. The inclusion of LY294002 to MMC cultures resulted in a 75% decrease in red fluorescence intensity, indicating Akt/PKB is necessary for IGF-1R preservation of $\Delta\psi_m$. An identical analysis with U0126 (Fig. 7C) revealed an extreme leftward shift, indicative of almost complete collapse of $\Delta\psi_m$. As shown in Fig. 7D, the inclusion of U0126 to MMC cultures resulted in barely detectable level of red fluorescence intensity, documenting a key role for ERKs in the preservation of $\Delta\psi_m$ by the IGF-1R. The aggregate data indicate Akt/PKB and ERKs are integral components of the IGF-1R signaling pathway that preserves $\Delta\psi_m$, but neither is sufficient alone to prevent dissipation of $\Delta\psi_m$.

Activated IGF-1R targets Bcl-2 family of proteins and p53. To determine whether IGF-1R-dependent signals target members of the Bcl-2 family and the proapoptotic transcription factor p53, immunoblot analyses were performed on lysates of MMC. As shown in Fig. 8A, the ratio of Bax/Bcl-2 was increased in MMC maintained at 25 mM glucose, an alteration that favors progression of the apoptosis program. Ligand activation of the IGF-1R prevented the upregulation in the Bax/Bcl-2 ratio. Interestingly, IGF-1 did not affect the Bax/Bcl-2 ratio in MMC maintained at 5 mM glucose. Because Bax is a target gene for p53 (10, 29, 30), we examined the phosphorylation status of the p53 protein. Ser392 is located at the COOH terminus of p53 and linked to transcriptional activation (10). Phosphorylation of Ser392 was upregulated in MMC exposed to 25 mM glucose (Fig. 8B). This modification of the p53 protein was not detected in the presence of IGF-1, suggesting p53 as a target for IGF-1R-dependent signals in this system.

The phosphorylation status of Bad (Fig. 8, C and D) was also altered in MMC maintained at 25 mM glucose. Phosphorylation at Ser112 and Ser136 is recognized as a mechanism of inactivating the proapoptotic function of Bad (17, 24, 27). ERKs phosphorylate Ser112 of the Bad protein (2), whereas Akt/PKB phosphorylates Ser136 (22). As shown in Fig. 8, C and D, phosphorylation at Ser112 and Ser136 was markedly attenuated in MMC maintained at 25 mM glucose. The addition of IGF-1 to culture media enhanced phosphorylation at Ser112 and Ser136 of the Bad protein. IGF-1R-dependent phosphorylation at Ser112 was markedly attenuated by the MEK inhibitor U0126, whereas the PI-3K inhibitors wortmannin and LY294002 blocked phosphorylation at Ser136.

**Effect of activated IGF-1R on phospho-ERK expression in mitochondria.** ERK signaling modules have been reported to inhibit the mitochondrial apoptosis program via the phosphorylation of Bad (2). To determine if IGF-1R-activated ERK signaling modules target mitochondrial pools of Bad in our system, mitochondria-enriched fractions of MMC were prepared (21) and probed with phospho-ERK antibody. As shown in Fig. 9, A and C, phospho-ERK content was decreased in mitochondria of MMC maintained at 25 mM glucose. IGF-1 restored the depressed phospho-ERK content detected at 25 mM glucose. Total ERK content remained constant under control and experimental conditions (Fig. 9B). We next asked whether IGF-1R-dependent restoration of phospho-ERK content was coupled with increased phosphorylation of the mitochondrial Bad protein. As shown in Fig. 10, A and C, phosphorylation at Ser112 was downregulated in MMC maintained at 25 mM glucose. The inclusion of IGF-1 to the culture media of MMC maintained under identical conditions restored the depressed phosphorylation at
Ser112. Conversely, IGF-1 had no detectable effect on the phosphorylation status of Ser136 (Fig. 10, D and F). Consistent with this result, immunoblot analyses did not detect the presence of phospho-Akt/PKB in mitochondrial preparations (data not shown). Total Bad content remained constant under control and experimental conditions (Fig. 10, B and E). Taken together, IGF-1R-dependent signals alter the expression and phosphorylation status of the Bcl-2 family of proteins. IGF-1R-activated ERK signaling modules selectively target Ser112 of the mitochondrial Bad protein to promote the survival phenotype.

**Effect of activated IGF-1R on cytochrome c release.** The release of cytochrome c from mitochondria is tightly regulated by protein-protein interactions among Bcl-2, Bax, and Bad (14, 50). To determine whether IGF-1R-induced perturbations in the Bcl-2 family inhibit cytochrome c release, immunoblots were performed on cytosolic fractions of MMC. As shown in Fig. 11, A and B, a twofold increase in the content of cytochrome c was detected in cytosol of MMC maintained at 25 mM glucose. The addition of IGF-1 reduced the glucose-induced increment in cytochrome c release to control values. Coomassie blue-stained gels (Fig. 11C) are shown that document equal loading conditions. Taken together, the aggregate data indicate IGF-1R-dependent signals target mitochondria as part of the survival program, preserving membrane potential and inducing alterations in the Bcl-2 family that inhibit cytochrome c release.

**DISCUSSION**

In the present study, we demonstrate that ligand activation of the IGF-1R protects NHMC and MMC from the apoptotic stimulus of high ambient glucose concentration. IGF-1R cytoprotection was dependent on the recruitment of Akt/PKB and ERK signaling pathways, both functioning in a codependent manner to promote cell survival. In addition, evidence is presented for the first time in a resident glomerular cell line that the activated IGF-1R suppresses glucose-induced ROS generation and preserves \( \Delta\psi_{m} \). Finally, we demonstrate that IGF-1R-dependent signals induce perturbations in the expression and phosphorylation status of the Bcl-2 family of proteins, which inhibit cytochrome c release and promote the survival phenotype.

**Activated IGF-1R protects against glucose-induced mesangial cell apoptosis.** The activated IGF-1R protects several cell lines from a wide range of apoptotic stimuli (8, 27, 29, 30, 33, 35, 46). Here, we report for the first time the activated IGF-1R protects NHMC and MMC from glucose-induced apoptosis. The observation that IGF-1 is cytoprotective in our system appears to conflict with reports indicating a pathogenetic role for growth hormone and IGF-1 in diabetic nephropathy (11, 12, 15). The activated IGF-1R is a powerful inhibitor of apoptosis caused by a variety of stimuli including c-myc overexpression (18), serum withdrawal (33), anticancer drugs (9, 49), osmotic...
shock (41), and ionizing radiation (35). The complex milieu of the diabetic glomerulus is characterized by the activation of multiple cytokines and growth factors (6, 54). The mechanism(s) by which resident glomerular cells are lost in the diabetic kidney has not been explored. In particular, the process of glomerular occlusion, in which glomeruli disappear or are no longer identifiable, remains a mystery (5). Cell death by apoptosis does not result in sclerosis or residual scarring and in the absence of immunocytochemical analysis cannot be detected morphologically (19). Although the application of an in vitro system to study the fate of resident glomerular cells has limitations, our results clearly document IGF-1 activates a survival program that protects mesangial cells from the apoptotic stimulus of high extracellular glucose concentration. Furthermore, the data are in accord with recent observations, indicating only IGF-1 and Bcl-2 are capable of suppressing initiation of apoptosis, whereas caspase inhibitors can only arrest completion of the apoptosis program (32, 41). Taken together, our finding that IGF-1 protects NHMC and MMC from glucose-induced apoptosis offers a new perspective on the potential role of this pleiotropic growth factor in the diabetic glomerulus.

**IGF-1R-dependent survival pathways.** In the present study, ligand activation of the IGF-1R was coupled with increased Akt/PKB and ERKs activities in lysates of MMC. Selective inhibitors were used to determine whether Akt/PKB and/or ERKs alone are necessary and sufficient to protect against glucose-induced apoptosis. The results indicate both pathways are required but neither is sufficient alone to inhibit the glucose-induced apoptosis program. Akt/PKB and ERKs share a common downstream target, phosphorylating and inactivating the proapoptotic protein Bad. Moreover, Akt/PKB has also been reported to target several other genes implicated in the transmission of pro- and antiapoptosis signals, including caspase-9, IkB, forkhead genes, and endothelial nitric oxide synthase (23). Recent work has provided compelling evidence for the

![Fig. 9. Effect of activated IGF-1R on phospho-ERK expression in mitochondria. MMC were maintained under one of the following conditions for 16 h: C, H, HI, or CI. A: phospho-ERK content in mitochondrial enriched subfractions. B: total content of ERK. C: ratio of phospho-ERK/ERK. Data are presented as means ± SE and represent 8 independent experiments. * or + P < 0.05. *C vs. H; +H vs. HI or CI.](image)

![Fig. 10. Effect of activated IGF-1R on phosphorylation of mitochondrial Bad protein. MMC were maintained under one of the following conditions for 16 h: C, H, HI, or CI. A: immunoblot analysis of phospho-Bad Ser112 in mitochondrial enriched subfractions. C: ratio of phospho-Bad Ser112/Bad. D: immunoblot analysis of phospho-Bad Ser136 in mitochondrial enriched subfractions. F: ratio of phospho-Bad (Ser136)/Bad. B and E: total content of Bad in mitochondrial preparations. Data are presented as means ± SE and represent 4–6 independent experiments. *, +, or #P ≤ 0.05. *C vs. H; +H vs. HI or CI; #HI vs. CI.](image)
downstream targets that participate in the expression of the survival phenotype, while quenching the generation of ROS. For example, ERK-dependent signals have been reported to phosphorylate and activate the antiapoptosis Bcl-2 protein (20, 39). Several mechanisms by which Bcl-2 may inhibit mitochondrial ROS generation have been proposed (31): 1) Bcl-2 may directly scavenge ROS; 2) Bcl-2 may inhibit electron transfer from complexes I to III; and 3) Bcl-2 protects cytochrome c from inactivation. It seems reasonable to infer that the increased availability of Bcl-2 in IGF-1-treated MMC may serve the dual role of promoting cell survival and protecting against oxidant stress.

Current concepts indicate that the collapse of \( \Delta \psi_{\text{mit}} \) is a key event in triggering the apoptosis program (14, 36). Our results strongly suggest IGF-1R-dependent signals inhibit opening of the mitochondrial permeability transition pore in NHMC and MMC maintained at high ambient glucose concentration. The coadministration of LY294002 or U0126 with IGF-1 in each case resulted in a dissipation of \( \Delta \psi_{\text{mit}} \) by the IGF-1R in this system, the effect of U0126 was proportionately greater than LY294002. This observation is in keeping with the effect of U0126 on glucose-induced apoptosis described above, in the presence and absence of IGF-1. Taken together, IGF-1R-dependent signals block glucose-induced generation of ROS and prevent the collapse of \( \Delta \psi_{\text{mit}} \), thereby preventing activation of the mitochondrial apoptosis program.

**Activated IGF-1R targets Bcl-2 family of proteins and p53.** In the present study, ligand activation of the IGF-1R decreased the Bax/Bcl-2 ratio in MMC maintained at 25 mM glucose, a modification that is directionally opposed to progression of the apoptosis program (52). Conversely, MMC maintained under identical conditions in the absence of IGF-1 exhibit an upregulation of the Bax/Bcl-2 ratio and a 50% increase in apoptotic death. Homodimers of the Bcl-2 protein stabilize the mitochondrial membrane, whereas heterodimers of Bcl-2 and Bax neutralize the antiapoptotic function of Bcl-2 (19). A potential mechanism by which IGF-1R-dependent signals may downregulate the Bax/Bcl-2 ratio involves the tumor suppressor protein p53, which is a transcriptional regulator of the Bax gene (10, 29, 30). Transcriptional activation of the p53 protein has been linked to phosphorylation at Ser392 (10). IGF-1 markedly attenuated phosphorylation at Ser392, suggesting p53 is a target for IGF-1R antiapoptotic signals. This notion is supported by previous investigations (29, 30), demonstrating that IGF-1 upregulates the expression of the p53-dependent protein Mdm2, which by means of protein:protein interaction inhibits p53 DNA-binding activity. Finally, a direct link between the activated IGF-1R and the mitochondrial survival program was also documented, by demonstrating a functional coupling between IGF-1R-activated ERK signaling modules and phosphorylation of mitochondrial pools of Bad (2). Taken together, the

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**Fig. 11.** Effect of activated IGF-1R on cytochrome c (Cyt. c) release. MMC were maintained under one of the following conditions for 16 h: C, H, HI, or CI. Data are presented as means ± SE and represent 5 independent experiments. *, +, or #P ≤ 0.05. *C vs. H; +H vs. HI or Cl; #HI vs. Cl.
activated IGF-1R promotes shifts in the expression and phosphorylation status of the Bcl-2 family, which are directionally opposed to initiation of the terminal apoptosis cascade.

**Effect of activated IGF-1R on cytochrome c release.** The mitochondria are key determinants of cell death and cell survival (1, 14, 36). Emerging concepts suggest the release of cytochrome c from mitochondria commits a cell to die by either apoptosis or necrosis (14). In the present study, cytochrome c was detected in the cytosolic fractions of MMC maintained at high glucose concentration. Ligand activation of the IGF-1R prevented glucose-induced cytochrome c release, blocking activation of the terminal apoptosis program. In a previous communication from our laboratory (21), the free radical scavengers NAC and DPI exerted a similar effect, providing a strong rationale for glycol-oxidant stress as the signal that triggers mitochondrial dysfunction and apoptosis in this system.

The present study has certain limitations, among which was the relatively brief exposure of mesangial cells to high glucose concentration, compared with chronic in vivo models of hyperglycemia. Moreover, although oxidative stress has also been reported in the diabetic kidney (38), the application of an in vitro system may not mimic the in vivo condition. Finally, the approach and relevance of mesangial cell rescue in the in vitro setting remain to be established.

In summary, the activated IGF-1R protects against glycol-oxidative stress and apoptosis in NHMC and MMC maintained at high glucose concentration. IGF-1R cytoprotection was mediated by activation of Akt/PKB- and ERK-dependent signals but could not be conferred by either signaling pathway alone. The IGF-1R survival program targeted distinct proteins, inducing posttranslational modifications in the Bcl-2 family that favor cell survival and inhibit the glycol-oxidant death signal. In keeping with evolving concepts, in which the majority of cell death signals engage mitochondria, IGF-1R-dependent signals stabilized this organelle by inhibiting ROS generation, preserving $\Delta\Psi_{m}$, phosphorylation/inactivation of Bad, and preventing the release of cytochrome c. The application of therapeutic interventions directed at selective expression of one or more of the molecular components of the IGF-1R survival program may offer a novel approach to protect against cell death and arrest the progression of diabetic glomerulopathy.

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