Redox dependence of glomerular epithelial cell hypertrophy in response to glucose

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Redox dependence of glomerular epithelial cell hypertrophy in response to glucose. Am J Physiol Renal Physiol 290: F741–F751, 2006. First published October 18, 2005; doi:10.1152/ajprenal.00313.2005.—Podocytes or glomerular epithelial cells (GECs) are important targets of the diabetic microenvironment. Podocyte foot process effacement and widening, loss of GECs and hypertrophy are pathological features of this disease. ANG II and oxidative stress are key mediators of renal hypertrophy in diabetes. The cellular mechanisms responsible for GEC hypertrophy in diabetes are incompletely characterized. We investigated the effect of high glucose on protein synthesis and GEC hypertrophy. Exposure of GECs to high glucose dose dependently stimulated [3H]leucine incorporation, but not [3H]lysine incorporation. High glucose resulted in the activation of ERK1/2 and Akt/PKB. ERK1/2 pathway inhibitor or the dominant negative mutant of Akt/PKB inhibited high glucose-induced protein synthesis. High glucose elicited a rapid generation of reactive oxygen species (ROS). The stimulatory effect of high glucose on ROS production, ERK1/2, and Akt/PKB activation was prevented by the antioxidants catalase, diphenylene iodonium, and N-acetylcysteine. Exposure of the cells to hydrogen peroxide mimicked the effects of high glucose. In addition, ANG II resulted in the activation of ERK1/2 and Akt/PKB and GEC hypertrophy. Moreover, high glucose and ANG II exhibited additive effects on ERK1/2 and Akt/PKB activation as well as protein synthesis. These additive responses were abolished by treatment of the cells with the antioxidants. These data demonstrate that high glucose stimulates GEC hypertrophy through a ROS-dependent activation of ERK1/2 and Akt/PKB. Enhanced ROS generation accounts for the additive effects of high glucose and ANG II, suggesting that this signaling cascade contributes to GEC injury in diabetes.

high glucose; angiotensin II; podocytes; reactive oxygen species; protein synthesis; hypertrophy

Podocytes, or visceral glomerular epithelial cells (GECs), are highly specialized cells covering the exterior glomerular basement membrane surface of the glomerular capillary (4, 36). In addition to abnormalities in the glomerular endothelium and mesangium, recent data suggest that diabetic nephropathy is accompanied by changes in GECs (49). The diabetic milieu, represented by hyperglycemia, causes broadening and effacement of GEC foot processes associated with the loss of podocytes themselves (30, 49). Because GECs do not usually proliferate, GEC loss results in an increase in size due to hypertrophy and additional widening/effacement of the foot processes to cover the denuded glomerular basement membrane (32, 52). While this is primarily compensatory, GEC hypertrophy is likely maladaptive and contributes to glomerulosclerosis (23, 32, 52).

Components of the local renin-angiotensin systems (RAS) are upregulated in diabetes in proximal tubular epithelial cells, mesangial cells, and GECs (6, 49). GECs can generate ANG II and do express the AT1 receptor (47, 49, 54). High glucose concentration induces ANG II formation in GECs through upregulation of angiotensinogen expression (36, 49). Angiotensin-converting enzyme inhibition or AT1 receptor blockade attenuates GEC foot process broadening/effacement and hypertrophy and prevents loss of GEC in animal models of diabetes or in cells exposed to high glucose, supporting a role for ANG II in GEC injury in diabetes (21, 36, 49, 54).

A growing body of evidence indicates that oxidative stress is a major mechanism of cellular dysfunction in diabetes and contributes to diabetic complications, including diabetic nephropathy (5, 27, 28). It has been suggested that overproduction of reactive oxygen species (ROS) is the main initiating and progression factor involved in the development of diabetic nephropathy (5, 27, 28). Increased ROS generation induces GEC dysfunction in glomerular diseases such as Heymann nephritis (34, 36, 49). However, to our knowledge, no data related to the role of ROS in GEC injury in diabetes have been reported.

In this study, we explored the effects of high glucose and ANG II on oxidant generation and hypertrophy of GECs. We describe an essential role for oxidative stress in high glucose-induced GEC hypertrophy. We provide the first evidence that in these cells high glucose stimulates protein synthesis via a redox-dependent activation of the protein kinases ERK1/2 and Akt/PKB. Finally, our data show that high glucose and ANG II elicit additive responses to activate these signals and to induce GEC hypertrophy.

MATERIALS AND METHODS

Cell culture and transfections. Rat visceral GECs (podocytes) were isolated and characterized as described by Kreisberg et al. (26). These cells were used between passages 10 and 12. Cells were maintained in DMEM supplemented with antibiotic/antifungal solution and 10% fetal bovine serum. GECs were transiently transfected with plasmid DNA [15 µg of vector alone or hemagglutinin (HA)-Akt(K179M)] via electroporation (Gene pulser, Bio-Rad) as previously described (14). The HA epitope-tagged mammalian expression construct HA-Akt(K179M) was a generous gift of Dr. T. F. Franke (Harvard Institutes of Medicine, Boston, MA).

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Immunoprecipitation and ERK1/2 and Akt/PKB activity assay. GECs were grown to near confluency in 60- or 100-mm dishes and serum-deprived for 48 h. All incubations were carried out in serum-free DMEM at 37°C for a specified duration. The cells were lysed in radioimmune precipitation buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1% NP-40] at 4°C for 30 min. The cell lysates were centrifuged at 10,000 g for 30 min at 4°C. Protein was determined in the cleared supernatant using the Bio-Rad method. Immunoprecipitation and ERK1/2 and Akt/PKB activity assays were performed as described (14, 16). Anti-ERK1/2 or Akt/PKB immunoprecipitates were used in an immune complex kinase assay using myelin basic protein as a substrate. For immunoprecipitation, rabbit anti-ERK1/2 (Santa Cruz Biotechnology) or sheep anti-Akt/PKB (Upstate Biotechnology) was used. The bands corresponding to phosphorylated myelin basic protein were quantitated by densitometry and/or PhosphorImager analysis.

Immunoblotting. GEC lysates were prepared as described above for ERK1/2 and Akt/PKB activity assay. For immunoblotting, proteins (25–50 µg) were separated using 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with mouse polyclonal phosphospecific ERK (1:1,000), rabbit anti-ERK1/2 (1:2,000) or rabbit polyclonal anti-Akt1/PKB (1:1,000, Cell Signaling Technology), and the primary antibodies were detected using horseradish peroxidase-conjugated IgG (1:2,500 or 1:5,000). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using National Institutes of Health Image software.

Detection of intracellular ROS. The peroxide-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescin diacetate (Molecular Probes) was used to assess the generation of intracellular ROS as described previously (14, 44). This compound is converted by intracellular esterases to 2',7'-dichlorodihydrofluorescin, which is then oxidized by intracellular ROS to the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF). Cells were grown in 120- or 24-well plates and serum-deprived for 48 h. Immediately before the experiments, cells were washed with HBSS and loaded with 50 µM 2',7'-dichlorodihydrofluorescein diacetate dissolved in HBSS for 30 min at 37°C. They were then incubated for 30 min with different inhibitors (see RESULTS) followed by the addition of a selected agonist or vehicle for various time periods. Subsequently, DCF fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, and measured with a multiwell fluorescence plate reader (Wallac 1420 Victor2, PerkinElmer).

Measurement of DNA synthesis. DNA synthesis is measured as the incorporation of [3H]thymidine into TCA-insoluble material as described previously (14, 16). Briefly, confluent GECs were washed with phosphate-buffered saline and incubated in serum-free medium for 48 h. Agonists were then added for an additional 48 h before being pulsed with 1 µCi/ml [3H]thymidine for 4 h. The medium was...
removed, and the cells were washed twice with ice-cold 5% TCA to remove unincorporated [3H]thymidine. Cells were solubilized by adding 0.75 ml of 0.25 N NaOH and 0.1% SDS. A 0.5-ml volume of this cell lysate was neutralized and counted in a scintillation counter.

[3H]leucine incorporation. [3H]leucine incorporation was measured as previously shown (14, 16). GECs were grown in six-well dishes, made quiescent in serum-free medium for 48 or 72 h. GECs were then incubated with or without agonist for 48 h. Six hours before being harvested, GECs were pulsed with 2 μCi/ml [3H]leucine. At the end of this incubation period, cells were washed three times with phosphate-buffered saline and solubilized overnight with 1.5 ml of 0.1% SDS. The content of two wells was pooled and transferred into a tube containing 60 μl of 10% bovine serum albumin. Proteins were precipitated with 300 μl 20% TCA and left overnight at 4°C. Samples were then centrifuged at 2,000 g for 30 min at 4°C, the supernatant was discarded, and the pellet was resuspended in 0.5 N NaOH.

Fig. 2. Effects of high glucose concentration on ERK1/2 and Akt/PKB activity in GECs. A and C: time courses of ERK1/2 (A) or Akt/PKB (C) activation by high glucose. Serum-deprived GECs were treated with 25 mM glucose for the time periods indicated. B and D: dose-responses of ERK1/2 (B) and Akt/PKB (D) activation by glucose. Cells were treated with various concentrations of glucose (10–30 mM) for 15 min. ERK1/2 or Akt/PKB immunoprecipitates were incubated with myelin basic protein (MBP), and phosphorylation of the substrate was assayed. Top: MBP phosphorylation by glucose. Middle: immunoblot analysis of cell lysates using specific ERK1/2 or Akt/PKB antibodies. Bottom: histogram representing the ratio of the radioactivity incorporated into the phosphorylated MBP quantified by PhosphorImager analysis factored by the densitometric measurement of the ERK1/2 or Akt/PKB band. Values are means ± SE of 3 independent experiments expressed as percentage of control, where the ratio in the control was defined as 100%. *P < 0.05 and **P < 0.01 vs. control. E: serum-deprived GECs were treated with high glucose (25 mM) or mannitol (20 mM) for 15 min, and measurement of ERK1/2 and Akt/PKB activity was performed as described above. Total amounts of ERK1/2 and Akt/PKB in cell lysates are also shown. Autoradiograms and immunoblots are representative of 3 independent experiments.
Duplicate aliquots (0.5 ml) were removed and counted in a scintillation counter.

Statistical analysis. Results are expressed as means ± SE. Statistical significance was assessed by Student’s unpaired t-test. Significance was determined at P < 0.05.

RESULTS

High glucose concentration induces protein synthesis and hypertrophy of GECs. To study GECs, hypertrophy was measured as the ratio of the incorporation of [3H]leucine, a measure of protein synthesis, to the incorporation of [3H]thymidine, a measure of DNA synthesis. Exposure of quiescent confluent GECs to various concentrations of glucose (5–30 mM) dose dependently stimulated [3H]leucine incorporation. The maximal effect occurred at 25–30 mM and represented a 1.5- to 1.9-fold increase in [3H]leucine incorporation compared with cells treated with normal glucose concentration (5 mM) (Fig. 1A). Exposure of the cells to high glucose concentration (25 mM) had no significant effect on [3H]thymidine incorporation (Fig. 1B), suggesting that high glucose induces GEC hypertrophy. FCS (10% vol/vol), which served as a positive control, significantly stimulated GEC DNA synthesis. Figure 1C shows that no change in protein synthesis was observed in cells exposed to mannitol (20 mM) as an osmotic control.

Effects of high glucose on ERK1/2 and Akt/PKB activity in GECs. The MAPK family members ERK1/2 and the serine-threonine kinase Akt/PKB play a critical role in cell growth and hypertrophy in various cell types, including renal cells (11, 14, 16, 19, 20). We postulated that ERK1/2 and Akt/PKB activation contributes to glucose-induced protein synthesis and hypertrophy in GECs. To examine the effect of glucose on the activity of ERK1/2 and Akt/PKB, cultured GECs were incubated with either 25 mM glucose for different periods of time or with various concentrations of glucose (0.001–1 μM) for 15 min. Anti-ERK1/2 or anti-Akt/PKB immunoprecipitates were used in an immune complex kinase assay using myelin basic protein as a substrate. As shown in Figs. 2, A and C, high glucose concentration caused a rapid increase in ERK1/2 and Akt/PKB activity in a time-dependent manner, an effect that peaked at 15 min and subsided by 180 min. Stimulation of GECs with different concentrations of glucose showed dose-dependent increase in ERK1/2 and Akt/PKB activity, with a threshold at 20 mM and a maximal effect occurring at 25–30 mM (Fig. 1, B and D). Mannitol (20 mM) did not affect ERK1/2 or Akt/PKB activity (Fig. 2E). Glucose had no significant effect on ERK1/2 and Akt/PKB protein levels, as determined by immunoblotting (Figs. 1, A–D, middle).

Role of ERK1/2 and Akt/PKB in high glucose-induced GEC hypertrophy. The above-mentioned studies suggest that high glucose is a strong activator for ERK1/2 and Akt/PKB; therefore, we postulated that these kinases might be involved in glucose-induced protein synthesis. Inhibition of the ERK1/2 pathway by treatment of the cells with MEK inhibitor PD-98059 abrogated high glucose-induced [3H]leucine incorporation (Fig. 3A). To assess the role of Akt/PKB in GEC protein synthesis, we tested the effect of HA-tagged kinase-inactive Akt/PKB with a point mutation in the ATP-binding site [HA-Akt(K179M)] on high glucose concentration-stimulated [3H]leucine incorporation. As shown in Fig. 3B, inhibition of Akt by transient transfection of the cells with HA-Akt(K179M) abolished glucose-induced [3H]leucine incorporation. Of note, the dose-responses of high glucose-induced [3H]leucine incorporation paralleled the dose-responses of high glucose-induced ERK1/2 and Akt/PKB activation. Taken together, these findings are consistent with a critical role for ERK1/2 and Akt/PKB in the hypertrophic response of GECs to high glucose.

Effect of high glucose on ROS production in GECs. High glucose has been reported to increase ROS production in vascular cells as well as in renal cells including tubular epithelial cells or mesangial cells but not in GECs (5, 25, 27, 28). To evaluate the role of high glucose concentration as a signal leading to oxidative stress in GECs, we examined their effect on the generation of ROS. The production of intracellular ROS by GECs in response to high glucose concentration was demonstrated with a fluorescence-based assay using peroxide-

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Fig. 3. Role of ERK1/2 and Akt/PKB in high glucose-induced protein synthesis. A: serum-deprived GECs were treated with 5 mM (filled bars) or 25 mM (open bars) glucose for 48 h in the presence or absence of the MEK inhibitor PD-98059 (50 μM for 1 h). Protein synthesis was measured using [3H]leucine as described in MATERIALS AND METHODS. Values are means ± SE of 3 independent experiments expressed as percentage of control, where the cpm/well in the cells treated with 5 mM glucose (control) was defined as 100%. **P < 0.01 compared with control. **P < 0.01 compared with treatment with 25 mM glucose. B: GECs were transfected with hemagglutinin (HA)-tagged inactive Akt/PKB mutant [HA-Akt(K179M)] or vector as control and treated with 5 mM (open bars) or 25 mM (filled bars) glucose for 48 h. Bottom: immunoblot of cells transfected with vector or the dominant negative form of Akt/PKB using anti-HA antibody. Values are means ± SE of 3 independent experiments expressed as percentage of control, where the cpm/well in the cells treated with 5 mM glucose (control) was defined as 100%. **P < 0.01 compared with control. **P < 0.01 compared with treatment with 25 mM glucose + vector.
Fig. 4. **A**: effect of high glucose concentration on the production of reactive oxygen species (ROS) in GECs. GECs were serum-starved and then treated with 25 mM glucose for the indicated time periods. 2',7'-Dichlorodihydrofluorescein (DCF) fluorescence, reflecting the relative levels of intracellular ROS, was measured with a multiwell fluorescence plate reader as described in MATERIALS AND METHODS. Values are the means ± SE of 3 independent experiments. *P < 0.05 and **P < 0.001 vs. control. **B**: serum-deprived GECs were preincubated with or without catalase (300 U/ml), diphenylene iodonium (DPI; 10 μM), or N-acetylcysteine (NAC; 20 mM) for 30 min before treatment with 25 mM glucose for 10 min, and DCF fluorescence was measured with a multiwell fluorescence plate reader as described in MATERIALS AND METHODS. Values are means ± SE of 3 independent experiments. **P < 0.01 compared with control. ##P < 0.01 compared with treatment with 25 mM glucose. C: serum-deprived GECs were treated with high glucose (25 mM) or mannitol (20 mM) for 10 min, and measurement of ROS generation was performed and expressed as described above. D and E: time course of ERK1/2 (D) and Akt/PKB (E) activation by H2O2. Serum-deprived GECs were treated with 100 μM H2O2 for the indicated time periods. Measurement of ERK1/2 and Akt/PKB activity was performed as described in Fig. 2. Total amounts of ERK1/2 and Akt/PKB in cell lysates are also shown. Autoradiograms and immunoblots are representative of 3 independent experiments. H: effects of H2O2 on protein synthesis in GECs. Serum-deprived GECs were treated with the indicated concentrations of H2O2 for 48 h. I: effects of antioxidants on high glucose-induced protein synthesis in GECs. Serum-deprived GECs were treated with 5 mM (open bars) or 25 mM (filled bars) glucose for 48 h in the presence or absence of the indicated inhibitors (catalase, 300 U/ml for 30 min; DPI, 5 μM for 15 min; NAC, 20 mM for 30 min). In H and I, values are means ± SE of 3 independent experiments. **P < 0.01 compared with control. ###P < 0.01 compared with treatment with 25 mM glucose.
sensitive fluorophore DCF. Quantification of DCF fluorescence was performed with a multiwell fluorescence plate reader. Incubation of GECs with 25 mM glucose resulted in a rapid and time-dependent increase in DCF fluorescence; a significant increase was seen as early as 2.5 min after treatment (Fig. 4A). Catalase (300 U/ml) completely blocked the oxidative effect of high glucose, suggesting that intracellular hydrogen peroxide \((H_2O_2)\) is primarily responsible for the fluorescence signal (Fig. 4B). Moreover, high glucose-induced ROS production was almost completely blocked by diphenylene iodonium (DPI; 10 \(\mu M\)), an inhibitor of flavoprotein-containing enzymes, such as the NAD(P)H oxidase systems, or by the antioxidant N-acetylcysteine (NAC; 20 mM), (Fig. 4B). Exposure of the cells to mannitol (20 mM) had no effect on ROS generation (Fig. 4C).

Collectively, these results demonstrate that high glucose elicits ROS production in GECs. Furthermore, it appears that the time courses of intracellular ROS generation in response to glucose are consistent with a potential role for these ROS in downstream signaling events and, in particular, regulation of redox-sensitive protein kinases.

**Role of ROS in high glucose-induced ERK1/2 and Akt/PKB activation and in GEC hypertrophy.** It has been reported that the activity of ERK1/2 and Akt/PKB is redox sensitive in various cells type including renal proximal tubular epithelial cells or mesangial cells (7, 14, 16, 19, 46). The correlation between the time courses of high glucose-induced ERK1/2 and Akt/PKB activation and the kinetics of oxidant production in response to glucose (compare Figs. 2, A and B, and 4A) indicates that ROS may mediate the effects of high glucose on ERK1/2 and Akt/PKB. To test this hypothesis, we studied the effect of \(H_2O_2\) on ERK1/2 and Akt/PKB activation in GECs. As shown in Fig. 4, D and E, 100 \(\mu M\) \(H_2O_2\) induced robust activation of ERK1/2 and Akt/PKB. The kinetics were consistent with the time courses of high glucose-induced ERK1/2 and Akt/PKB activation by high glucose. Next, we examined the effect of NAC and DPI on high glucose-induced ERK1/2 and Akt/PKB activation. As shown in Fig. 4, F and G, NAC (20 mM) and DPI (10 \(\mu M\)) significantly inhibited both high glucose-induced ERK1/2 and Akt/PKB activation. These data strongly suggest that high glucose concentration-induced ERK1/2 and Akt/PKB activation is mediated by ROS.

Treatment of GECs with \(H_2O_2\) stimulates \([H]leucine incorporation\) (Fig. 4H), indicating that ROS induce protein synthesis. Exposure of GECs to 300 U/ml of catalase, 20 mM NAC or 5 \(\mu M\) DPI, concentrations that blocked the activation of ERK1/2 and Akt/PKB and ROS generation, inhibited the increase in \([H]leucine incorporation induced by high glucose\) (Fig. 4J). Moreover, these data provide additional evidence for the involvement of a ROS-dependent mechanism in the high glucose-induced ERK1/2 and Akt/PKB activation and implicate ROS as mediators of the signaling pathway activated by high glucose, leading to GEC hypertrophy via ERK1/2 and Akt/PKB.

**Additive effect of high glucose and ANG II on the redox-, ERK1/2-, and Akt/PKB-dependent pathways modulating GEC hypertrophy.** Under hyperglycemic conditions, the vasoactive peptide ANG II has been shown to contribute to the alteration of vascular and renal cell function that leads to hypertrophy and fibrosis, the two major manifestations of diabetic vasculopathy or nephropathy (6, 49, 52). Activation of ERK1/2 and Akt/PKB pathways plays a key role in ANG II-induced deleterious effects such as cell hypertrophy (14, 19, 28, 46). Furthermore, hyperglycemia enhances the signaling pathways triggered by ANG II in various cell types (1, 2, 33, 40, 45, 50). Whether high glucose concentrations and ANG II exert additive effects on the hypertrophic cascade involving ROS, ERK1/2, and Akt/PKB, however, has not been previously investigated in GECs. First, we evaluated the effect of treatment of GECs with ANG II on ERK1/2 and Akt/PKB activation. As shown in Fig. 5, A and B, ANG II (1 \(\mu M\)) caused an increase in ERK1/2 and Akt/KB activity in a time-dependent manner, an effect that peaked at 5 min and returned to basal levels within 180 min. ANG II had no significant effect on ERK1/2 or Akt/PKB levels as determined by immunoblotting. This indicates that, similar to what is observed with high glucose concentration, ANG II stimulates the two kinases in GECs. We next investigated whether an additive or a synergistic interaction exists between high glucose and ANG II and assessed its potential role in the regulation of the redox-, ERK1/2- and Akt/PKB-dependent hypertrophic cascade accounting for GEC hypertrophy. To evaluate the effect of high glucose and ANG II on ERK1/2 and Akt/PKB activity, GECs were incubated for 5 days in media containing either normal

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**Fig. 5.** Effects of ANG II on ERK1/2 and Akt/PKB activity in GECs. A and B: time course of ERK1/2 and Akt/PKB activation by ANG II. Serum-deprived GECs were treated with 1 \(\mu M\) ANG II for the time periods indicated. ERK1/2 and Akt/PKB activities were measured as in Fig. 2. Values are means ± SE of 3 independent experiments. \(*P < 0.05\) and \(**P < 0.01\) vs. control.
glucose (5 mM) or high glucose (25 mM) before exposure to ANG II (1 μM) for 5 min. As depicted in Fig. 6, A and B, high glucose alone or ANG II under normal glucose increased ERK1/2 and Akt/PKB activity. Importantly, high glucose significantly enhanced ANG II-induced ERK1/2 and Akt/PKB activation (Fig. 6, A and B), demonstrating that high glucose and ANG II displayed an additive response. Similarly, both high glucose and ANG II increased protein synthesis. Combined exposure of GECs to high glucose and ANG II caused an augmentation in [3H]leucine incorporation greater than that observed with high glucose or ANG II alone (Fig. 6C). These data indicate that high glucose and ANG II also act in an

![Fig. 6. Additive effects of high glucose and ANG II. A and B: GECs were grown for 5 days under normal glucose (5 mM) or high glucose (25 mM) concentration and then treated for 15 min with ANG II. ERK1/2 and Akt/PKB activities were measured as in Fig. 2. Values are means ± SE of 3 independent experiments. **P < 0.01 compared with control. ###P < 0.01, compared with treatment with 25 mM glucose or 1 μM ANG II alone. C: GECs were treated with 5 or 25 mM glucose in the presence or the absence of ANG II for 48 h. Protein synthesis was measured by [3H]leucine incorporation. Values are means ± SE of 3 independent experiments. **P < 0.01 compared with control. ##P < 0.01 compared with treatment with 25 mM glucose or 1 μM ANG II alone. D: GECs were grown for 5 days under normal glucose (5 mM) or high glucose (25 mM) concentration and then treated for 15 min with ANG II in the presence or absence of the indicated inhibitors (catalase, 300 U/ml for 30 min; DPI, 10 μM for 15 min; NAC, 20 mM for 30 min). Total amounts of ERK1/2 and Akt/PKB in cell lysates are also shown. Autoradiograms and immunoblots are representative of 3 independent experiments. E: GECs were grown for 5 days under normal glucose (5 mM) or high glucose (25 mM) concentration and then treated for 15 min with ANG II in the presence or absence of the indicated inhibitors (catalase, 300 U/ml for 30 min; DPI, 10 μM for 15 min; NAC, 20 mM for 30 min). Protein synthesis was measured by [3H]leucine incorporation. Values are means ± SE of 3 independent experiments. **P < 0.01 compared with control. ###P < 0.01 compared with treatment with 25 mM glucose or 1 μM ANG II alone. @@P < 0.01 compared with 25 mM glucose + 1 μM ANG II.}
additive manner to induce protein synthesis. Finally, the redox dependency of this additive effect was tested. Figure 6D showed that treatment with catalase, DPI, or NAC totally abrogated the additive effect of high glucose and ANG II on ERK1/2 and Akt/PKB activity. Moreover, the induction of ERK1/2 and Akt/PKB activity by high glucose or ANG II alone is also mediated by ROS. Similarly, catalase and DPI abolished the additive action of high glucose and ANG II on protein synthesis as well as the increase in \[^{3}H\]leucine incorporation due to high glucose or ANG II by themselves (Fig. 6E). This suggests that the additive response to high glucose concentration and ANG II is a redox-sensitive process and that augmented ROS generation may account for the amplified ERK1/2 and Akt/PKB activation and subsequent GEC protein synthesis and hypertrophy.

DISCUSSION

In this study, we provide evidence that exposure of GECs to high glucose activates MAPK ERK1/2 and the serine-threonine protein kinase Akt/PKB through generation of ROS. We also demonstrate that this redox-dependent activation of these signaling molecules mediates high glucose-induced GEC protein synthesis and cell hypertrophy. Furthermore, high glucose concentration and ANG II have additive effects on ERK1/2 and Akt/PKB activation and protein synthesis in GECs. The data indicate that oxidative stress contributes to podocyte injury in diabetes.

In a number of cells, high glucose promotes an increase in ERK1/2 and Akt/PKB activity (12, 18, 38, 41, 42). In GECs, we found that the increase in the activity of the two kinases was observed after a short (5–180 min) as well as prolonged exposure (5 days) to high glucose. Prolonged exposure of GECs results in ERK1/2 activation (22). However, this is the first report of activation of the MAPK after short-term exposure to high glucose. It is also the first report of Akt/PKB activation by high glucose in these cells. Hence, activation of the ERK1/2 and Akt/PKB pathways represents a very proximal step in the intracellular signaling triggered by high glucose in GECs. Despite the demonstration that high glucose stimulates protein synthesis and hypertrophy in cultured GECs (22, 54) and that, in vivo, GEC hypertrophy contributes to glomerular enlargement in diabetic nephropathy (49), the mechanisms underlying these processes or the profile of growth of these cells in a diabetic milieu remains largely unknown. Understanding the events involved in GEC hypertrophy is important.

Indeed, it is postulated that GECs do not replicate, most likely because of the constitutive overexpression of cell cycle-arresting proteins, which include cyclin-dependent kinase inhibitors such as p21Cip1 or p27Kip1 (36, 49). Therefore, GECs typically cope with injury in many instances by undergoing hypertrophy (23, 32, 52). Recent evidence suggests that GECs play a critical role in the early functional and structural changes of diabetic kidney disease. Hypertrophy of GECs with broadening and effacement of their foot processes, associated with a decrease in their number per glomerulus, occurs in type 1 and 2 diabetes (13, 31, 35, 41, 43, 44). Hypertrophy eventually leads to a degenerative process in GECs that accounts for the development of proteinuria and glomerulosclerosis in diabetes (8, 13, 20, 27, 29, 35, 43, 44). GEC hypertrophy occurs early in diabetes and accompanies glomerular enlargement (9, 20, 29).

At this stage, broadening of the foot process occurs to cover the expanded basement membrane (9, 20, 29). The development of diabetic nephropathy in experimental animal models as well as in diabetic patients is associated with a decrease in the number of GECs (13, 35, 41, 43). Given that the GECs are unable to replicate, remaining GECs expand and broaden their foot processes to cover the denuded basement membrane (8, 13, 31, 35, 41, 43, 44). Moreover, Wiggins et al. (48), using a rat model of aging, provided evidence that there are recognizable stages of hypertrophy through which GEC pass en route to GEC loss and consequent glomerulosclerosis. These authors postulated that similar stages of GEC hypertrophy occur in association with glomerulonegaly caused by diabetes.

The present study provides evidence that ERK1/2 and Akt/PKB mediate high glucose-induced protein synthesis and establish a basis for the cellular mechanisms of the hypertrophic response to high glucose. It is known that Akt/PKB participates in the hypertrophy of renal cells, including mesangial cells and proximal tubular epithelial cells (14, 15, 39) as well as GECs, on stimulation with mechanical stretch (37). However, to the best of our knowledge, the kinase has not been identified as an intermediate in the signaling cascade linking high glucose to GEC hypertrophy. The involvement of the MEK/ERK1/2 pathway corroborates the report from Hoshi et al. (22) demonstrating that glucose-induced activation of ERK1/2 is responsible, at least partially, for GEC hypertrophy. Hyperglycemia and high glucose concentration induce several growth factors and upregulate their receptors in renal cells, which, in turn, result in growth and the production of extracellular matrix proteins (52). The vasoactive peptide ANG II is of obvious biological relevance as a major pathogenic mediator of diabetic nephropathy (52). In addition to its hemodynamic properties, ANG II exerts potent effects on several renal cell types to stimulate hypertrophy and synthesis of extracellular matrix proteins (6, 49, 52). Our present findings demonstrate for the first time that ANG II also elicits a robust activation of ERK1/2 and Akt/PKB in GECs. Interestingly, we found that high glucose and ANG II elicited additive responses on ERK1/2 and Akt/PKB activation and subsequent GEC hypertrophy. This supports the contention that high glucose and ANG II exert additive hypertrophic and prosclerotic effects in vascular and renal cells. For instance,
high glucose and ANG II additively induce hypertrophy in renal tubular cells (50). Furthermore, additive effects of high glucose and ANG II on ERK1/2 and Janus-activated kinase/signal transducers and activators of transcription pathway activation are key mechanisms for the increased vascular smooth muscle cell or mesangial cell growth under high-glucose conditions (1, 2, 33, 40, 45).

The identification of ERK1/2 and Akt/PKB as mediators of GEC hypertrophy is consistent with the current hypothesis that high glucose- or ANG II-induced hypertrophy requires the cyclin-dependent kinase inhibitors p21Cip1 or p27Kip1 in several renal cells including podocytes. Indeed, it has been proposed that activation of ERK1/2 and Akt/PKB by ANG II mediates p27Kip1 upregulation, leading to hypertrophy of proximal tubular epithelial and mesangial cells (19, 56). Similarly, ERK1/2-dependent increased expression of p27Kip1 plays a key role in high glucose-induced mesangial cell hypertrophy (51). Moreover, blockade of the ANG II AT1 receptor lowers p27Kip1 expression in GEC exposed to high glucose (54). These observations suggest that a related signaling cascade exists in GECs.

There is now considerable evidence that ROS function as classic second messenger molecules (10). It has been reported that ERK1/2 and Akt/PKB are activated by oxidative stress in a variety of cell types including renal cells (7, 14, 16, 19, 46). Oxidative stress has been implicated in the development of diabetic complications (5, 27, 28). High glucose induces ROS generation in vascular cells and renal cells (5, 27, 28). However, the effect of high glucose on ROS in GEC has not been investigated. Exposure of GECs to high glucose elicits a rapid increase in intracellular ROS production. This early effect supports the contention that ROS mediate early signaling events in response to high glucose. Importantly, we show that ROS, and specifically H2O2, directly induce ERK1/2 and Akt/PKB activation, as well as protein synthesis. Moreover, the effect of high glucose on ERK1/2 and Akt/PKB is markedly reduced by the antioxidants catalase, NAC, and DPI. Finally, the same antioxidants nearly abrogated high glucose-induced protein synthesis. Collectively, these findings indicate that ROS are signaling molecules responsible for ERK1/2 and Akt/PKB activation initiated by high glucose and resulting in protein synthesis. Although oxidants derived from a mitochondrial source were shown to be predominant in certain cell types in response to high glucose (5), others have reported that activation of NAD(P)H oxidases play a pivotal role in high-glucose-induced ROS generation (3, 24, 25, 27, 28). We find that the generation of intracellular ROS, the activation of ERK1/1 and Akt/PKB, as well as the stimulation of protein synthesis in response to high glucose are abolished by DPI, an inhibitor of flavin-containing oxidases, such as NAD(P)H oxidase. In addition, the kinetics of oxidant generation together with the fact that exogenously applied catalase inhibits DCF fluorescence indicate that ROS are rapidly released from GECs. This strongly suggests that ROS generation happens most likely through enhanced NAD(P)H oxidase activity. While the NAD(P)H oxidase components p47phox, p67phox, p22phox, and gp91phox/Nox2 are known to be expressed in GECs (17, 34), the precise structure and the mechanism of activation of the various oxidases responsible for generation of ROS in these cells remain to be determined.

Interestingly, the antioxidants catalase, NAC, and DPI prevented the additive effects of high glucose and ANG II on ERK1/2 and Akt/PKB activation and on protein synthesis. This strongly suggests that increased ROS generation and the consequent augmentation in ERK1/2 and Akt/PKB activity constitute the molecular basis of the additive response exhibited by high glucose and ANG II. Importantly, the action of high glucose or ANG II per se on the two kinases and on protein synthesis are redox dependent as well. Therefore, it is tempting to propose that high glucose and ANG II signaling may converge at the level of ROS, resulting in the enhancement of ANG II-stimulated ERK1/2 and Akt/PKB activation as well as GEC hypertrophy in response to high glucose. Such regulation has been reported in adipocytes, where high glucose enhances insulin-stimulated ROS generation and augments its signaling (53). In aortic endothelial cells, the effects of leptin on ROS production are also additives to those of high glucose (55).

Our results unveil the pivotal role of ERK1/2 and Akt/PKB activation through ROS by conditions mimicking hyperglycemia in GEC protein synthesis and hypertrophy. Moreover, this study provides evidence that, in conditions characterized by activation of the renin-angiotensin system such as diabetes, high glucose and ANG II may exert additive effects on GEC hypertrophy, thereby altering cell function (Fig. 7). ROS generation has been implicated in GEC dysfunction in inflammatory diseases of the kidney (34, 36, 49). Therefore, understanding the GEC response to oxidative stress and the identification of the source(s) of ROS in diabetes may have direct therapeutic implications.

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