Mesangial cell signaling cascades in response to mechanical strain and glucose

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Background. Elevated glucose levels and glomerular hypertension (Pgc) are considered to contribute to the elaboration of matrix protein by mesangial cells (MCs) in diabetic glomeruli. MCs grown in 30 mM of glucose produce excessive matrix protein, as do MCs exposed to cyclic strain, and the combination of the two exacerbates this. Tight glucose control or reduction in Pgc clinically delays progression of diabetic nephropathy. MC c-fos is induced in response to either application of strain or high ambient glucose, inducing increases in activated protein-1 transactivation activity and extracellular matrix production. Stimuli that lead to c-fos induction pass through the three mitogen-activated protein (MAP) kinase pathways: p44/42, SAPK/JNK, and p38/HOG. We studied MAP kinase activation in MCs exposed to mechanical strain and a high glucose.

Methods. MCs (passage 5 through 10) cultured for 96 hours on type 1 collagen-coated flexible-bottom plates in either 5.6 or 30 mM glucose were exposed to 5, 10, or 30 minutes of cyclic strain (60 cycles per min) by computer-driven generation of vacuums of $-14$ kPa, inducing 20% elongation in the diameter of the surface. Control MCs were grown on both coated rigid and flexible-bottom plates. Protein levels (by Western blot) and activity assays for all three kinase cascades were performed at baseline and after 5, 10, and 30 minutes. All experiments were performed in triplicate.

Results. MAP kinase signaling was seen in response to stretch, and high ambient glucose affected the pattern of activation. Both p44/42 and p38HOG kinase activities showed small increases to a maximum of 2.5- to 3.5-fold greater than static MCs at 10 minutes. Activity in both kinase cascades was slightly suppressed by 30 mM glucose. In contrast, SAPK/JNK activity was present at a very low level in static MCs and increased markedly by 10 minutes of stretch. Thirty micromolars of glucose augmented this effect by a factor of six over MCs cultured in 5.6 mM glucose after 10 minutes of stretch. Neither glucose concentration nor mechanical strain had any effect on the protein expression of any of the kinases by Western blot.

Conclusions. MAP kinase cascade signaling is seen when physical force is applied to MCs, and glucose affects the pattern of activity. Thirty micromolars of glucose markedly increase the level of SAPK/JNK activation. This may have implications in diabetic signal transduction and matrix protein production.

Clinical and experimental diabetic nephropathy is characterized by kidney and glomerular enlargement and initial increases in the single nephron glomerular filtration rate (GFR) [1], followed by glomerular capillary basement membrane thickening and accumulation of matrix components [2]. Diffuse and nodular diabetic glomerulosclerosis is the end result of this injurious process, leading eventually to renal failure.

The study of the pathogenesis of this disease has concentrated on the roles of glucose and glomerular hypertension (elevated Pgc). Several lines of evidence implicate glucose in the pathogenesis of diabetic glomerular disease. Kreisberg et al showed that high glucose increased the synthesis of the extracellular matrix (ECM) proteins fibronectin, laminin, and type IV collagen in mesangial cell (MC) cultures [3, 4]. Others have shown increases in the production and activity of transforming growth factor-$\beta_1$ (TGF-$\beta_1$), a cytokine important in the stimulation of matrix protein production, in MCs exposed to high glucose concentrations [5]. The Diabetes Control and Complications Trial showed that intensive insulin therapy in type 1 diabetes mellitus could delay the onset and slow the progression of diabetic nephropathy, implicating glucose as an important mediator in diabetic kidney injury [6].

Similarly, elevated Pgc plays a role in the genesis of diabetic kidney injury. Micropuncture data have shown rises in Pgc in diabetic rat glomeruli [7], and prevention of this by blockade of the renin-angiotensin system (RAS) ameliorates glomerular injury experimentally [1] and clinically [8]. Taken together, these studies suggest that hemodynamic factors are also important in the development of diabetic renal disease. Recently, the cellular basis for this interaction has been explored by Riser et al. They showed that mechanical strain and high glucose had additive effects on MC TGF-$\beta_1$ and collagen production [9]. The signaling pathways responsible for such an interaction have not been elucidated.
Early studies by Akai et al showed that mechanical strain increased c-fos expression in MCs [10], whereas Kreisberg et al demonstrated that high glucose could also result in MC c-fos and c-jun induction [11]. Our laboratory has observed early induction of c-fos and c-jun in diabetic rat glomeruli [12]. The c-fos and c-jun proteins, along with other jun proteins such as junB and junD, are members of the activated protein-1 (AP-1) family of transcription factors, and a heterodimeric interaction between fos and jun proteins allows for binding to consensus DNA sequences in the regulatory elements of certain genes, called AP-1 or tetradecanoylphorbol acetate (TPA)-responsive sites [13]. Wilmer and Cosio postulated that the increase in TGF-β1 synthesis was mediated through the induction of AP-1 transcription factor activity and showed increases in AP-1 binding in MCs grown in 30 mM of glucose [14].

We have recently reported early mitogen-activated protein kinase (MAPK) pathway activation in MCs exposed to cyclic strain, but the interaction between glucose and strain has not been extensively studied. Accordingly, we examined activation of the three MAP kinase cascades: p44/42 MAPK, p38HOG MAPK, and stress activated protein kinase (SAPK/JNK) in MCs stretched in normal-glucose (5.6 mM) and high-glucose (30 mM) conditions.

METHODS

Cell culture

Sprague-Dawley rat MCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum (FCS; GIBCO BRL, Grand Island, NY, USA), streptomycin (100 μg/ml), penicillin (100 U/ml), and 2 mM glutamine at 37°C in 95% air/5% CO2 as described previously [15]. Experiments were carried out in cells between passages 5 through 10.

Application of stretch/relaxation

Mesangial cells (2 x 10^5/well) were plated onto six-well plates with either a rigid or flexible bottom coated with bovine type I collagen (Flexcell International Corp., McKeesport, PA, USA). Cells were grown to confluence for 72 hours in either 5.6 mM or 30 mM and were then rendered quiescent by incubation for 24 hours in DMEM with 0.5% FCS under the same glucose conditions. Some of the flexible-bottom plates were then exposed to cycles of stretch/relaxation for periods of 5, 10, and 30 minutes by exposure to a cyclic vacuum generated by a computer-driven system (Flexercell Strain Unit 2000; Flexcell Co.). Plates were exposed to continuous cycles of stretch/relaxation, with each cycle being 0.5 seconds of stretch and 0.5 seconds of relaxation, for a total of 60 cycles per minute. The vacuum was −14 kPa and induced a 20% elongation in the diameter of the surface. Control (un-stretched) cells were studied after the same growth and quiescence protocols, using both rigid and flexible-bottom type I collagen-coated plates.

Protein isolation

Cellular levels of p44/42, JNK/SAPK, and p38 MAP kinase proteins were determined in stretched and unstretched control cells at baseline (after a total of 96 hr in the respective glucose environments) and then at 5, 10, and 30 minutes after the application of stretch. Briefly, at the end of each time point, media were removed, and the cells were washed once with ice-cold phosphate-buffered saline. Phosphate-buffered saline was then removed and cells were harvested under non-denaturing conditions on ice by incubation for five minutes with 0.5 ml 1 x ice-cold cell lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM egtazic acid (EGTA), 1% triton, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na orthovanadate, 1 μg/ml leupeptin] and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then scraped into microcentrifuge tubes on ice and sonicated four times for five seconds each. After microcentrifugation at 14,000 r.p.m. for 10 minutes at 4°C, the supernatant was transferred to a fresh microcentrifuge tube. The protein concentration was measured with the Bio-Rad (Richmond, CA, USA) assay kit as mentioned earlier in this article.

Western blotting for mitogen-activated protein kinase, SAPK/JNK, and p38HOG

Forty micrograms of sample were then separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis to a nitrocellulose membrane (Protran, Schleicher and Schuell, Keene, NH, USA), membranes were incubated for three hours at room temperature with 25 ml of blocking buffer (1 x TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk) and then overnight at 4°C with p44/42 MAPK (thr 202/tyr 204) polyclonal antibody (1:1000), SAPK/JNK polyclonal antibody (1:1000), or p38 MAP polyclonal antibody (1:1000; all New England Biolabs, Beverly, MA, USA) in 10 ml of antibody dilution buffer [1 x TBS, 0.05% Tween-20 with 5% bovine serum albumin (BSA)] with gentle rocking overnight at 4°C. Membranes were then washed three times with TTBS and then incubated with horseradish peroxidase-conjugated antirabbit secondary antibody (1:2000) in 10 ml of blocking buffer for 45 minutes at room temperature. After three further Tris-buffered saline washes, the membrane was incubated with Lumiglo reagent (KPL Inc., Gaithersburg, MD, USA) and then exposed to x-ray film (X-OMAT; Eastman Kodak, Rochester, NY, USA).

Mitogen-activated protein kinase activity assays

Immunoprecipitation. After protein isolation from total cell lysate, as discussed earlier in this article, 200 μg
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Fig. 1. Representative autoradiographs of p44/42 mitogen-activate protein (MAP) kinase protein expression by Western blot. Bands migrated to the expected 42 and 44 kD and, consequently, are difficult to separate. Densitometry of the Western blot revealed no significant difference in p44/42 MAP kinase protein levels at any time point (data not shown).

“Pull-down” SAP kinase assay

After protein isolation, as discussed earlier in this article, 2 μg of c-Jun fusion protein beads (New England Biolabs) were added to 250 μg of cell lysate protein and incubated overnight at 4°C. Lysate was then centrifuged for 30 seconds to recover the beads and was washed twice with 1 × lysis buffer as discussed earlier in this article. The pellet was then resuspended in kinase buffer and boiled as previously. Twenty microliters of sample were run on a 12% SDS-PAGE gel. Blotting and detection were as discussed earlier in this article, except that the primary antibody was phospho-specific c-Jun (ser63) at a 1:1000 dilution.

RESULTS

p44/42 mitogen-activated protein kinase expression and activity

Mesangial cells were grown in 5.6 mM or 30 mM glucose for 72 hours prior to growth arrest. After 24 hours of growth arrest in 0.5% FCS, cells were subjected to mechanical strain designed to elicit 20% elongation. Western blot analysis of p44/42 protein showed that preincubation with 30 mM glucose did not influence p44/42 MAPK expression (Fig. 1), and neither did the application of mechanical strain. Cell lysates were then subjected to immunoprecipitation with p44/42 MAPK (thr202/tyr204) monoclonal antibody, and p44/42 MAPK activity was measured by Western blot analysis of the phosphorylation of a target Elk-1 fusion protein by the immunoprecipitate using a phospho-specific anti–Elk-1 (ser383) antibody. Preincubation with 30 mM glucose for 96 hours did not lead to an increase in basal p44/42 activity in MCs grown on type 1 collagen on either rigid or flexible-bottom plates (Fig. 2). As expected, 20% elongation led to a twofold to threefold increase in p44/42 MAPK activity in 5.6 mM glucose, peaking 10 minutes after the initiation of strain (Fig. 2). A smaller increase in p44/42 activity was observed in response to mechanical strain when MCs were grown in 30 mM glucose, although the time course was the same, with activity peaking at 10 minutes (Fig. 2). Densitometry of these activity values are presented graphically in Figure 3.
Fig. 2. Representative autoradiographs of p44/42 MAP kinase activity by Western blot of immunoprecipitates from cell lysates after incubation with an Elk-1 fusion protein. The expected molecular weight of the phosphorylated substrate, Elk-1, is 40 kD. (Left panel) In 5.6 mM glucose, application of −14 kPa mechanical strain (20% elongation) to mesangial cells led to an increase in p44/42 MAP kinase activity of 2.5-fold over baseline at 10 minutes. (Right panel) In 30 mM glucose, application of −14 kPa mechanical strain (20% elongation) to mesangial cells led to an increase in p44/42 MAP kinase activity to about twofold over baseline at 10 minutes. Absolute levels of activity were lower in 30 mM glucose at all time points when compared with 5.6 mM glucose. Experiments were performed in triplicate.

Fig. 3. Densitometry of p44/42 MAP kinase activity data. Bars show p44/42 MAP kinase activity in mesangial cells stretched at −14 kPa (20% elongation) in either 5.6 mM (□) or 30 mM (■) glucose (N = 3 for each experiment).

p38HOG MAP kinase expression and activity

We next sought to determine the effects of stretch and glucose on p38HOG MAP kinase activity. MCs were grown in 5.6 mM or 30 mM glucose for 72 hours prior to growth arrest. After 24 hours of growth arrest in 0.5% FCS, cells were subjected to mechanical strain designed to elicit 20% elongation. Western blot analysis of p38 protein showed that preincubation with 30 mM glucose did not influence p38 HOG MAPK expression (Fig. 4), and neither did the application of mechanical strain. Cell lysates were then subjected to immunoprecipitation with p38 MAPK monoclonal antibody, and p38 MAPK activity was measured by Western blot analysis of the phosphorylation of a target ATF-2 fusion protein by the immunoprecipitate using a phospho-specific anti–ATF-2 antibody. Preincubation with 30 mM glucose for 96 hours did not lead to an increase in basal p38 HOG MAPK activity in MCs grown on type 1 collagen on either rigid or flexible-bottom plates (Fig. 8). A marked and significant increase (P < 0.01 when compared with baseline) in SAPK/JNK activity was found in cells stretched in 30 mM glucose, which appeared at 5 minutes and peaked at 10 minutes (Fig. 8). Although a similar time course of changes was seen in MCs stretched in 5.6 mM glucose, the quantitative increase in activity was much less than that observed in MCs grown in high (30 mM) glucose media (P < 0.01 at all time points after stretch application). These densitometry data are presented graphically in Figure 9.

DISCUSSION

The regulation of gene transcription in cells of the diabetic glomerulus has not been fully elucidated, but a series of recent studies has focused on the proto-oncogenes c-fos and c-jun, which together constitute the AP-1 transcription factor. We have shown sustained c-jun tran-
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Fig. 4. Representative autoradiographs of p38-HOG kinase protein expression by Western blot. The product appears as a 38 kD band. Densitometry of the Western blot revealed no significant difference in p38 HOG kinase protein levels at any time point (data not shown).

Fig. 5. Representative autoradiographs of p38-HOG MAP kinase activity by Western blot of immunoprecipitates from cell lysates after incubation with an ATF-2 fusion protein. The expected molecular weight of the phosphorylated substrate, ATF-2, is 35 kD. (Left panel) In 5.6 mm glucose, application of −14 kPa mechanical strain (20% elongation) to mesangial cells led to an approximate fivefold increase in p38HOG MAPK activity by 10 minutes, although absolute values were small. (Right panel) In 30 mm glucose, application of −14 kPa mechanical strain (20% elongation) to mesangial cells resulted in no increase in p38 HOG kinase activity through to 30 minutes. Experiments were performed in triplicate.

Fig. 6. Densitometry of p38 HOG MAP kinase activity data. Bars show p38 HOG MAP kinase activity in mesangial cells stretched at −14 kPa (20% elongation) in either 5.6 mm (●) or 30 mm (■) glucose (N = 3 for each experiment).

found that the application of mechanical strain to MCs led to increases in c-fos transcripts by 30 minutes [10]. Following this, increases in types I and IV collagen, laminin, and fibronectin synthesis were demonstrated in stretched MCs, linking physical forces to the production of ECM protein [16]. We have reported that p44/42 MAPK and p38 MAPK, but not SAPK/JNK, were activated in response to cyclic stretch for up to two hours following the initiation of strain in a physiologic (5.6 mmol) glucose medium [17].

Taken together, these studies provide a rationale for studies of metabolic and hemodynamic determinants of glomerular injury in diabetes. Our hypothesis was that metabolic (high glucose) and hemodynamic (cyclic stretch) factors play an important role in the pathogenesis of diabetic glomerulopathy. In this regard, Akai et al

scriptural induction in diabetic kidney after one week [12] and transient c-fos induction, suggesting that AP-1 may play an important role in the initial regulation of gene expression in the early phase of diabetic glomerular hypertrophy. In accord with this observation, Kreisberg et al found c-jun transcription to be sustained in vitro with exposure of MCs to 30 mm glucose [11].

Hyperglycemia is necessary, but not sufficient, for the development of diabetic nephropathy. Experimental [7] and clinical [8] studies support the view that hemodynamic factors play an important role in the pathogenesis of diabetic glomerulopathy. In this regard, Akai et al
phorylation on threonine and tyrosine residues by upstream dual-specificity kinases [18]. In general, these pathways consist of three modules: an ERK, activated by a MEK, which in turn is activated by a MEK kinase (MEKK). The archetypal pathway, p44/42, is strongly activated by growth factors. Phosphorylation of ERK in the p44/42 cascade in turn leads to phosphorylation of ternary complex factor (Elk-1), resulting in formation of the ternary complex and induction of c-fos transcription [19]. Upstream of the ERKs and responsible for their phosphorylation is MEK-1/-2 (MAP kinase/ERK kinase) [20, 21], which is, in turn, activated by the serine threonine kinase Raf-1 [21], at the MEKK level. Raf-1, in turn, appears to be activated by a conformational change induced by interaction with membrane bound Ras [22]. Raf-1 may also be activated in the absence of interaction with Ras via protein kinase C (PKC), which can phosphorylate Raf-1 directly [23]. PKC activation has been demonstrated in MCs cultured in high glucose and diabetic glomeruli [24]. The induction of AP-1 DNA binding in response to high glucose in MCs was at least partially PKC dependent, lending experimental support to this proposed series of events [14]. The time course is important: c-fos transcripts are elevated up until 24 hours in MCs exposed to high glucose [11] or in diabetic glomeruli [12], but they return to normal or below baseline by 48 hours to 5 days [25]. However, AP-1 DNA binding is still increased at five days and later [14], suggesting that effects on c-jun, the other member of the AP-1 heterodimer, might be important.

The c-Jun-N-terminal (JNK) kinase cascade, or stress-activated protein kinase (SAPK) pathway, also displays proline specificity in the dual phosphorylation of threonine and tyrosine residues [20]. At the ERK level, SAPK (JNK) mediates translational activation of c-Jun by phosphorylating it at serines 63 and 73 in a specific process involving a stable SAPK:c-Jun complex [26]. The induction of c-jun transcription is also SAPK/JNK dependent via a cis-TRE element, which is constitutively occupied by c-Jun protein and ATF-2 as a heterodimer [27], and transcription is stimulated when these proteins are phosphorylated in situ following exposure of the cell to stimuli that activate SAPK/JNK as described earlier in this article [28]. ATF-2 is also phosphorylated in its N-terminal domain by SAPK/JNK pathway activation, which potentiates the transcription inducing activity of ATF-2 [29].
C-Jun/ATF-2 complexes appear to prefer CRE (TGAC GTCA) sequences [20]. Upstream of SAPK lies the MEK kinase called SEK [30], which, in turn, is activated by MEKK-1 at the MEKK level [31].

The relationship between the effect of stretch and high glucose on SAPK/JNK activation and the regulation of gene expression was not investigated in this study, although the interaction of stretch and glucose on ECM protein synthesis has been reported by Riser et al [9]. The combination of stretch and high glucose was found to result in greater MC TGF-β1 and ECM production than either in isolation. The TGF-β1 promoter has AP-1 consensus sites [15, 32]. Given the recent report that Jun and Fos co-operate with SMAD proteins in directing TGF-β1–induced transcription of target genes [33], it is tempting to speculate that SAPK signaling may not only stimulate TGF-β1 production by MCs, but may also modulate TGF-β1–directed ECM production in the diabetic milieu. Extracellular stimuli known to activate SAPK such as oxidants [34, 35] and osmotic stress [36, 37] exist in the diabetic milieu, and the addition of mechanical strain as a further stimulus to activation increases the likelihood that SAPK signaling may play a pathogenic role in the diabetic glomerulus. A number of potentially pathogenic genes might be up-regulated by the preferential CRE transactivating activity of c-Jun/ATF-2 such as endothelin-1 [20] and the adhesion molecule E-selectin [38]. SAPK activation has also been implicated in the negative modulation of the mitogenic response to growth factors [31], potentially an important role in the diabetic glomerulus, which is characterized by resident cell hypertrophy rather than proliferation.

In conclusion, we have shown that in rat MCs exposed to 30 mm glucose and cyclic stretch of −14 kPa, early SAPK/JNK kinase cascade activation is seen. These data indicate that both metabolic and hemodynamic factors play a role in the genesis of intracellular signaling changes in the diabetic milieu. Further studies will be necessary to more closely define the downstream consequences of this change in kinase signaling.

References

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