High glucose-enhanced activation of mesangial cell p38 MAPK by ET-1, ANG II, and platelet-derived growth factor

EVA GTANIA, POLI LEKAS, I. GEORGE FANTUS, JOHN DLUGOSZ, and CATHARINE WHITESIDE. High glucose-enhanced activation of mesangial cell p38 MAPK by ET-1, ANG II, and platelet-derived growth factor. Am J Physiol Endocrinol Metab 282: E161–E169, 2002.—Mitogen-activated protein kinase (MAPK) p38 is activated in response to stress stimuli and growth factors relevant to the pathogenesis of diabetic nephropathy. We postulated that mesangial cells exposed to high glucose and to endothelin-1 (ET-1), angiotensin II (ANG II), and platelet-derived growth factor (PDGF) demonstrate enhanced p38 activity and subsequent activation of the cAMP responsive element binding (CREB) transcription factor. Primary rat mesangial cells exposed to 5.6 (NG) or 30 mM glucose (HG) or NG plus 24.4 mM sorbitol (osmotic control) for 4 days were acutely stimulated with ET-1, ANG II, or PDGF. After 3 days of HG, p38 phosphorylation and kinase activity increased twofold (P < 0.05 vs. NG, n = 5). No change in p38 activity was observed with sorbitol. In HG, activation of p38 by ET-1, ANG II, or PDGF was enhanced compared with NG and was protein kinase C (PKC) independent. In HG, CREB phosphorylation in response to ET-1, ANG II, and PDGF stimulation was enhanced compared with NG and was abolished by p38 inhibition with SB202190. To conclude, in HG, mesangial cell p38 is activated, which in turn stimulates CREB phosphorylation. Furthermore, in HG, mesangial cell p38 responsiveness to ET-1, ANG II, and PDGF and consequent CREB phosphorylation are enhanced through a PKC-independent pathway, which may contribute to the pathogenesis of diabetic nephropathy.

IN DIABETIC HUMANS and animal models susceptible to progressive nephropathy, high glucose causes glomerular mesangial cells to increase production and decrease degradation of extracellular matrix proteins, resulting in mesangial expansion (36, 47, 49, 61). Within hours to days, exposure of mesangial cells to high glucose causes activation of specific diacylglycerol (DAG)-sensitive protein kinase C (PKC) isozymes (2, 30, 32) and mitogen-activated protein kinase (MAPK) (13, 19, 24) signaling pathways. Downstream responses include increased expression of c-fos and c-jun (48), transforming growth factor (TGF)-β (34), collagen IV (60, 61), and fibronectin (50). Because progressive nephropathy occurs in 30–40% of individuals with diabetes (33, 45), high glucose is necessary but not sufficient to cause glomerulosclerosis. Also implicated in the pathogenesis of human and experimental diabetic nephropathy are peptide growth factors, including angiotensin II (ANG II) (37) and endothelin-1 (ET-1) (12, 51). In rat models, platelet-derived growth factor (PDGF) is an important factor in the pathogenesis of nondiabetic progressive glomerulosclerosis (27).

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Materials and Methods

Reagents. Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, fetal calf serum (FCS), trypsin, and human plasma fibronectin were purchased from Gibco Life Technologies (Burlington, ON, Canada). Phosphospecific p38 antibody and affinity-purified polyclonal anti-p38 antibodies, used for immunoblotting and immunoprecipitation, and activating transcription factor 2 (ATF-2) fusion protein were purchased from New England Biolabs (Mississauga, ON, Canada). Phosphospecific and total CREB antibodies used for immunoblotting were purchased from New England Biolabs (Mississauga, ON, Canada). Horseradish peroxidase-conjugated anti-rabbit IgG was from Bio-Rad (Hercules, CA), peroxidase-conjugated goat anti-mouse secondary antibodies and FITC-conjugated goat anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Specific fluorescence probes, FITC-secondary antibody, and SlowFade antifade reagent were purchased from Molecular Probes (Eugene, OR). Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (San Diego, CA). The apoptosis assay kit and human PDGF were purchased from Boehringer Mannheim (Laval, QC, Canada). ET-1, ANG II, and all other chemicals were purchased from Sigma Chemical (St. Louis, MO). The Bio-Rad DC protein assay kit was obtained from Bio-Rad (Mississauga, ON, Canada). BSA was obtained from ICN Pharmaceuticals (Montreal, QC, Canada).

Mesangial cell culture. Mesangial cells were obtained from glomeruli of 150-g male Sprague-Dawley rats, and primary cultures were established as previously described (2, 13, 30). Cell culture surfaces were evenly coated with human plasma fibronectin dissolved in sterile water to a concentration of 50 μg/ml. The surfaces were left to dry and used within 1 wk. Cells were grown on fibronectin-coated surfaces including 6-well plates for experiments using total cell lysates, 100-mm dishes for p38 activity assay, or glass coverslips for confocal microscopy. Mesangial cells, passage 10, were used for all experiments and were cultured in medium, pH 7.4, containing DMEM (5.6 mM glucose), 10 mM HEPES, 44 mM NaHCO3, 12.5 U/ml penicillin G, 12.5 U/ml streptomycin, and 20% FCS, which was changed every 2 days. Subcultures of passage 10 were used for all of the immunoblotting and p38 activity experiments and for confocal imaging studies. At confluence, the cells were growth arrested by exposing them to DMEM containing 0.5% FCS in the presence of 5.6 or 30 mM glucose, or 5.6 mM glucose plus 24.4 mM sorbitol. Stimulation of the cells with ET-1, PDGF, ANG II, or sorbitol (400 mM) was performed at 37°C with agonist concentrations, and duration is indicated in RESULTS.

Western immunoblot analysis. At the end of treatment, cells were washed and harvested for total protein by use of previously described methods (2, 19). Cell were lysed in boiling 2× SDS sample buffer, passed through a 26-gauge needle five times, and boiled for 5 min. Cell lysates were assayed for total protein by the modified micro-Lowry method with the use of the Bio-Rad DC kit with BSA as the standard. Sample buffer was added. Equal amounts of protein (15 μg) from each sample, along with molecular weight standards (Hiillex, Scarborough, ON, Canada) and C6 glioma cell positive control samples, were separated by 10% SDS-PAGE at 100 V for 2 h and blotted onto an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) overnight at 32 V by means of a Bio-Rad Trans blot apparatus. The membrane was blocked for 1 h in Tris-buffered saline containing Tween 20 (TBST: 10 mM Tris-HCl, 150 mM NaCl, 0.1% (vol/vol) Tween 20) and 5% nonfat dry milk powder at room temperature. Phosphorylated and total cellular p38 were detected using p38-specific antibodies at 1:2,000 dilution for 2 h at room temperature. The phosphospecific p38 antibody detects the dual-phosphorylated threonine and tyrosine present in the TGY motif of p38, which is associated with its activation. All antibodies were diluted in TBST containing 5% BSA. After being washed with TBST, the membranes were incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated IgG (1:5,000). After being washed with TBST three times, a chemiluminescence kit (Kirkgaard & Perry Laboratories, Gaithersburg, MD) and Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) were used for development and visualization of immunoreactive bands according to the manufacturer’s instructions. To determine the uniformity of loading and transfer, PVDF membranes were stained with Ponceau-S. To analyze the density of the bands, the immunoblots were digitized using the White/UV Transilluminator Gel Documentation System and UV Image Store 5000 (UVP Diamed Lab Supplies, Mississauga, ON, Canada). National Institutes of Health (NIH) Image (1.62) software was used to obtain densitometry measurements. Relative quantities of total and phosphorylated p38 or phosphorylated ATF-2 fusion protein were compared after normalization to the respective values in 5.6 mM glucose on the same gels arbitrarily assigned a value of 1.0.
p38 Activity assay. The cultured mesangial cells were treated as described for the p38 phosphorylation studies. At the end of the treatment period, the cells were rinsed once with ice-cold PBS, and then lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] was added, and the plates were left on ice for 5 min. The cells were scraped off the plate, sonicated (4 times for 5 s each with a Branson sonicator), and microcentrifuged for 10 min at 4°C, and the supernatant (cell lysate) was collected and assayed for protein by use of the modified Lowry protein assay. An equal amount of protein (200 μg) was taken from each sample, and specific p38 polyclonal antibody (1:100 dilution) was added for immunoprecipitation. After overnight incubation at 4°C, protein A-Sepharose beads were added, and the incubation was continued for 3 h. The immunoprecipitates were microcentrifuged for 30 s at 4°C, the pellets were washed twice with lysis buffer and twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl2), and then suspended in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg of ATF-2 fusion protein followed by incubation for 30 min at 30°C. The reaction was terminated by the addition of 25 μl of 3× SDS sample buffer. The samples were boiled for 5 min, vortexed, and microcentrifuged. The same volume of each sample (20 μl) was loaded on a 12% SDS-PAGE gel. Immunoblotting was performed as described, with the use of a phosphospecific antibody. The immunoblot is representative of 3 separate experiments. HG, high glucose.

Apoptosis assessment. Terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-fluorescein end labeling (TUNEL) analysis was performed by using an in situ cell death detection kit (Boehringer Mannheim) according to the manufacturer's protocol. In brief, after exposure of the cells on fibronectin-coated glass coverslips to normal glucose or high glucose for 3 days or to normal glucose for 3 days followed by 15 min of ultraviolet light, 3 h later, they were washed with 3× PBS at room temperature, fixed, and per-
Table 1. Dose-dependent phosphorylation of mesangial cell p38 after 10 min of stimulation with ET-1 or PDGF or 5 min with ANG II

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>Fold Increase in p38 Phospho/Total vs. Control</th>
<th>P Value vs. Control</th>
</tr>
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<tbody>
<tr>
<td>ET-1</td>
<td>100</td>
<td>5.56 ± 1.48</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.00</td>
<td></td>
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<tr>
<td></td>
<td>1.0</td>
<td>2.24 ± 0.16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>ANG II</td>
<td>1000</td>
<td>2.24 ± 0.22</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.02</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.53 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>PDGF</td>
<td>50</td>
<td>4.40 ± 0.75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>2.82 ± 0.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

Values of fold increase are means ± SE. Concentrations for endothelin-1 (ET-1) and ANG II are nanomolar and for platelet-derived growth factor (PDGF) are nanograms per milliliter. NS, not significant.

RESULTS

Effects of high glucose on p38 activation. Exposure of mesangial cells to 30 mM glucose for 3 days resulted in a twofold increase \((P < 0.05\) vs. normal glucose) in p38 phosphorylation (Fig. 1A). The levels of total p38 were not changed in high glucose (Fig. 1A). Ponceau-S staining confirmed that, within each experiment, the individual lanes were loaded with equal amounts of protein (data not shown). Also, high glucose did not alter total cellular protein recovery \((2.4 ± 0.2 \text{ mg/ml}} in 30.0 \text{ mM glucose vs. } 2.5 ± 0.2 \text{ mg/ml in 5.6 mM glucose). Glucose levels in the normal- and high-glucose incubation media, as measured by a Beckman autoanalyzer, remained unchanged over 4 days (data not shown). To confirm p38 activation after 3 days of high-glucose exposure, phosphorylation of an ATP-2 fusion protein substrate by immunoprecipitated p38 was observed (Fig. 1B).

Effect of osmolarity. To examine whether changes in osmolarity accounted for the altered p38 activity, cells were incubated in sorbitol at 400 mM as a positive control and in 5.6 mM glucose plus 24.6 mM sorbitol to mimic the high glucose concentration and duration of treatment. Acute stimulation of cells with 400 mM sorbitol resulted in a fourfold increase in p38 phosphorylation, whereas exposure to 5.6 mM glucose plus 24.6 mM sorbitol over 3 days had no effect (data not shown).

ET-1, ANG II, and PDGF stimulation. Stimulation of mesangial cells in 5.6 mM glucose with 100 nM ET-1 for 10 min, 1 \mu \text{M ANG II for 5 min, or } 50 \text{ ng/ml PDGF for 10 min} caused p38 phosphorylation (Fig. 2). Further analysis of the p38 response to the three agonists by immunoblotting revealed a concentration-dependent increase in phosphorylation (Table 1). To determine whether p38 activation by these agonists was enhanced in high glucose, a submaximal concentration of each (10 nM ET-1, 100 nM ANG II, and 5 ng/ml PDGF) was used to compare the p38 response in normal vs. high glucose. A significant potentiation of p38 phosphorylation by high glucose was observed for all three agonists (Fig. 3).

We (2, 30) and others (8, 32, 49) have shown that high glucose causes increased expression and activation of mesangial cell DAG-responsive PKC isozymes. Furthermore, the increased response of ERK1/2 to ET-1 in high glucose appeared to be PKC dependent (13). The DAG-sensitive PKC isozymes, which are increased in glomerular mesangial cells exposed to high glucose (30), can be acutely activated by phorbol ester. PMA acutely stimulated the phosphorylation and activity of p38 (Fig. 4). DAG-sensitive PKC isozymes-α, -β, and -ε were downregulated by chronic PMA in high glucose, whereas PKC-ζ, resistant to DAG/phorbol ester...
ter activation, remained unchanged after 24-h PMA exposure (Fig. 5A). p38 activation by high glucose alone was not significantly decreased by downregulation of PKC (Fig. 5B). Similarly, the stimulation of phosphorylation and activation of p38 by ET-1, ANG II, or PDGF were not altered by chronic pretreatment with PMA in high glucose (Fig. 6) or normal glucose (data not shown).

**Activated p38 and CREB phosphorylation.** Activation of the p38 MAPK signaling pathway may mediate phosphorylation of the transcription factor CREB (25, 55). To examine this possible downstream effect of p38 activation, first it was established that the p38 inhibitor SB202190 abolished anisomycin activation of ATF-2 (Fig. 7). Then, we assessed CREB phosphorylation by ET-1, ANG II, and PDGF stimulation under normal and high glucose conditions with and without SB202190. CREB phosphorylation was enhanced in cells exposed to high vs. normal glucose (Fig. 8A) or normal glucose (data not shown).

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in both normal and high glucose (Fig. 8B). The concentration of SB202190 utilized in this study had no effect on ERK1/2 activity (data not shown). Figure 8C illustrates that the PKC inhibitor calphostin C failed to inhibit ET-1, ANG II, and PDGF activation of p38 and CREB phosphorylation in both normal and high glucose.

Apoptosis assay. Mesangial cell apoptosis was examined using an in vitro cell death detection assay. Cells exposed to ultraviolet light for 15 min demonstrated significant nuclear labeling with fluorescein, indicating apoptosis. Cells in 5.6 or 30 mM glucose for 3–5 days demonstrated no nuclear labeling, suggesting that apoptosis did not occur under these conditions. Additionally, Wright-Giemsa staining (which detects damaged nuclear DNA) revealed no difference in the intensity of nuclear staining between normal and high glucose-exposed cells (arbitrary units of pixel intensity per nucleus 51 ± 2 and 50 ± 3, respectively, images not shown).

DISCUSSION

High glucose alters mesangial cell phenotype through signaling events that regulate gene expression. We report that, in 30 mM glucose, glomerular mesangial cells demonstrate not only increased basal activity of p38 compared with cells in 5.6 mM glucose but also enhanced responsiveness of p38 to ET-1, ANG II, and PDGF. Activation was demonstrated by increased phosphorylation of p38 and by enhanced phosphorylation of an ATF-2 fusion protein by immunoprecipitated p38. Apoptosis was not associated with these findings. These observations are consistent with the recent report that high-glucose exposure of vascular smooth muscle cells also leads to increased p38 activity and enhanced responsiveness to ANG II (41). Although 400 mM sorbitol enhanced p38 activity as expected, 24.6 mM sorbitol did not alter p38 activity, indicating that the effect of high glucose was not due to increased osmolarity. In pathological states such as diabetes, mesangial cells are likely exposed to peptide growth factors.
factors, including ET-1, ANG II, and PDGF, all of which have been implicated in the pathogenesis of diabetic nephropathy (10, 12, 37, 51). ET-1 stimulates p38 in cardiac myocytes (6, 42) and pulmonary artery smooth muscle (56). Additionally, ANG II (35, 41) and PDGF (39, 44) stimulate vascular smooth muscle p38 activity. However, ours is one of the first studies to show that ET-1, ANG II, and PDGF activate p38 in mesangial cells, p38 is known to phosphorylate transcription factors including ATF-2 (26), CCAAT/enhancer-binding protein homologous protein-1 (54), nuclear factor-κB (53), myocyte-specific enhancer binding factor 2C (18), Ets-like transcription factor-1 (43) and CREB (55). Natarajan et al. (41) demonstrated that the combination of ANG II and high glucose had additive effects on AP-1 activity of vascular smooth muscle cells. In the present study, we demonstrate that nuclear CREB phosphorylation is enhanced in high glucose and further augmented during stimulation by ET-1, ANG II, and PDGF compared with the responses in normal glucose. Glucose-dependent CREB phosphorylation, in the absence of peptide agonist stimulation, appears to be entirely dependent on p38 and independent of DAG-sensitive PKC. These findings are consistent with a major role for p38 in high glucose-enhanced CREB activation with or without peptide agonist action.

The stimulation of p38 by cytokines, ultraviolet radiation, and hyperosmolarity is well described in many cell types including mesangial cells (16, 17). We demonstrated that PMA, presumably through DAG-sensitive PKC activation, increases p38 phosphorylation, suggesting a potential link between PKC and p38. Several lines of evidence have implicated PKC as a key player in the renal pathological effects of high glucose. We have previously demonstrated increased PKC activity and enhanced membrane-association of PKC-α, -δ, and -ε in high glucose (30). We have also found that, in high glucose during ET-1 stimulation, the pattern of PKC-δ and -ε translocation was altered compared with the response in normal glucose (13). ANG II stimulation of mesangial cells causes translocation of PKC-δ and -ε with a smaller effect on PKC-α (unpublished results). These data suggest PKC as a possible link in the high glucose and/or agonist stimulation of p38 activation. However, downregulation of the PKC isoforms with PMA for 24 h or calphostin C for 1 h did not reduce phosphorylation of p38 stimulated by high glucose alone or by an agonist. Recently, Igarashi et al. (24) reported an increase in p38 activity in vascular smooth muscle cells, which was partly dependent on both PKC and osmolarity. The different findings between the latter study and our own may be due to cell type specificity or fibronectin attachment in our report.

Another mechanism that may activate mesangial cell p38 MAPK in high glucose is TGF-β1 possibly through the tyrosine-activated kinase. After 48 h or more exposure to high glucose, mesangial cells express and secrete excess TGF-β1, which acts as an autocrine factor (21). Other possible upstream signaling proteins include Cdc42 and Pak 1 (59).

In general, MAPKs are activated by phosphorylation on tyrosine and threonine residues and inactivated by dephosphorylation. MAPK phosphatase-1 (MKP-1), a dual-specificity protein tyrosine phosphatase that exhibits catalytic activity toward both regulatory sites on MAPKs, may deactivate MAPKs including p38 (5). Therefore, another possible mechanism of p38 activation in high glucose may be inhibition or downregulation of MKP-1, which is responsible for increased ERK1/2 activity in high glucose (1).

In summary, primary cultured rat mesangial cells exposed to high glucose demonstrate increased p38 activity and, consequently, CREB phosphorylation within 3 days. ET-1, ANG II, and PDGF stimulate mesangial cell p38 and CREB phosphorylation, and in high glucose, significantly augmented responses to all 3 agonists were observed. High glucose-enhanced p38 activity does not involve PKC isozymes or increased osmolarity. The enhanced activation of p38, and in turn CREB, may contribute to the altered mesangial cell phenotype leading to progressive diabetic nephropathy.

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