Stretch-induced mesangial cell ERK1/ERK2 activation is enhanced in high glucose by decreased dephosphorylation

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Stretch-induced mesangial cell ERK1/ERK2 activation is enhanced in high glucose by decreased dephosphorylation. Am J Physiol Renal Physiol 279: F688–F697, 2000.—Glomerular hypertension and hyperglycemia are major determinants of diabetic nephropathy. We sought to identify the mechanisms whereby stretch-induced activation of mesangial cell extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2) is enhanced in high glucose (HG). Mesangial cells cultured on fibronectin Flex I plates in normal glucose (NG; 5.6 mM) or HG (30 mM), were stretched by 15% elongation at 60 cycles/min for up to 60 min. In HG, a 5-min stretch increased ERK1/ERK2 phosphorylation by 6.4 ± 0.4/4.3 ± 0.3-fold (P < 0.05 vs. NG stretch). In contrast, p38 phosphorylation was increased identically by stretch in NG and HG. Unlike many effects of HG, augmentation of ERK activity by HG was not dependent on protein kinase C (PKC) as indicated by down-regulation of PKC with 24-h phorbol ester or inhibition with bisindolylmaleimide IV. In both NG and HG, pretreatment with arginine-glycine-aspartic acid peptide (0.5 mg/ml) to inhibit integrin binding or with cytochalasin D (100 ng/ml) to disassemble filamentous (F) actin, significantly reduced phosphorylation of ERK1/ERK2 and p38. To determine whether the rate of mitogen-activated protein kinase dephosphorylation is affected by HG, cellular kinase activity was inhibited by depleting ATP. Post-ATP depletion, phosphorylation of ERK1/ERK2 was reduced to 36 ± 9/51 ± 14% vs. 9 ± 5/7 ± 6% in NG (P < 0.05, n = 5). Thus stretch-induced ERK1/ERK2 and p38 activation in both NG and HG is β1-integrin and F-actin dependent. Stretch-induced ERK1/ERK2 is enhanced in high glucose by diminished dephosphorylation, suggesting reduced phosphatase activity in the diabetic milieu. Enhanced mesangial cell ERK1/ERK2 signal in response to the combined effects of mechanical stretch and HG may contribute to the pathogenesis of diabetic nephropathy.

cyclic-strain; p38; β1-integrin; arginine-glycine aspartic acid peptide; dephosphorylation; extracellular signal-regulated kinase 1 and 2

DIABETIC NEPHROPATHY IS CAUSED by high glucose (25) and accelerated by glomerular hypertension (31). In diabetes, glomerular mesangial cells demonstrate excessive growth and extracellular matrix (ECM) protein synthesis (36). High glucose induced activation of mesangial cell protein kinase C (PKC) (12, 20) and mitogen-activated protein kinases (MAPKs) (12, 14), and subsequent gene expression, have been implicated. Increased glomerular pressure precedes mesangial cell growth and ECM production in diabetes (42) and could contribute to extracellular signal-regulated kinase (ERK) activation by high glucose. However, at present, the combined effects of high glucose and cell stretch in mesangial cells are poorly understood.

Members of the MAPK family, such as ERK and p38, are regulated by a variety of stimuli, including PKC and phosphatases. High glucose causes activation of mesangial cell PKC (22), ERK1/ERK2 (10), and p38 (14, 21), but not all the effects of high glucose on MAPK are attributable to PKC. We have observed that the enhanced response of ERK1/ERK2 to endothelin-1 (ET-1) in high glucose is PKC dependent (10) whereas enhanced p38 activation is PKC independent (40). Mechanical stretch also activates ERK (34). This has been reported to occur through multiple mechanisms including PKC, Ca2+, and src kinase-dependent signaling pathways (37). The activity of MAPK family members is tightly controlled not only by the rate of phosphorylation but also by dephosphorylation. Inactivation of MAPK signaling is mediated by serine/threonine protein phosphatases, protein tyrosine phosphatases (24) and a class of 9 dual specificity protein phosphatases that include mitogen-activated protein phosphatase-1 (MKP-1). MKP-1 is expressed in both cultured mesangial cells (4, 38) and in isolated rat glomeruli (2) and its expression is inhibited in mesangial cells exposed to high glucose for 5 days in a PKC-dependent manner (2). In rat vascular smooth muscle cells (VSMC), exposure to 12- and 24-h high glucose decreases basal MKP-1 protein by 75% via a p38-dependent pathway (3). Therefore, we postulated that high glucose increases activation of mesangial cell ERK1/ERK2 and p38 MAPK by mechanical stretch through mechanisms involving both PKC and phosphatases.
The composition of extracellular matrix modifies the ERK response to mechanical stretch. For instance, rat cardiac fibroblasts (28) and rat VSMC (32) require fibronectin for stretch-induced activation of ERK1/ERK2. In vivo, mesangial cells attach to mesangial matrix proteins including collagen IV, laminin, perlecan and fibronectin via αβ-specific integrins (8). This study is the first to examine the effects of mechanical stretch and high glucose on MAPKs in mesangial cells attached to fibronectin.

To analyze the effects of high glucose and mechanical stretch together, activation of ERK1/ERK2 and p38 were analyzed by immunoblot of total cell content of phospho- and total MAPKs, and by immunoprecipitation of phospho-ERK1/ERK2 and p38, followed by phosphorylation of an Elk-1 and ATF-2 fusion proteins, respectively. The roles of PKC-dependent and -independent pathways in the stretch-activation of ERK1/ERK2 and p38 were studied by inhibition of signaling via PKC, Ca²⁺ and tyrosine kinase phosphorylation. Confocal fluorescence imaging was used to examine focal adhesion complexes and filamentous (F)-actin, with and without inhibitors of attachment and F-actin assembly. The change in stretch-induced ERK1/ERK2 and p38 phosphorylation after ATP depletion in normal and high glucose was used to unmask the rate of MAPK dephosphorylation. These experiments demonstrated a role for integrins, the actin cytoskeleton, and MAPK dephosphorylation. These experiments demonstrated a role for integrins, the actin cytoskeleton, and PKC in the enhancement of stretch-activated ERK by high glucose on fibronectin.

Materials and Methods

Materials. Dulbecco’s modified Eagle medium (DMEM), penicillin, streptomycin and trypsin were purchased from Gibco Life Technologies (Burlington, ON). Fetal bovine serum (FBS) was purchased from Wisent (St. Bruno, QC). Phorbol 12-myristate 13-acetate (PMA), arginine-glycine-aspartic acid (RGD), cytochalasin D, leupeptin, pepstatin A, aprotinin, bensamidine, Tween-20, sodium-orthovanadate, diithiothreitol (DTT), rotenone, 2-deoxyglucose, and ATP solution were made up fresh as needed. Aprotinin, bensamidine, Tween-20, sodium-orthovanadate, diithiothreitol (DTT), rotenone, 2-deoxyglucose, and ATP solution were made up fresh as needed. protease, cells were either pretreated with 1 μM BIM IV for 1 h or with 0.1 μM PMA for 24 h prior to stretch. To chelate Ca²⁺, cells were pretreated with 25 μM BAPTA for 10 min prior to stretch. To inhibit protein tyrosine kinases, cells were either pretreated with 10 μM Herb A or 10 μM PP2 for 18 h prior to stretch. To inhibit PI 3-kinase, cells were pretreated for 1 h with 1 μM wortmannin. To inhibit β₁-integrin binding, cells were pretreated with 0.5 mg/ml cytochalasin D for 3 h. Phosphotyrosine immunoblots were used to validate the effectiveness of the protein tyrosine kinase inhibitors. Immunoblotting of ERK1/ERK2, p38, and phosphorylated tyrosine. Stretched and unstretched cells were quickly washed twice with ice-cold phosphate buffered saline (PBS) before the addition of 125 μl boiling 2× SDS sample buffer/ well containing 130 mM Tris-base, pH 6.8, with HCl, 4% SDS, and 20% glycerol. The cells were scraped and lysates were processed. The samples were then boiled for 2 min and clarified by centrifugation at 15,000 g for 10 min. Protein concentration was determined using the modified micro Lowry detergent-compatible protein assay (Bio-Rad) with bovine serum albumin (BSA, BioShop, Toronto, ON) as the standard. Equal amounts of protein (15 μg) from each sample were then separated by SDS-PAGE. Anisomycin-treated C-6 glioma cell extract (New England Biolabs) served as a positive control. Gels were equilibrated in transfer buffer at room temperature (RT) for 10 min and then transferred overnight at 4°C to polyvinyliden difluoride (PVDF) membranes (Millipore, Bedford, MA).

PVDF membranes were rinsed with PBS and blocked with 5% skim milk powder in Tris-buffered saline (TBS), pH 7.5, containing 0.05% Tween-20 (TTBS) for 2 h at RT. The blots were washed in TBS containing 0.1% Tween-20, 5% nonfat dry milk and 0.01% Tween-20 between each wash. The blots were incubated overnight at 4°C with primary antibodies in 5% skim milk or 5% BSA TTBS with dilutions as follows: 1:3,000 for phosphorylated ERK1/ERK2, 1:2,000 for phospho-ERK1/ERK2, 1:1,000 for phospho-p38, 1:500 for phospho-ERK1/ERK2, 1:1,000 for phospho-p38, 1:500 for phospho-p38, and 1:2,000 for phospho-p38. After 3–5 min washes with TTBS, secondary antibodies, either goat anti-mouse HRP or goat anti-rabbit HRP, were used at 1:5,000 dilution in 5% skim milk TTBS for 20 min at RT and were visualized using enhanced chemiluminescence (ECL; Kirkegaard & Perry Laboratories, Gaithersburg, MD). The blots were exposed to Kodak X-Omat Blue film (Eastman Kodak, Rochester, NY) for 10–60 s.

The same membranes were then reprobed to detect total ERK1/ERK2 and total p38 protein. For ERK1/ERK2, the
membranes were exposed to 15% hydrogen peroxide in TBS for 20 min at RT. For total p38, the membranes were stripped in 0.1 M glycine, pH 2.9, for 20 min at RT. After two 5-min washes, the membranes were reblocked with 5% skim milk TTBS, and the immunoblots were repeated using a polyclonal anti-ERK1/ERK2 antibody (1:3,000) and polyclonal anti-p38 MAPK antibody (1:2,000), respectively. This was followed by a 20-min incubation at RT with an HRP-conjugated goat anti-rabbit secondary.

Densitometry was performed using National Institutes of Health Image software (version 1.62, National Institutes of Health, Bethesda, MD) on a Macintosh 7200/100 computer. Densitometry was performed on both sets of blots and the results were expressed as a ratio of phosphorylated to total MAPK. All results were expressed as a ratio compared with unstretched cells in 5.6 mM glucose.

**Analysis of ERK1/ERK2 and p38 activation.** Basal and stretch-induced ERK1/ERK2 and p38 activities were measured using immunoprecipitation and phosphorylation of Elk-1 and ATF-2 fusion protein following the manufacturer’s instructions (New England Biolabs). Briefly, cells were rinsed twice with ice-cold PBS and then lysed with 100 μl of radioimmunoprecipitation buffer (RIPA). The lysate was clarified by centrifugation at 15,000 g for 10 min at 4°C, and 20-μl aliquots were collected for protein assay as described above. Fifteen microliters of either an immobilized monoclonal anti-phospho-ERK1/ERK2 antibody or an immobilized anti-phospho p38 antibody were added to 200 μl of cellular protein overnight at 4°C. The MAPK immunocomplexes were then microcentrifuged for 30 s at 4°C, washed twice with 200 μl RIPA buffer and twice with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2, suspended in 50 μl kinase buffer supplemented with 200 μM ATP and either 2 μg ATP-2 for p38 activity measurement or 2 μg Elk-1 fusion protein, followed by incubation for 30 min at 30°C. The reaction was terminated by addition of 25 μl of 3× SDS sample buffer and boiling for 5 min. Reaction products were separated by 10% SDS-PAGE and transferred onto PVDF membranes overnight as described above. After blocking in 5% skim milk TTBS for 2 h at RT, the membranes were incubated with phospho-specific ATF-2 or Elk-1 antibodies (1:2,000 in 5% BSA TTBS) overnight at 4°C. Active ERK1/ERK2 (New England Biolabs) and anisomycin-treated cells served as positive controls. Fusion protein phosphorylation was detected by ECL, and densitometry was performed as described above.

**Inhibition of MAPK activation and measurement of MAPK dephosphorylation.** To inhibit MAPK activation, mesangial cells were depleted of ATP by preincubation in PBS preheated to 37°C supplemented with 5 μM rotenone and 10 mM 2-deoxyglucose for 10 and 15 min after stretch (29). To verify ATP depletion, cellular ATP content was measured using an ATP bioluminescent somatic cell assay kit (Sigma) and normalized to cellular protein. Mesangial cells were lysed in 75 μl boiling 2× SDS sample buffer, and total cellular protein was determined as described above. Lysates were electrophoresed, and membranes were subsequently immunoblotted with anti-phospho-ERK1/ERK2, anti-phospho-p38 and total ERK1/ERK2 and total p38 antibodies. The change in the amount of phosphorylated ERK1/ERK2 or phosphorylated p38 between successive time points after ATP depletion was used to determine the extent of MAPK dephosphorylation after mechanical stretch.

**Confocal imaging.** Mesangial cells were growth-arrested for 2 days in 0.5% PBS DMEM on coverslips coated with fibronectin (50 μg/ml). Cells were then serum starved for 3 h in DMEM prior to labeling of F-actin and vinculin with rhodamine-phalloidin and FITC-conjugated goat anti-mouse IgG according to our previously published methods (43). Cells were either pretreated for 1 h with 0.5 mg/ml RGD peptide or for 3 h with 100 ng/ml cytochalasin D. Briefly, cells were washed three times with PBS, fixed in 3.7% formaldehyde, and permeabilized with 100% methanol at −20°C for 10 min. After washing in PBS, cells were blocked in 1% goat serum plus 0.1% BSA in PBS for 1 h at RT. Monoclonal anti-vinculin antibody was diluted 1:4 in blocking solution and 200 μl were added to each coverslip. After 1-h incubation at 37°C, cells were washed three times in PBS then exposed to 200 μl of a combination of 0.165 M rhodamine-phalloidin and FITC-conjugated goat anti-mouse secondary antibody, 1:160, in blocking solution. After a 1-h incubation at 37°C, cells were washed and mounted on glass slides with Immuno-fluorescent mounting media (ICN Biomedical, Costa Mesa, CA) and imaged with a dual channel confocal laser scanning microscope (Zeiss LSM 410, Düsseldorf, Germany). The following controls were performed: 1) incubation with FITC-conjugated secondary antibody alone, which demonstrated no significant labeling; and 2) preincubation of the primary antibody with specific blocking peptide, which prevented fluorescence labeling of vinculin.

**Statistical analyses.** All results are expressed as means ± SE. Statistical analysis was performed using InStat 2.01 statistics software (Graph Pad, Sacramento, CA). The means of three or more groups were compared by the one-way ANOVA. If significance of P < 0.05 was obtained in the ANOVA, the Tukey multiple comparison posttest was applied. If the Bartlett’s test indicated non-Gaussian distribution then the nonparametric Kruskal-Wallis test, followed by Dunn’s multiple comparison or the Mann-Whitney test was utilized. Differences described as significant in the text are P < 0.05 unless otherwise stated.

**RESULTS**

**Mechanical stretch stimulates ERK1/ERK2.** Cyclic stretch stimulated the phosphorylation of ERK1/ERK2 in a time-dependent manner as observed in Fig. 1. The responses were rapid and transient with peak phosphorylation at 5 min of stretch, returning to basal values by 30–60 min. Immunoblotting of cell extracts with anti-ERK1/ERK2 antibody revealed no change in total levels of ERK1/ERK2 protein.

Incubation of mesangial cells for 2 days in 30 mM glucose did not alter basal levels of ERK1/ERK2 (Fig. 1B). In 30 mM glucose, 5 min of stretch increased phosphorylation of ERK1/ERK2 by 6.4 ± 0.4/4.3 ± 0.3-fold (n = 8, P < 0.01 vs. normal glucose static) and was enhanced compared with 5.6 mM glucose (3.3 ± 0.3/3.0 ± 0.3-fold, P < 0.05, normal vs. high glucose).

In Fig. 1C, 5 min of stretch stimulated ERK1/ERK2 activity, as measured by phosphorylation of Elk-1 fusion protein, 11 ± 1-fold (n = 5, P < 0.01 vs. normal glucose static). The response was enhanced in high glucose (17 ± 3-fold, n = 5, P < 0.05, high vs. normal glucose).

**Mechanical stretch stimulates p38.** Cyclic stretch also stimulated the phosphorylation of p38 in a time-dependent manner (Fig. 2A). Phosphorylation of p38 was significantly increased by 2 min and was maximal
at 5–10 min. The response was transient, returning to static basal levels by 30–60 min.

High glucose did not alter basal levels of p38 phosphorylation (Fig. 2B). Stretch for 5 min increased p38 phosphorylation by 5.4 ± 0.4-fold (n = 8, P < 0.01 vs. normal glucose static) and was unchanged in high glucose (4.6 ± 0.3 fold, n = 8, P > 0.01 vs. normal glucose static).

In Fig. 2C, 5 min of stretch stimulated p38 activity, as measured by phosphorylation of ATF-2 fusion protein, 10 ± 2-fold (n = 3, P < 0.01 vs. normal glucose static). The stretch response was unchanged in high glucose (7.4 ± 1.2-fold, n = 3).

Effect of osmolarity. Cells were growth-arrested for 2 days in normal glucose supplemented with either 24.4 mM L-glucose or 24.4 mM mannitol to serve as osmotic controls. Static or stretch-activated ERK1/ERK2 was not enhanced in the presence of either L-glucose or mannitol (Fig. 3A). Similar results were observed for p38 (Fig. 3B).

Inhibiton of PKC. To examine the role of PKC in mechanical stretch-induced ERK1/ERK2 activation, cells were pretreated for 1 h with the PKC inhibitor BIM IV (1 μM) or downregulated with 24-h PMA (0.1 μM). Inhibition or downregulation of PKC did not alter stretch-activated ERK1/ERK2 or p38 phosphorylation in normal or high glucose. Total ERK1/ERK2 and p38 protein were not affected by PKC inhibition (Fig. 4). In translocation experiments with PKC-isoform-specific
antibodies, high glucose or stretch did not alter cytosol, membrane or particulate fraction PKC-\(\alpha\),-\(\delta\),-\(\epsilon\), and -\(\zeta\) content (data not shown).

Inhibition of potential upstream signaling pathways. Tyrosine phosphorylation of stretched cells in normal and high glucose was examined with immunoblotting using a PY-20 anti-phosphotyrosine antibody (Fig. 5A). On fibronectin, mesangial cells displayed a high basal level of tyrosine phosphorylation of proteins with molecular masses of \(\sim 125, 150, 90,\) and \(70\) kDa. Mechanical stretch stimulated tyrosine phosphorylation of two additional proteins with a molecular mass of \(44\) and \(42\) kDa, consistent with ERK1/ERK2. To confirm the effectiveness of tyrosine phosphorylation inhibitors, erbstatin, herbimycin A, and PP2 pretreated cells (\(10\) \(\mu\)M for \(18\) h) were challenged with \(50\) ng/ml platelet-derived growth factor (PDGF) for \(10\) min. In untreated cells, PDGF markedly stimulated the phosphorylation of ERK1/ERK2 (Fig. 5B). Only PP2 prevented the PDGF-induced activation of ERK1/ERK2 phosphorylation (Fig. 5B). Total levels of ERK1/ERK2 were not affected by any of the inhibitors.

As shown in Table 1, inhibition of tyrosine phosphorylation with PP2, chelation of \(Ca^{2+}\) with BAPTA for \(10\) min, or inhibition of PI 3-kinase with wortmannin (\(1\) \(\mu\)M for \(1\) h) all failed to prevent stretch-activated ERK1/ERK2 and p38 phosphorylation in normal or high glucose.

Effect of RGD peptide and cytochalasin D on ERK1/ERK2 and p38. Cells in normal and high glucose were pretreated for \(1\) h with \(0.5\) mg/ml RGD peptide. In the static condition, RGD peptide did not affect basal levels of either ERK1/ERK2. RGD pretreatment reduced stretch-induced ERK1/ERK2 phosphorylation to \(72 \pm 9/62 \pm 7\%\) of stretch \((n = 5, P < 0.05\) vs. no RGD) (Fig. 6A), which was no different in high glucose, \(82 \pm 6/79 \pm 9\%\) of stretch.

The effects of RGD peptide on p38 phosphorylation are shown in Fig. 6B. Basal levels of p38 were not altered by RGD, but \(5\) min stretch-induced activation of p38 phosphorylation was reduced to \(43 \pm 3\) and \(40 \pm 4\%\) of stretch \((n = 4, P < 0.01\) vs. no RGD) in normal and high glucose, respectively.

The effects of cytochalasin D on ERK1/ERK2 phosphorylation are shown in Fig. 7A. In normal and high glucose, basal levels of ERK1/ERK2 were not altered by cytochalasin D, but \(5\) min stretch-induced ERK1/ERK2 phosphorylation was reduced to \(56 \pm 9/49 \pm 6\%\) of stretch \((n = 4, P < 0.01\) vs. no cytochalasin D). The effect of cytochalasin D on ERK1/ERK2 was no different in high glucose \((46 \pm 2/54 \pm 4\%).

Figure 7B shows the effects of cytochalasin D on p38 phosphorylation. In normal and high glucose, cytochalasin D pretreatment significantly increased basal levels of p38 by \(1.9 \pm 0.2\)-fold and \(2.5 \pm 1.0\)-fold, \((n = 4, P < 0.01\) vs. no cytochalasin D).
P, 0.01 vs. basal), respectively. Stretch-induced p38 phosphorylation was reduced to 34 ± 6 and 26 ± 4% of stretch (n = 4, P < 0.01 vs. no cytochalasin D) in normal and high glucose, respectively.

To demonstrate that RGD peptide disrupted cell adhesion, cells cultured on fibronectin-coated coverslips in normal glucose were visualized for F-actin and vinculin using confocal microscopy, in the absence and presence of RGD peptide. As shown in Fig. 8A, in the absence of RGD peptide, vinculin was localized in focal adhesion complexes. F-actin filled the cytoplasm and was organized in a bundled pattern (Fig. 8D). In the presence of cytochalasin (CD) (Fig. 8B), decreased intensity of both vinculin and F-actin staining was observed (Fig. 8E). In the presence of RGD, (Fig. 8C) the intensity of vinculin staining was markedly reduced, and was accompanied by reduction in F-actin labeling (Fig. 8F).

The combined effect of CD and RGD was also investigated. Pretreatment markedly reduced both

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Table 1. The effects of inhibitors on ERK1/ERK2 and p38 MAPK activation by mechanical stretch in normal and high glucose

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Mechanisms of Action</th>
<th>ERK1/ERK2 and p38 Activity</th>
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<tbody>
<tr>
<td>BAPTA/AM (25 μM, 10 min)</td>
<td>Chelation of Ca²⁺</td>
<td>←</td>
</tr>
<tr>
<td>PP2 (10 μM, 18 h)</td>
<td>Inhibition of src-family kinases</td>
<td>←</td>
</tr>
<tr>
<td>BIM IV (1 μM, 1 h)</td>
<td>Inhibition of PKC</td>
<td>←</td>
</tr>
<tr>
<td>PMA (0.1 μM, 24 h)</td>
<td>Downregulation of PKC</td>
<td>←</td>
</tr>
<tr>
<td>RGD Peptide (0.5 mg/ml, 1 h)</td>
<td>Decreased fibronectin binding</td>
<td>↓</td>
</tr>
<tr>
<td>Cytochalasin D (100 ng/ml, 3 h)</td>
<td>Disruption of F-actin</td>
<td>↓</td>
</tr>
<tr>
<td>Wortmannin (1 μM, 1 h)</td>
<td>Inhibition of PI 3-kinase</td>
<td>←</td>
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</table>

ERK1 and ERK2, extracellular signal-regulated kinase 1 and 2, respectively; PP2, 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine; BAPTA, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid; BIM IV, bisindolylmaleimide IV; PMA, phorbol 12-myristate 13-acetate; RGD, arginine-glycine-aspartic acid. ↓, Decreased activity; ←, No change in activity.
vinculin and F-actin labeling and resulted in spontaneous detachment of a large proportion of the cultured cells, preventing the analysis of stretch (data not shown).

The effects of high glucose on ERK1/ERK2 and p38 dephosphorylation. The overall phosphatase activity on ERK was examined using the method of Meriin et al. (29). Rotenone and 2-deoxyglucose were used to rapidly inhibit cellular kinase activity, and the rate of ERK dephosphorylation was then followed by immunoblotting. Pretreatment of mesangial cells with rotenone and 2-deoxyglucose led to a 10-fold decrease in cytosolic ATP concentration in 10 min in both normal and high glucose (10.4 ± 0.3 and 5.2 ± 0.8%, P < 0.01 vs. normal glucose time 0, n = 3) (Fig. 9A). ERK1/ERK2 phosphorylation levels after ATP depletion are shown in Fig. 9B. In the static condition, 10-min ATP depletion did not alter basal levels of ERK1/ERK2 in normal and high glucose, but by 15 min, ERK1/ERK2 phosphorylation was undetectable. In normal glucose, stretch-induced ERK1/ERK2 phosphorylation was reduced to 15 ± 3/20 ± 7% of stretch (P < 0.01, n = 5) 10 min after ATP depletion and to 9.4 ± 5/7.4 ± 6% of stretch (P < 0.01, n = 5) at 15 min. By contrast, in high glucose, ERK1/ERK2 dephosphorylation was reduced to 36 ± 9/51 ± 13% and 18 ± 9/22 ± 10% (P < 0.05 vs. normal glucose stretch, n = 5) after 10- and 15-min ATP depletion, respectively. Figure 9C shows the results of similar experiments performed with p38. In normal and high glucose, basal p38 levels were 58 ± 20 and 66 ± 15% (n = 5, P < 0.05 vs. basal) 10 min after ATP depletion. At 15 min, phosphorylated p38 was not detectable. In normal glucose, at 10 and 15 min after ATP depletion, stretch-induced p38 dephosphorylation was reduced to 38 ± 15 and 8 ± 7% of stretch (P < 0.01, n = 5), respectively. The effect was no different in high glucose (32 ± 12 and 7 ± 6% of stretch, respectively).

Fig. 7. The effects of cytochalasin D (CD) on stretch-activated ERK1/ERK2 and p38. A: representative immunoblots of mesangial cell phosphorylated ERK1/ERK2 and total ERK1/ERK2 in 5-min stretched cells pretreated with 100 ng/ml CD for 3 h to disrupt F-actin. +, An anisomycin-treated positive control. Zero indicates the basal static condition and 1 indicates stretch in the absence of CD, in NG and HG. B: representative immunoblots of mesangial cell phosphorylated p38 and total p38 in 5-min stretched cells pretreated with 100 ng/ml CD for 3 h.

Fig. 8. Confocal imaging of F-actin and vinculin. A: representative image of basal mesangial cell vinculin staining in NG. B: representative image of mesangial cell vinculin staining after cytochalasin D (CD; 100 ng/ml) pretreatment for 3 h. C: representative image of mesangial cell vinculin staining in NG after RGD peptide (0.5 mg/ml) pretreatment for 1 h. D: representative image of basal mesangial cell F-actin staining in NG. E: representative image of mesangial cell F-actin staining in the presence of CD (100 ng/ml) pretreatment for 3 h. F: representative image of mesangial cell F-actin staining after RGD peptide (0.5 mg/ml) pretreatment for 1 h.
DISCUSSION

In glomerular mesangial cells cultured on fibronectin, exposure to 15% stretch rapidly and transiently activates ERK1/ERK2 and p38. ERK1/ERK2, but not p38, was activated by stretch (28). High surface pressure directly applied to mesangial cells on collagen I also rapidly and transiently activated ERK1/ERK2 (23). Ingram et al. (16) stretched mesangial cells 20 and 30% for 30–120 min on collagen I. They observed, at 20% stretch, activity of ERK1/ERK2, but not p38. At 30% stretch, ERK1/ERK2 and p38 stimulation and proliferation were observed (16). In a later study, the same group showed activation of p38, ERK1/ERK2, and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) with 20% stretch for 10 min (17). Ishida et al. (19) report that mesangial cells on collagen I demonstrate a rapid, transient, and stretch intensity-dependent activation of ERK1/ERK2 and SAPK/JNK, whereas p38 was not examined. Taken together, these observations indicate that mesangial cell ERK1/ERK2 and p38 are more stretch responsive when attached to fibronectin compared with collagen I.

In our study, activation of ERK1/ERK2 and p38 was not likely due to autocrine factor release in response to stretch, since conditioned media from stretch plates failed to stimulate both ERK1/ERK2 and p38 in static controls (data not shown). The transient response of ERK1/ERK2 and p38 may be partly explained by the rapid activation of mitogen-activated protein kinase phosphatases (MKPs). Li et al. (26) recently demonstrated that as little as 8- to 30-min cyclic strain in rat VSMC grown on collagen I stimulated simultaneous rapid expression of MKP-1 mRNA and protein, which was dependent on prior activation of both ERK1/ERK2 and p38 MAPK (26). In rat VSMC, angiotensin II and PMA also stimulate ERK1/ERK2, accompanied by enhanced MKP-1 activity (5). Simultaneous stimulation of MKP-1 may be a negative feedback mechanism regulating ERK1/ERK2 and p38 MAPK.

An advantage of the present experiments is that culture of cells on fibronectin more closely mimics in vivo conditions compared with plastic. After 2 days exposure to high glucose, basal levels of ERK1/ERK2 and p38 were not changed in the current experiments. By contrast, we have reported previously that mesangial cells cultured on plastic demonstrate increased basal activity of ERK1/ERK2 (10). Consistent with these findings, 3 days of high glucose did not alter basal levels of p38, ERK1/ERK2, and SAPK/JNK in mesangial cells cultured on collagen I (17). We have previously reported that culturing cells in high glucose on plastic for 2 days increases PKC activity and PKC-α, -δ, and -ε membrane translocation (22). In the current study, neither 2 days of high glucose, nor stretch stimulated PKC isoform translocation in mesangial cells on fibronectin (data not shown). This suggests that fibronectin alters responsiveness of PKC isoforms to high glucose.

In human keratinocytes (39) and in Caco-2 intestinal epithelial cells (11) cultured on collagen I, stretch stimulated transient translocation of PKC-α and PKC-δ within 5 min (39) and PKC-α and PKC-ε within 30 s (11). It is possible that in our study, transient activation of some PKC isoforms occurred prior to the earliest 2-min time point. Nevertheless, neither BIM IV nor...
24-h PMA, prevented stretch-induced ERK1/ERK2 and p38 activation. This is in contrast to the work of Ingram et al. (18) who showed that 28% stretch-induced p38 activation was attenuated by 24-h PMA. Akai et al. (1) observed enhanced membrane PKC activity in mesangial cells on collagen I after 12-h stretch, but the individual isoforms involved were not examined. ERK1/ERK2 activation stimulated by either direct pressure to mesangial cells grown on collagen I (23) or stretch (19), was also PKC independent.

In our study, mesangial cells plated on fibronectin in either normal or high glucose displayed a higher basal level of protein tyrosine phosphorylation than those cultured on plastic (data not shown). Stretch stimulated the appearance of only two additional tyrosine phosphorylated protein bands at 44 and 42 kDa, consistent with ERK1/ERK2. MacKenna et al. (28) also reported no dramatic changes in the phosphotyrosine profile of cardiac fibroblasts stretched on fibronectin. In our study, only PP2, a src-kinase inhibitor partially reduced basal tyrosine phosphorylation on fibronectin and completely attenuated PDGF stimulation of ERK1/ERK2. PP2 did not inhibit the stretch-induced activation of either ERK1/ERK2 or p38. By contrast, in mesangial cells attached to collagen I, ERK1/ERK2 activation by either direct pressure (23) or stretch (19), was totally prevented by protein tyrosine kinase inhibition with either 10 μM genistein or 10 μM herbimycin A. These inhibitors, used at the same concentration in our study, had only a slight inhibitory effect on PDGF-stimulated ERK1/ERK2 phosphorylation and had no effect on mechanical stretch-induced MAPK activation.

Integrins are implicated in the activation of ERK1/ERK2 and the SAPK/JNK pathways (28). The precise mechanisms are not known, but p21 ras-dependent (35) and ras-independent pathways (6) are proposed. In our study, preincubation of cells with RGD peptide markedly attenuated stretch-activated p38 and blunted ERK1/ERK2 activation. This is in contrast to rat cardiofibroblasts, where RGD alone had no effect on stretch-activated ERK1/ERK2 (28), whereas various β-integrin antibodies were more effective in preventing stretch-activated ERK1/ERK2 (28). These differences in integrin-dependent responses may be due to cell phenotype or the RGD concentration and duration of exposure. Interestingly, in rat VSMC, RGD peptide prevented stretch-induced proliferation, which was also blocked by β3- and α,β3-integrin antibodies but not by an antibody to β1-integrins (41), and ERK1/ERK2 activation was not measured (41).

Confocal imaging revealed the presence of an intact cytoskeleton (normal F-actin assembly) in the presence of RGD, despite reduced focal adhesion. The actin cytoskeleton may play a direct role in integrin signaling (27). In our cells, maintenance of F-actin assembly, despite reduced focal adhesion contacts in the presence of RGD (33), suggests that a mechanism(s) other than integrin attachment maintains F-actin stress fibers. Disassembly of F-actin with cytochalasin D, which also appeared to reduce focal adhesion, inhibited stretch-induced ERK1/ERK2 and p38 activation, emphasizing that intact F-actin is necessary for ERK1/ERK2 and p38 activation.

In our experiments, mesangial cell β3-integrin content was not altered after 2 days high-glucose exposure (data not shown), and RGD appeared to have the same effect on MAPKs in normal and high glucose. Therefore, the high-glucose effect appears independent of β3-integrin.

In high glucose, stretch-induced ERK phosphorylation decayed at a slower rate than in normal glucose (Fig. 9). This indicates that a decrease in phosphatase activity may explain our findings of enhanced ERK1/ERK2 activation in high glucose. Awazu et al. (2) reported a PKC-dependent decrease in expression of MKP-1 in mesangial cells cultured in high glucose for 5 days and in isolated glomeruli from streptozotocin-induced diabetic rats after 1–3 wk of hyperglycemia. This was accompanied by increased basal phosphorylation of ERK1/ERK2 in high glucose (2). In our study, basal ERK1/ERK2 activity was unchanged after 2 days of high glucose and during stretch. PKC inhibition had no effect on high glucose-enhanced ERK1/ERK2 activity. Another candidate is MKP-3, a dual specificity cytosolic phosphatase that is specific for ERK1/ERK2 inactivation (30).

In summary, mechanical stretch of glomerular mesangial cells on fibronectin causes ERK1/ERK2 and p38 MAPK activation, which is in part β3-integrin and F-actin dependent. High-glucose enhancement of stretch-activated mesangial cell ERK1/ERK2 is mediated by reduced dephosphorylation. Increased mechanical stretch due to glomerular hypertension may contribute to progressive glomerulopathy through enhanced activation of mesangial cell ERK1/ERK2.

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