Chronic exposure of human mesangial cells to high glucose environments activates the p38 MAPK pathway

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Background. High glucose (HG) environments activate several protein kinase pathways in mesangial cells, including the mitogen-activated protein kinase (MAPK) pathway, ERK. The p38 MAPK pathway is activated by events that occur in the setting of diabetes, such as protein kinase C (PKC) up-regulation and cellular stresses (osmotic stress and redox changes). Substrates of activated p38 MAPK include transcription factors that are involved in the microvascular complications of diabetes. This current study investigated the mechanisms of HG-mediated activation of p38 MAPK in cultured human mesangial cells (HMCs) and the effects of p38 MAPK activation on the transcription factor activator protein-1 (AP-1).

Methods. HMCs were cultured in 5 mmol/L n-glucose [normal glucose (NG)] or 30 mmol/L n-glucose (HG) for seven days. Cells were also treated with HG for brief periods of time (0.5 to 4 hours) to assess the acute effects of HG on p38 MAPK. Using Western blotting of HMC lysates, changes in the tyrosine and threonine phosphorylation of p38 MAPK were measured. The kinase activity of immunoprecipitated p38 MAPK was determined by an in vitro assay that measured the phosphorylation and activation of MAPKAP kinase-2, an intermediary signaling protein downstream of p38 MAPK. To investigate the role of osmotic stress in HG activation of p38 MAPK, cells were acutely treated with mannitol (25 to 250 mOsm/L × 5 to 60 min) or were grown seven days in media supplemented with mannitol at concentrations iso-osmotic to HG media. To investigate the role of PKC in HG-mediated p38 MAPK activation, HMCs were treated with the PKC inhibitors GF 109203X, Ro 32-0432, or rottlerin during the last several hours of HG treatment. HG conditioned cells were also treated with the antioxidants L-N-acetylcysteine (L-NAC) or diphenylidonium (DPI) prior to harvest. To determine a functional significance of HG-mediated p38 MAPK activation, the DNA binding of the transcription factor complex AP-1 was measured by electrophoretic mobility shift assay.

Results. The p38 MAPK pathway was not activated by the acute addition of HG to the HMCs. However, activation of p38 MAPK in HMCs grown seven days in HG was demonstrated by increased tyrosine and threonine phosphorylation of p38 MAPK proteins and increased kinase activity of immunoprecipitated p38 MAPK. As assessed by a kinase assay, p38 MAPK activity in cells grown in HG for seven days exceeded that of NG cells by more than 250%. This difference was not due to differences in the amount of p38 MAPK protein between the treatment groups. Acute osmotic activation of p38 MAPK occurred at extremely high mannitol concentrations (250 mOsm/L) that exceeded the osmotic stress of acute HG. Furthermore, in cells grown for seven days in mannitol at concentrations similar to HG, p38 MAPK activity was similar to control values. Phorbol ester (PMA) treatment stimulated a twofold increase in p38 MAPK activity. The addition of GFX or Ro 32-0432 to HG cells, at concentrations that inhibited PMA activation of p38 MAPK, did not inhibit the glucose-mediated p38 MAPK activation. Rottlerin, a PKC δ inhibitor, also failed to reverse the HG-mediated p38 MAPK activation. Treatment of HG cells with L-NAC or DPI inhibited the HG-mediated p38 MAPK phosphorylation. As we have previously shown, DNA binding of the transcription factor complex AP-1 was increased in HG cells. This binding was reversed by treatment of the HG cells with the p38 MAPK inhibitor SB 203580.

Conclusions. Chronic exposure of HMC to HG concentrations activates the p38 MAPK pathway. This activation appears to be independent of changes in the amount of total p38 MAPK produced by the cells, independent of chronic osmotic stress and independent of PKC activation. The reversal of p38 MAPK by L-NAC and DPI suggests the glucose-mediated p38 MAPK activation may occur via reactive oxygen species. The activity of AP-1, a transcription factor complex that regulates several genes involved in diabetic nephropathy, is reversed when the p38 MAPK pathway is inhibited. These findings suggest the p38 MAPK pathway may be an important pathway involved in diabetic complications.

Several clinical studies have demonstrated that elevated blood glucose levels are a risk for the development of microvascular complications of diabetes, including diabetic nephropathy [1–4]. During the development of diabetic nephropathy, genes that alter cell growth and extracellular matrix (ECM) protein production are expressed differently than in the euglycemic setting. Much of this altered gene expression is the result of glucose-mediated activation of cell signaling pathways [5–7]. In
a variety of cells, high glucose (HG) concentrations up-regulate serine–threonine protein kinases, including several isoforms of the protein kinase C (PKC) pathway, and the mitogen-activated protein kinase (MAPK) pathway, ERK [8–13].

The ERK pathway is one of four MAPK pathways that also include the Jun N-terminal kinases (JNKs), p38 MAPKs, and the recently discovered Big MAPK pathway. These MAPK pathways regulate many transcription factors that control cell growth and ECM gene expression. Therefore, MAPKs are possible mediators of diabetic complications. Haneda et al first demonstrated a modest level of ERK pathway activation in mesangial cells grown in HG environments [13]. Since then, the p38 MAPK pathway has also been shown to be inducible by HG environments in vascular smooth muscle cells (VSMCs), pancreatic islet cells, and rat mesangial cells [14–17]. In contrast, we and others have reported that the JNK pathway is not inducible by HG concentrations in human or rat mesangial cells [16, 18]. This difference in glucose responsiveness between the MAPK pathways may be due to differences in the mode of activation of each pathway. ERKs are activated by growth factors and other mitogens that up-regulate PKC. In Haneda et al’s report, PKC activation was an intermediate step in the glucose-mediated activation of ERK [13]. In contrast, the JNK pathway is classically PKC independent in its stimulation by cell stress (osmotic stress, redox changes, pH shifts, or temperature changes) and pro-inflammatory cytokines [19–21]. The p38 MAPK pathway is uniquely activated by agonists that stimulate both the ERK and JNK pathways. p38 MAPK proteins are activated by cell stress [22–28] and by growth factors, cytokines, and other mitogens, including those that increase PKC activity [29–31].

When activated, the p38 MAPK proteins alter cellular functions at transcriptional and post-transcriptional levels. p38 MAPK regulates the phosphorylation and activation of several transcription factors (ATF2, CREB, CHOP, Elk-1, MEF2C) [32–35] that modulate a variety of genes, including genes involved in cell growth and ECM expression. Cell contractility is also regulated by p38 MAPK through the phosphorylation of small heat shock proteins [36–38]. p38 MAPK regulation of some transcription factors and cell contractility occurs via intermediary signaling proteins, such as the MAPKAP kinases [29, 36, 37, 39–41]. The p38 MAPK proteins also regulate gene transcription independently of transcription factor activation. For example, we previously reported that interleukin-1β (IL-1β)–induced expression of the monocyte chemotaxtractant protein-1 (MCP-1) gene requires p38 MAPK, although DNA binding of the transcription factor responsible for MCP-1 expression, nuclear factor-kB, is unaffected by p38 MAPK inhibition [42]. Other investigators have shown that p38 MAPK influences transcription through the stabilization of mRNA. For example, in models of inflammation, mRNAs of adhesion proteins and interleukins are stabilized by activated p38 MAPK [43–46].

That p38 MAPK can be activated by osmotic stress, PKC activation, or reactive oxygen species (ROS) suggests that this pathway may be an important signaling pathway in the development of diabetic complications. In rat aortic smooth muscle cells, HG concentrations activate the p38 MAPK pathway in a PKC-dependent and osmotic stress-dependent manner [14]. Based on these observations and evidence that genes that are p38 MAPK-dependent are expressed in diabetes [47], we hypothesized that HG concentrations could activate p38 MAPK in human mesangial cells (HMCs). Our current report shows that chronic HG exposure stimulates p38 MAPK activity in HMCs. However, this p38 MAPK activation is independent of PKC activation and independent of osmotic stress. A role for ROS in the glucose-mediated p38 MAPK activation is suggested by the reversal of p38 MAPK phosphorylation by antioxidant compounds. In these studies, we also show that glucose-mediated binding of activator protein-1 (AP-1), a transcription factor that regulates many ECM genes, is reversed by p38 MAPK inhibition.

METHODS

Human mesangial cell culture and conditioning

Human mesangial cells were isolated from cadaver kidneys deemed anatomically unsuitable for use in transplantation. Glomeruli were isolated from the renal cortex by mincing the tissue and then passing the tissue fragments through progressively smaller sieves (Bellco Glass Inc., Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum, 50 U/mL penicillin, and 50 mg/mL streptomycin (Sigma Chemicals, St. Louis, MO, USA) without supplemental d-glucose [5 mmol/L d-glucose, or normal glucose (NG)] or in media supplemented with 25 mmol/L d-glucose (Sigma) for a total d-glucose concentration of 30 mmol/L (HG). Media were changed every three days after cell passage. In parallel experiments, NG cells were treated with HG for 30 minutes to four hours prior to harvest. To evaluate the role of osmotic stress in p38 MAPK activation, cells were first placed in serum-free NG media [Media 199 + 0.25% bovine serum albumin (BSA)] for 18 hours and treated for 5 to 60 minutes with increasing concentrations of mannitol (25 to 250 mOsM/L). HMCs were also grown for seven days in serum-containing NG media supplemented with mannitol (Sigma) at a concentration iso-osmolar to HG media.
To investigate the role of PKC activation on the p38 MAPK pathway, HMCs were treated with the phorbol ester phorbol 12-myristate 13-acetate (PMA; 10 to 50 mmol/L; Sigma) for 30 minutes. To determine the role of PKC in HG activation of p38 MAPK, NG and HG cells were treated with the PKC inhibitors GF109203X (Bisindolylmaleimide HCl or GFX, 0.5 to 2 µmol/L; Calbiochem-Novabiochem, La Jolla, CA, USA), Ro 32-042 (1 to 10 µmol/L; BioMol, Inc., Plymouth Meeting, PA, USA), or rottlerin (1 to 5 µmol/L; BioMol Inc.) at varying time points (4 to 12 hours) prior to harvest, as we have previously described [18]. To determine the role of ROS in p38 MAPK activation, cells were treated with diamide (2 mmol/L; Sigma) for 10 minutes or H2O2, (100 to 500 µmol/L) for 30 minutes. NG and HG cells were also treated with the membrane permeable anti-oxidant L-N-acetylcysteine (L-NAC, 50 µmol/L; Sigma), the flavoprotein inhibitor diphenyliodonium (DPI, 10 µmol/L; Calbiochem) or the vehicle for DPI, dimethyl sulfoxide (DMSO; 0.01%), four hours prior to harvest. Prior to electrophoretic mobility shift assays (EMSAs), NG and HG cells were treated for four hours with the p38 MAPK inhibitor SB 203580 (5 to 15 µmol/L; Calbiochem) or with the vehicle of SB 203580, DMSO (0.015%). In some experiments, we treated cells with interleukin-1β (IL-1β; 1.1 ng/mL × 30 min, specific activity 5 × 10⁶ U/mg; Genzyme, Cambridge, MA, USA), which served as a positive control for p38 MAPK activation.

Immunoblotting

Conditioned HMCs were washed in PBS, pH 7.4, and rapidly lysed by scraping in a 4°C hypotonic HEPES buffer containing 20 mmol/L HEPES, pH 7.9, 1 mmol/L Na3VO4, 1 mmol/L egtazic acid (EGTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 20 µmol/L leupeptin, and 0.15 U/mL aprotinin, 0.1% NP-40. After clarification of the lysates, protein content was quantified by the bicinchonicoic method (BCA) using BSA as the standard (Pierce Analytical Research, Rockford, IL, USA). One milligram of protein was diluted in HEPES lysis buffer without detergent, and 2.0 µg polyclonal rabbit anti-p38 MAPK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added for overnight immunoprecipitation at 4°C. Protein G-Sepharose was then added for 45 minutes. The immunoprecipitate was pelleted and washed three times in phosphate-buffered saline (PBS). The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to 0.45 µmol/L nitrocellulose using a semidyne technique [51]. Following drying, the nitrocellulose was quenched with PBS 0.01% Tween (PBST), 2% BSA for 120 minutes and thereafter immunoblotted with a monoclonal antiphosphotyrosine antibody (Clone 4G10; Upstate Biotechnology Inc., Lake Placid, NY, USA) for 120 minutes at 27°C. The nitrocellulose was washed in PBST then incubated with appropriate biotinylated secondary antibodies (Zymed Laboratories, Inc., South San Francisco, CA, USA) and streptavidin-horseradish peroxidase. Bands of interest were demonstrated using an enhanced chemiluminescence (ECL) technique (Amersham, Inc., Arlington Heights, IL, USA). In other experiments, HMCs were rapidly lysed using an SDS lysis buffer containing 2% SDS, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L PMSF, 20 µmol/L leupeptin, and 0.15 U/mL aprotinin. Cell lysates were briefly sonicated at 4°C, and equivalent amounts were promptly separated by 10% SDS-PAGE under reducing conditions. After transfer, the nitrocellulose was probed overnight at 4°C with a rabbit polyclonal anti-phospho-p38 antibody (New England Biolabs, Beverly, MA, USA). This phospho-specific antibody was produced by inoculating rabbits with a peptide fragment representing the tyrosine and threonine phosphorylated form of p38 MAPK. In parallel experiments, HMC lysates obtained by SDS lysis were separated by 10% SDS-PAGE under reducing conditions. Following transfer, the nitrocellulose was immunoblotted with anti-p38 MAPK antibody (Santa Cruz Biotechnology), which recognizes phosphorylated and unphosphorylated p38 MAPK. Nonimmune rabbit isotype-specific IgG (Zymed Laboratories) served as control for the primary antibodies.

In vitro p38 MAPK assays

Human mesangial cells lysed using the HEPES buffer were clarified and the protein content normalized with HEPES lysis buffer without detergent to dilute the NP-40 concentration to <0.05%. Thereafter, 2.0 µg of C-terminus anti-p38 MAPK antibody (Santa Cruz Biotechnology) were mixed with the lysate overnight at 4°C. The immune complexes were precipitated with protein G-Sepharose and washed three times in cold PBS. The complexes were then resuspended in a kinase assay buffer containing 20 mmol/L MOPS, pH 7.2, 25 mmol/L β-glycerol phosphate, 5 mmol/L EGTA, 1 mmol/L Na3VO4, and 1 mmol/L dithiothreitol. The measurement of p38 MAPK activity involved a two-step phosphorylation assay (Upstate Biotechnology). To the immune complex mixture 100 µmol/L adenosine 5’-triphosphate (ATP), 15 mmol/L magnesium chloride and 200 ng of the fusion protein GST-MAPKAP kinase-2 were added. The activation of the MAPKAP kinase-2 by the immunoprecipitated p38 MAPK was allowed to proceed at 30°C for 15 minutes. To this mixture was then added [γ-32P] ATP and 0.12 mmol/L of a peptide sequence that corresponds to residues 1 through 9 of human glycogen synthase. The phosphorylation of the peptide fragment by activated MAPKAP kinase-2 was allowed to proceed at 37°C for 10 minutes and was terminated by the placement of the mixture onto phosphocellulose disks. The disks were allowed to
air dry for five minutes. They were then washed extensively in 0.75 mol/L phosphoric acid and finally in acetone. Scintillation counting of the disks was performed (Beckman Model LS 5801; Beckman Coulter, Inc., Fullerton, CA, USA).

**AP-1 electrophoretic mobility shift assay**

Nuclear proteins were harvested from NG and HG cells, and AP-1 EMSAs were performed as we have described [18, 52]. The cells were washed twice in cold PBS, pH 7.4, scraped, and centrifuged at 4°C. Nuclear protein extracts were obtained using the protocol of Dignam, Lebovitz, and Roeder as modified by Osborn, Kunkel, and Nabel with the addition to each buffer of the protease inhibitor leupeptin [53, 54]. Protein concentrations were measured by BCA method (Pierce Analytical Research) using BSA as the standard. Ten micrograms of nuclear protein were added to an incubation buffer containing 10 mmol/L Tris, 100 mmol/L NaCl, 1 mmol/L EDTA, 4% glycerol, and 2 mmol/L poly dI-dC. Double-stranded oligonucleotides containing a tandem repeat of the classical TPA-responsive element (TRE) and their complementary strands were annealed and end labeled with γ-[^32]P ATP by T4 polynucleotide kinase. Unlabeled P[^32]-ATP was separated from the oligonucleotide mixture with D-25 Sephadex columns and 0.2 ng of the labeled probe was added to the incubation buffer for 20 minutes at 27°C. The protein–DNA complexes were resolved at 4°C on 5% nondenaturing polyacrylamide gels in 0.25 × TBE buffer. Thereafter, the gels were dried and exposed to autoradiography film at −70°C. DNA–protein complexes were demonstrated as bands retarded in their migration through the gel. Specificity of the bands was confirmed by incubating the nuclear protein with 50× unlabeled oligonucleotide.

**Statistical analyses**

The means ± SE of the kinase assays were compared by the Student t test. Statistical significance was determined by P < 0.05.

**RESULTS**

**High glucose concentrations activate p38 MAPK in HMCs**

In these studies, p38 MAPK activation by glucose was measured after seven days of HG treatment. The rationale for investigating this time point is that at seven days the phenotype of cultured HMC simulates the phenotype in diabetic nephropathy. For example, relative to NG-grown cells, HMCs grown seven days in HG demonstrate lower proliferation rates and increased ECM protein production [5, 55] and increased activity of transcription factors that up-regulate ECM genes, such as AP-1 [18]. The activation of p38 MAPK by HG was assessed using several technical approaches. First, lysates from conditioned HMC were immunoprecipitated with a polyclonal anti-p38 MAPK antibody, and the immunocomplexes were separated by SDS-PAGE and immunoblotted with an antiphosphothreonine/tyrosine antibody. This antiphosphotyrosine antibody was used to determine the specificity of the immunoprecipitating antibody and to determine whether differences in p38 MAPK tyrosine phosphorylation, a marker of p38 MAPK activation, existed between cell groups. In these experiments, a protein with a molecular mass similar to p38 MAPK (molecular radius of 39 kD) was strongly tyrosine phosphorylated in the HG cells relative to NG cells (Fig. 1A). This increased tyrosine phosphorylation suggested increased p38 MAPK activation. However, similar to other MAPKs, p38 MAPK is activated when dual phosphorylated on tyrosine and threonine residues [22, 56, 57]. Therefore, using a rabbit polyclonal antiphospho-threonine/tyrosine p38 MAPK antibody, we also measured the amount of p38 MAPK dual phosphorylation. These Western blots demonstrated an increase in the dual phosphorylation of p38 MAPK in HG cells relative to NG cells (Fig. 1B). To verify equal protein loading of the gels, immunoblots were performed using a rabbit polyclonal anti-p38 MAPK antibody. No differences in the amount of total p38 MAPK protein were detected between the treatment groups. Finally, we confirmed an increase in the kinase activity of p38 MAPK using an in vitro assay. In these experiments, immunoprecipitated p38 MAPK proteins were used to phosphorylate the intermediary signaling protein, MAPKAP kinase-2. This activated MAPKAP kinase-2 then phosphorylated a peptide substrate that corresponds to the activation domain of human glycogen synthase, using ^32P-ATP as a phosphorus source. Results of this kinase assay are demonstrated in Figure 1C. A basal activation of p38 MAPK activity was apparent in NG cells, which is consistent with Western blotting that demonstrated a low level of p38 MAPK phosphorylation. Seven-day HG treatment stimulated p38 MAPK activity to a level 250% above that of NG cells. The amount of substrate phosphorylation (counts per minute mean ± SE) was 5986 ± 531 for NG and 15,618 ± 1720 for HG cells (P < 0.01). When time points earlier than seven days were studied, HG inconsistently activated p38 MAPK, and the level of activation was much lower than at seven days.

**HG activation of HMC p38 MAPK is independent of osmotic stress**

The p38 MAPK pathway is rapidly inducible by osmotic stress [24–26, 58], and a role for osmotic activation of p38 MAPK by HG has been shown in rat aortic smooth muscle cells [14]. We investigated the role of osmotic stress in p38 MAPK activation using the following experimental approach. First, we measured the activation of p38 MAPK following a brief exposure of the HMCs to increasing concentrations of mannitol to determine, in
a general context, how responsive cultured HMCs are to osmotic stress. To lessen basal p38 MAPK activity in these experiments, cells were placed overnight in serum-free media prior to mannitol treatment. As shown in Figure 2, brief exposures of HMC to increasing concentrations of mannitol resulted in up-regulated p38 MAPK phosphorylation only when extremely high concentrations (250 mOsm/L) were used. The p38 MAPK phosphorylation induced by mannitol treatment occurred by 15 minutes and persisted for 60 minutes (Fig. 2B). The kinase activity induced by mannitol paralleled the phosphorylation changes. When 250 mOsm/L of mannitol were used, p38 MAPK activity increased to 155% that of control cells (Fig. 2C), a level of activation considerably less than that of IL-1β activation of p38 MAPK.
Fig. 4. Phorbol esters activate HMC p38 MAPK. (A) Phospho-p38 MAPK Western blot. HMCs were placed overnight in serum-free media (Control) and treated for 30 minutes with increasing concentrations of PMA. The phospho-p38 MAPK levels (p-p38 MAPK) versus total p38 MAPK content (p38 MAPK) are demonstrated (N = 3). (B) p38 MAPK assay using cell lysates of control and PMA-treated cells (N = 4, *P = 0.01).

Fig. 3. Acute HG treatment and chronic mannitol treatment fail to activate HMC p38 MAPK. (A) Phospho-p38 MAPK Western blot demonstrating the acute effects of HG treatment (hours) on HMC phospho-p38 MAPK levels (p-p38 MAPK) relative to total p38 MAPK levels (p38 MAPK). Experiments were performed in cells placed overnight in serum-free media to lower basal p38 MAPK phosphorylation and in cells kept in serum-containing media, similar to seven-day treatments of HG. IL-1β treatment (1.1 ng/mL × 30 min) served as positive controls (N = 4). (B) Phospho-p38 MAPK Western using lysates of HMC treated for seven days in NG media or in media supplemented with D-mannitol at concentrations iso-osmolar to HG media (M; N = 4). (C) p38 MAPK assays demonstrated similar levels of p38 MAPK activity in seven-day NG and seven-day D-mannitol cells (M; N = 5).

(IL-1β activation was 315% that of control cells). Second, we measured the ability of HG media to acutely activate p38 MAPK. We performed these studies in part to determine whether acute HG treatment could induce p38 MAPK, even independent of osmotic stress, since in rat mesangial cells p38 MAPK is inducible following a 60-minute HG treatment [16]. We investigated p38 MAPK activation in HMC placed in serum-free media, to decrease basal p38 MAPK activity, as well as in serum-containing media, with the concerns that the HG effects on HMCs may require the presence of serum components [49]. Regardless of the serum content in the media, acute HG treatment failed to induce changes in p38 MAPK phosphorylation in the HMCs (Fig. 3A). Third, we compared p38 MAPK activation of HMCs grown for seven days in NG media with that of cells grown seven days in NG media that was supplemented with mannitol at concentrations iso-osmolar to HG media. As shown in Figure 3 B and C, p38 MAPK dual phosphorylation and kinase activity of cells treated with chronic mannitol was similar to that of NG cells and less than that of the HG cells shown in Figure 1. The amount of substrate phosphorylation (counts per minute mean ± SE) was 5986 ± 531 for NG and 4991 ± 873 for the mannitol-treated cells (87 ± 17% that of NG cell, P = NS).
HG activation of HMC p38 MAPK is independent of PKC

In some cells, p38 MAPK activity is stimulated by PKC activation [29–31]. Since HG concentrations activate the PKC pathway [8, 59], we investigated the role of PKC in the glucose-mediated p38 MAPK activation. To determine the responsiveness of HMC p38 MAPK to PKC activation, cells were treated with the phorbol ester PMA for 30 minutes. As demonstrated in Figure 4, PMA stimulated a dose-dependent increase in p38 MAPK dual phosphorylation and kinase activity. To ensure that this PMA effect was due to PKC up-regulation, cells were pretreated for 60 minutes with the specific PKC inhibitors GF 109203 X (GFX) or Ro 32-0432 (Ro) prior to PMA. These PKC inhibitors were able to inhibit the PMA-mediated activation of p38 MAPK (Figs. 5A and 6A). Noteworthy is the fact that the vehicle for Ro 32-0432, DMSO (0.01%), did not inhibit p38 MAPK activity induced by PMA (Fig. 6A). The down-regulation of p38 MAPK by these PKC inhibitors occurred in serum-free media as well as serum-containing media. This was an important observation, since in parallel sets of experiments GFX and Ro 32-0432 were added to the NG and HG cells that contained serum in their media. In this set of experiments, the inhibitors were added for the last 4 to 12 hours of the seven-day glucose treatment. GFX and Ro 32-0432, at concentrations that inhibited the PMA-mediated p38 MAPK phosphorylation, did not inhibit p38 MAPK phosphorylation in the HG cells (Figs. 5C and 6B).

Since PKCδ is activated by HG [60], we investigated the possibility that glucose-mediated p38 MAPK activation was due only to PKCδ up-regulation. Because the pan-PKC inhibitors may not inhibit the novel PKCδ isoform as well as they inhibit classic PKC isoforms, cells also were treated with the PKCδ inhibitor rottlerin. No inhibition of PMA-induced p38 MAPK activation by rottlerin was observed when concentrations were used that other investigators have shown is capable of suppressing PKCδ-mediated activation of p38 MAPK (data not shown) [61]. This suggests phorbol-ester activation of p38 MAPK does not require PKCδ. Similarly, no inhibition of HG-mediated p38 MAPK phosphorylation by rottlerin treatment was observed (Fig. 6C).

To ensure these Western blots correlated with p38 MAPK activity, kinase assays were performed. As assessed by the in vitro kinase assay, p38 MAPK activity induced by PMA indeed was inhibited by PKC inhibition (Fig. 5D), lysates. No difference in HG p38 MAPK activity was present when GFX (2 μmol/L) was added to the HG cells for the last 1 to 12 hours of treatment (N = 5; NS, not significant).
HG activation of HMC p38 MAPK is inhibited by antioxidants

The role of oxidant stress in glucose-mediated p38 MAPK activation also was studied. To determine the ability of ROS to induce p38 MAPK in HMCs, cells placed overnight in serum-free media were treated with the cell-permeable compound diamide or with hydrogen peroxide. We have previously shown these compounds activate the ERK and JNK pathways in HMC via ROS generation [62]. As shown in Figure 7A, these oxidants strongly increased p38 MAPK phosphorylation relative to the basal activity of control cells. With the evidence that HMC p38 MAPK can be activated by ROS, the ability of antioxidants to reverse the glucose-mediated activation of p38 MAPK was assessed. In these experiments, NG- and HG-conditioned HMCs were treated with L-NAC or DPI, two compounds that reverse ROS effects by different routes. These compounds or their vehicles were added to NG and HG cells for the last four hours of conditioning. As shown in Figure 7B, inhibition of HG-mediated p38 MAPK activity, but not basal p38 MAPK phosphorylation of NG cells, was apparent with the antioxidant treatment. The membrane-permeable antioxidant L-NAC decreased the p38 MAPK activation to a modest extent, and DPI, a potent flavoprotein inhibitor, strongly reversed the p38 MAPK activation. As assessed by trypan blue exclusion, these inhibitory effects of L-NAC and DPI were not due to cell toxicity.

HG activation of HMC p38 MAPK stimulates AP-1 binding

To determine a relevant effect of p38 MAPK activation by HG, we assessed AP-1 activation and DNA binding. AP-1 is a transcription factor complex that regulates the TGF-β1 gene as well as several ECM genes involved in glomerulosclerosis. We previously reported that HG environments increase AP-1 binding to DNA promoters [18]. Curiously, we reported that the HG effect on AP-1 binding was only partially due to PKC activation, since PKC inhibition did not fully reverse the increased AP-1 binding. We speculated that other mediators of AP-1 binding might be functional in the HG environment. Therefore, in these current experiments, we hypothesized that HG-induced AP-1 binding may also occur via p38 MAPK. AP-1 EMSAs were performed using nuclear proteins from NG and HG cells that had been treated for the last four hours of the seven-day glucose treatment with the p38 MAPK inhibitor SB 203580 or the vehicle of SB 203580, DMSO (0.015%). Figure 8 shows a representative AP-1 EMSA. AP-1 binding was greater in HG versus NG cells, and treatment with the SB 203580 compound, but not its vehicle, reversed the HG-induced binding of AP-1.
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Fig. 8. Activator protein (AP-1) activation by HG is attenuated by p38 MAPK inhibition. Prior to the AP-1 electrophoretic mobility shift assay (EMSA), NG and HG cells were treated with the vehicle DMSO (0.015%) or the p38 MAPK inhibitor SB 203580 (15 μmol/L) for four hours prior to nuclear protein harvest. AP-1 EMSAs were performed as described in the Methods section. The addition of 50× unlabeled AP-1 oligo to the nuclear proteins served as a competitor. AP-1–specific bands are demonstrated at the arrow. NS depicts the nonspecific bands (N = 3).

DISCUSSION

The phenotype of cultured mesangial cells grown in HG environments resembles the phenotype of cells in diabetic nephropathy [5, 55, 63–66]. Such phenotypic transformation from cells grown in normal glucose involves a series of transcriptional and post-transcriptional events that follow glucose-mediated activation of cell signaling pathways. Therefore, identifying these signaling pathways and their targets is critical to the discovery of interventions that slow the development of diabetic nephropathy. Our studies show that the p38 MAPK pathway is an important glucose-responsive pathway in HMCs. Evidence that p38 MAPK is activated in HMCs by HG includes the increased tyrosine phosphorylation of p38 MAPK immunoprecipitated from mesangial cell lysates, increased dual (tyrosine and threonine) phosphorylation of a protein with molecular mass similar to p38 MAPK, and increased kinase activity of p38 MAPK immunoprecipitated from mesangial cell lysates. These latter experiments using an in vitro kinase assay demonstrated immunoprecipitated p38 MAPK is elevated more than twofold in HMCs grown in HG compared with cells grown in NG media.
Our results corroborate studies that show a glucose-mediated activation of p38 MAPK in VSMCs [14, 17] and rat mesangial cells [16]. However, our current studies suggest the timing of p38 MAPK activation by glucose differs between cell type and between culture conditions. p38 MAPK induction by glucose in pancreatic islet cells is rapid and transient [15]. Similarly, Kang et al reported very rapid and transient glucose-mediated activation of p38 MAPK in rat mesangial cells grown on collagen I matrix to glucose treatment [16]. In rat [14] and porcine [17] VSMCs, p38 MAPK is activated within 24 hours of HG treatment. It is interesting that we were unable to document such acute activation of the pathway in HMCs, and could not consistently demonstrate p38 MAPK activation prior to seven days of glucose treatment. This difference in the timing of p38 MAPK activation may be related to intrinsic differences in rate of growth of cultured human versus rodent or porcine cells or to differences in the type of matrix used to culture the cells.

Differences in the mode of p38 MAPK activation may also explain this difference in the timing of activation. The up-regulation of signaling pathways by HG can be generalized to those that are direct effects of the HG environment (for example, osmotic stress) and those that occur through indirect effects regulated by signaling intermediaries, as occurs in PKC activation via diacylglycerol synthesis. Therefore, we attempted to determine whether glucose-mediated activation of p38 MAPK occurred via a direct osmotic influence or an indirect route. Although the p38 MAPK pathway is activated by osmotic stress, no changes in p38 MAPK activity were observed in HMCs grown for seven days in manniot at concentrations that were similar to HG media. This is not surprising since p38 MAPK or JNK activation generally requires extremely elevated osmotic gradients [24], as we confirmed by demonstrating that p38 MAPK is activated in HMC by manniot only when very high concentrations are present. These observations contrast those made in cultured rat VSMCs in which relatively low levels of osmotic stress, equivalent to a hyperglycemic environment (manniot concentrations of 16 mOsm/L), acutely induce the pathway. Differences by which rat VSMC and HMC sense and/or protect against osmotic stress, or any other form of cell stress, may explain these observations.

PKC is an intermediary of p38 MAPK activation by glucose in rat VSMCs [14]. Similarly, Haneda et al demonstrated a PKC-dependent up-regulation of ERK in cultured murine mesangial cells grown in HG and in glomeruli obtained from diabetic rats [13]. Compared with p38 MAPK activation in our studies, this ERK activation was modest (~40% increase in ERK activity over the NG baseline) but reversible with a six-hour treatment of Calphostin C. We therefore investigated the role of PKC in the p38 MAPK activation by glucose. Because the ability of PKC to activate p38 MAPK appears cell specific, first the ability of phorbol esters to activate p38 MAPK in the HMCs was studied. Treatment with the phorbol ester PMA strongly activated p38 MAPK, and this event occurred via PKC up-regulation since the PKC inhibitors GFX and Ro 32-0432 reversed the p38 MAPK activation. Despite these observations, the glucose-mediated activation of p38 MAPK appeared to be PKC-independent because treatment of HG cells with these PKC inhibitors did not alter HG-mediated p38 MAPK activity. Noting that pan-PKC inhibitors may not fully suppress PKC isoform activity and HG conditions can activate PKC δ [60], we also used the novel PKC inhibitor rottlerin, at concentrations shown to inhibit p38 MAPK induced via PKC δ in other cell systems [61]. Rottlerin treatment failed to inhibit glucose-mediated p38 MAPK activation. Collectively, these observations suggest that in HMCs, HG induces p38 MAPK independently of PKC.

This difference between human and rodent cells might be explained by cell-specific differences in p38 MAPK isoform expression. Several p38 MAPK isoforms have been identified, and their abilities to be activated by PKC vary [67–71]. What p38 MAPK isoforms are present in human and rodent cells and particularly in mesangial cells remains to be determined. That p38 MAPK is activated in HMCs after several days of HG also suggests intermediaries such as growth factors that are known to also activate p38 MAPK [for example, transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF), endothelin] may be responsible. If growth factors produced in HG environments are the mechanism of the HG-mediated p38 MAPK activation, an indirect role of PKC may still exist. That is, in these current studies, a direct PKC activation of p38 MAPK was not demonstrated; however, an earlier PKC activation by HG that stimulates growth factor production that then activates p38 MAPK in a PKC-independent manner may be a plausible mechanism. Further study is needed to determine the role of growth factors in the glucose-mediated p38 MAPK activation.

The p38 MAPK pathway is also activated by oxidant stress [22, 72]. Several authors have demonstrated that diabetes or HG states increase oxidant stress [73–78], and we speculated that the induction of p38 MAPK activity in HG occurs through changes in the redox potential of HMCs. We demonstrated that diamide and H₂O₂, potent stimulators of ROS, could activate p38 MAPK in HMCs. We have previously shown these agonists activate the ERK and JNK pathways in HMCs [62]. When HG cells were treated with reducing agents for the last several hours of glucose treatment, p38 MAPK phosphorylation was reduced. The membrane-permeable antioxidant and thiol reagent L-NAC was able to lessen substantially the glucose-mediated p38 MAPK effects and the flavopro-
tein inhibitor DPI strongly inhibited p38 MAPK activation. DPI is a potent inhibitor of NADPH/NADH oxidase [79, 80], and the results of these studies suggest these enzymes, which generate H₂O₂, may be involved in the altered cell signaling of diabetes. It is noteworthy that other authors have recently shown in VSMCs that DPI and L-NAC reverse p38 MAPK signaling induced by the oxidant stress of PDGF-BB treatment [81]. Further work to document the mechanisms of ROS generation in HMCs will be important extensions of these observations. An important point that needs to be resolved is the difference in ROS sensitivity between the p38 MAPK and JNK pathways, since we previously reported that the JNK pathway, which also is inducible by oxidant stress [82–84], is not activated by HG concentrations in HMCs [18].

Our previous observations that HG environments fail to induce JNK raise interesting questions about the MAPK-mediated induction of TGF-β1 and ECM proteins in diabetic nephropathy. The TGF-β1 gene and several ECM genes are regulated by TPA-responsive elements (TREs) in their gene promoters [85–88]. TREs are bound and activated by the transcription factor complex AP-1. AP-1 dimers, which are usually comprised of Jun and Fos phosphoproteins, bind the TRE when the Jun protein is dephosphorylated at its carboxyl-terminus. However, transcription of TRE-dependent genes cannot occur until the amino-terminus of Jun is phosphorylated [89]. The PKC and ERK pathways can induce AP-1 binding, and inhibition of these pathways can inhibit TRE gene expression. For example, it has recently been shown that ERK inhibition prevents HG-induced production of TGF-β1 [90]. However, in the setting of HG concentrations, ERK activation alone should not be sufficient to induce TRE-dependent genes like TGF-β1. If JNK, the classic regulator of Jun amino-terminus phosphorylation, is not activated by HG, then another glucose-sensitive signaling pathway that controls AP-1 must exist.

Recently, the p38 MAPK pathway has been shown to regulate TRE gene expression and AP-1 binding to DNA [91–95]. The mechanisms by which the p38 MAPK pathway influences TRE genes remain to be fully determined. Nevertheless, we investigated whether glucose-mediated p38 MAPK activation affected AP-1 binding in HMC. These studies in part were based on our earlier observations that HG concentrations increase AP-1 binding in HMCs but that PKC and ERK inhibition does not fully reverse the HG effect [18]. We speculated that another pathway likely exists that controls the HG-mediated AP-1 binding, and potentially subsequent TRE gene transcription. Because oxidant stress increases AP-1 binding [96, 97], we also speculated that an ROS-regulated pathway would be involved. When HG cells were treated with the p38 MAPK inhibitor SB203580, a decrease in AP-1 binding was observed that did not exist when NG cells were similarly treated or when cells were treated with the vehicle of SB203580, DMSO. These observations strongly argue that a link between p38 MAPK and AP-1 exists in the HG environment. Such a link was recently suggested in a study that showed a decline in TGF-β1 production by glucose-conditioned porcine mesangial cells following SB203580 treatment [86]. Given the absence of JNK activation in HG environments, the activation of p38 MAPK likely completes TRE gene expression of bound AP-1. The p38 MAPK pathway therefore represents an extremely important pathway through which diabetic nephropathy may evolve.

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APPENDIX

Abbreviations used in this article are: AP-1, activator protein-1; BCA, bicinchoninic acid; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DPI, diphenylinodinonium; ECL, enhanced chemiluminescence; ECM, extracellular matrix; EGTA, ethyleneglycol tetraacetic acid; GFP, G 109203 X; HG, high glucose; HMC, human mesangial cells; IL, interleukin; JNK, Jun N-terminal kinase; L-NAC, L-n-acetylcysteine; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; NG, normal glucose; Ro, Ro 32-0432; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; TRE, TPA-responsive element; VSMC, vascular smooth muscle cells.

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