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Effect of glucose on stress-activated protein kinase activity in mesangial cells and diabetic glomeruli

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Background. We have reported that hyperglycemia increases c-*jun* mRNA levels in isolated glomeruli of diabetic rats. The transcriptional activity of c-*jun* can be modified by phosphorylation of serine residues in the regulatory domain of the protein by stress-activated protein kinases (SAPKs), but the effect of high glucose concentrations on SAPK expression and activity is unknown. Accordingly, we studied p42/44 MAPK, p38 MAPK, and SAPK expression and activity in primary mesangial cells exposed to high glucose concentrations, as well as SAPK expression and activity in glomeruli of normal and streptozotocin-induced diabetic rats.

Methods. Mesangial cells were incubated in 40 mM glucose for 30 and 60 minutes and 6, 12, 24, and 48 hours, whereas glomeruli of streptozotocin-induced diabetic rats were isolated one day and one and two weeks after the onset of hyperglycemia (blood glucose levels more than 15 mmol/liter), and were compared with age-matched normal rats. Cell lysates were subjected to Western blot analysis of SAPK and phosphorylated SAPK and an *in vitro* SAPK assay using recombinant c-*jun.*

Results. Western blot analysis revealed that SAPK was expressed, but unphosphorylated, in unstimulated mesangial cells and whole glomerular lysates from normal rats. In accord with these observations, no SAPK activity was detected in lysates from mesangial cells or whole glomeruli from normal rats, although mesangial cell SAPK activity was readily induced *in vitro* by sorbitol. High glucose concentrations did not increase SAPK activity or lead to detectable phosphorylated SAPK either *in vitro* or *in vivo.* In contrast, short-term exposure to 40 mM of glucose activated both p42/44 MAPK and p38 MAPK.

Conclusions. We conclude that high glucose concentrations do not activate SAPK in primary cultured mesangial cells or in diabetic glomeruli during the early phase of diabetic renal hypertrophy.

Early diabetic nephropathy is characterized by glomerular enlargement [1] and tubuloepithelial cell hypertrophy [2], but the progressive accumulation of extracellular matrix in mesangial areas leads to mesangial expansion, which is associated with tubular epithelial cell atrophy and interstitial fibrosis, and progressive renal failure [3, 4]. Strict control of hyperglycemia delays the onset and slows the progression of diabetic renal injury [5]. Glucose influences many aspects of renal cellular function, including sodium-glucose cotransport [6], protein glycosylation [7], cellular hypertrophy [8], synthesis of transforming growth factor-β [9], and extracellular matrix protein accumulation [10], and the exposure of mesangial cells to a high glucose medium has been used to study the cellular effects of glucose on the kidney glomerulus [11, 12].

Growth-related protooncogenes, including c-*fos*, c-*jun*, and c-*myc*, encode proteins that regulate gene transcription and are typically expressed transiently by cells in response to a stimulus [13–17]. Wolf et al have shown that c-*myc* is expressed when mesangial cells are exposed to high glucose concentrations [18]. More recently, we reported that the early phase of glomerular growth following the onset of hyperglycemia in diabetic rats is accompanied by increases in c-*fos* and c-*jun* expression [19], an effect that has also been observed *in vitro* [20].

Post-translational modification plays an important role in the regulation of c-*jun* activity [21]. Specifically, phosphorylation of two serine residues in the transactivation domain of c-*jun* (Ser 63 and Ser 73) enhances the transcriptional activity of *c-jun* [22]. The stress-activated protein kinases (SAPKs), also known as c-*jun* NH2-terminal kinases (JNKs), are responsible for this phosphorylation of c-*jun* [23–26]. Classically, SAPK/JNKs are activated by ultraviolet irradiation, heat shock, extracellular osmolarity, and cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1) [23, 24, 27–29].

The effect of high glucose concentrations on SAPK/JNK activity in mesangial cells *in vitro* and in diabetic glomeruli *in vivo* has not been studied. Accordingly, we sought to determine if high glucose concentrations increased SAPK/JNK activity in mesangial cells *in vitro*,

Key words: hyperglycemia, diabetic renal hypertrophy, SAPK, MAPK, tubuloepithelial cell injury.

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and/or in glomeruli from streptozotocin-induced diabetic rats during the early phase of diabetic renal hypertrophy.

METHODS

Animal model

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) weighing 312 to 353 g received streptozotocin, 60 mg/kg body wt, via a single tail vein injection. Age-matched normal rats served as the control group. Blood samples from tails were obtained every six hours after the administration of streptozotocin for determination of blood glucose levels with an Ames Accutest Glucometer. After the onset of hyperglycemia (defined as blood glucose levels greater than 15 mmol/liter), the blood glucose level was measured daily. Rats were excluded if their blood glucose levels failed to rise above 15 mmol/liter. All of the animals were allowed free access to standard food and water. Diabetic rats were studied at one day ($N = 5$), one week ($N = 5$), and two weeks ($N = 5$) after the onset of hyperglycemia. Age-matched normal rats served as the control group ($N = 5$).

Glomerular isolation

At each time point studied, five rats from each group were anesthetized intraperitoneally with Brietal® (50 mg/kg), and the kidneys were rapidly removed. Perirenal fat was trimmed off. After the renal capsule was removed, the cortex was separated from the medulla. Cortical tissue from each rat was pooled, placed in ice-cold phosphate-buffered saline (PBS) buffer (pH 7.4), and cut into small pieces. The glomeruli were isolated from the pooled cortical tissue by the technique of differential sieving, as previously described [30]. The tissue was first passed through a 250-micron sieve and resuspended in ice-cold PBS before centrifugation at 1000 r.p.m. for five minutes at 4°C. The pellet was resuspended in ice-cold PBS and drawn up through a 21 gauge needle, discharged three times, and then centrifuged at 1000 r.p.m. for five minutes at 4°C. The tissue pellet was resuspended in ice-cold PBS, and passed through a 106-micron sieve and then a 75-micron sieve. Glomeruli collected on the 75-micron sieve were finally resuspended in ice-cold PBS. The purity of the final suspension was determined by light microscopic examination. On average, there were fewer than five tubular fragments per 100 glomeruli. The suspension of glomeruli was then used for protein isolation.

Mesangial cell culture

Glomeruli from male Sprague-Dawley rats weighing 100 to 150 g were isolated by standard sieving methods as described previously [30, 31]. The isolated glomeruli were cultured in a 100 mm wells containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 10,000 U/ml penicillin, and 10,000 U/ml streptomycin at 37°C under 5% CO_2. Mesangial cells between passages 7 and 9 were used. Subconfluent cells (80%) were growth arrested by reducing the FBS concentration (0.5%) in DMEM. Cells were then incubated with either sorbitol (400 mM) for 15, 30, and 60 minutes or glucose (40 mM) at 37°C for 30 and 60 minutes and 6, 12, 24, and 48 hours. The reactions were terminated by a rapid aspiration of medium and washing twice with ice-cold PBS on ice.

Protein kinase assay

The activation of SAPK was confirmed by a nonradioactive kinase assay using a SAPK/JNK assay kit (#9810; New England Biolabs, Beverley, MA, USA). Cells were lyzed in a buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM egtazic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM α-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride], and the lysates were incubated with glutathione sepharose beads coated with N-terminal c-Jun fusion protein. After washing the nonspecifically bound proteins, the kinase reaction was initiated by incubation of the protein extract with a kinase buffer [25 mM Tris, pH 7.5, 5 mM α-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$] supplemented with 100 μmol cold adenosine 5′-triphosphate (ATP). The reaction was terminated by adding sample buffer [62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM DTT, 0.1% bromophenol blue] and boiling for five minutes. Phosphorylation of c-Jun at Ser63 was detected by Western blot analysis using polyclonal phospho-c-Jun (Ser63) antibody (New England Biolabs).

Western blot analysis

Samples of cultured mesangial cells or isolated glomeruli were lyzed in RIPA buffer (PBS, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.01% phenylmethylsulfonyl fluoride, 3% aprotinin, and 1 mM sodium orthovanadate). Extracts were incubated on ice for 30 minutes and were then centrifuged ($12,000 \times g$ for 20 min at 4°C). The protein concentration of the supernatant was measured with a Bio-Rad Protein Assay Kit (Ontario, Canada). The supernatant was mixed with equal volumes of $2 \times$ sample buffer (114 mM Tris, pH 6.8, 9% glycerol, 2.7% SDS, 0.02% bromophenol blue, 4.5% mercaptoethanol), boiled for five minutes, subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto nitrocellulose membranes (Millipore, Marbolo, MA, USA). Nonspecific sites were blocked by TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) supplemented with 5% skim milk for one hour at room temperature. The membranes were

probed with one of two polyclonal antibodies (1:500) against either SAPK/JNK or phospho-specific SAPK/JNK for 16 hours at 4°C. SAPK/JNK antibody (#9252; New England Biolabs) was produced by immunizing rabbits with a full-length p54 SAPK/JNK fusion protein. Phospho-specific SAPK/JNK antibody (#9251S; New England Biolabs) was produced by immunizing rabbits with a synthetic phospho-peptide against residues 179 to 193 of human SAPK, and therefore, it detected SAPK/JNK only if it was activated by phosphorylation at Thr183/Tyr185. After extensive washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (1:5000) for one hour at room temperature and washed in TBST. Immunoreactive proteins were visualized with enhanced chemiluminescence detection system (KPL Inc., Gaithersburg, MD, USA), followed by exposure to Kodak x-ray film.

Western blotting for mitogen-activated protein kinase, p38/HOG kinase and mitogen-activated protein kinase phosphatase-1

Forty micrograms of cell lysate was separated on a 12% SDS-PAGE gel. After electroblotting to a nitrocellulose membrane (Protran; Schleiter and Schuell, Keene, NH, USA), membranes were incubated for three hours at room temperature with 25 ml of blocking buffer (1 × TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk) and then overnight at 4°C with p42/p44 MAP kinase (thr 202/tyr 204) polyclonal antibody (1:1000; New England Biolabs), or p38 MAP kinase polyclonal antibody (1:1000; New England Biolabs; or MKP-1 polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 10 ml of antibody dilution buffer (1 × TBS, 0.05% Tween-20 with 5% bovine serum albumin) with gentle rocking overnight at 4°C. Membranes were then washed three times with TTBS and then incubated with HRP-conjugated antirabbit secondary antibody (1:2000) in 10 ml of blocking buffer for 45 minutes at room temperature. After three Tris-buffered saline washes, the membrane was incubated with LumiGlo reagent (KPL Inc.) and then exposed to x-ray film (X-OMAT; Kodak, Rochester, NY, USA).

Immunoprecipitation

After protein isolation from total cell lysates, 200 μg of protein were incubated with either p42/p44 MAP kinase (thr 202/tyr 204) polyclonal antibody (1:200) or p38 MAP kinase polyclonal antibody (1:100) with gentle rocking overnight at 4°C. Protein sepharose A beads (20 μl of 50% beads) were then added, and gentle rocking continued for three more hours. Lysate was then microcentrifuged for 30 seconds at 14,000 r.p.m. to recover the beads, and the pellet was washed twice with 0.5 ml of 1 × lysis buffer.

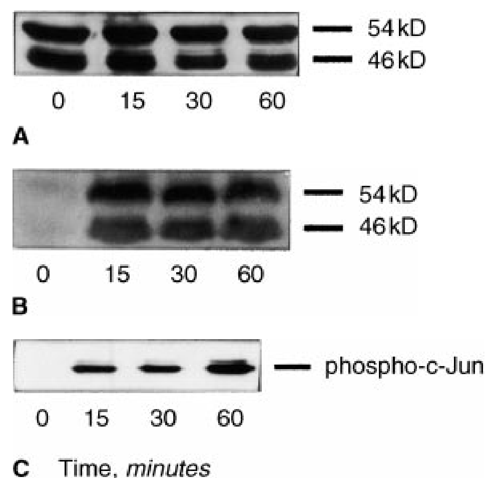

Fig. 1. Effect of 400 mM of sorbitol on stress-activated protein kinase (SAPK; *A*), SAPK phosphorylation (*B*), and SAPK activity (*C*) in primary cultured mesangial cells. (A) Western blot analysis of SAPK at baseline, 15, 30, and 60 minutes after the addition of 400 mM of sorbitol. Forty-six and 54 kDa isoforms of SAPK were detected. (B) Western blot analysis of phosphorylated SAPK at baseline, 15, 30, and 60 minutes after the addition of 400 mM of sorbitol. Phosphorylated SAPK was detected 15, 30, and 60 minutes after the addition of sorbitol. (C) Western blot analysis of a phosphorylated N-terminal *c-jun* fusion protein. SAPK activity was measured by determining the ability of cell lysates to phosphorylate an N-terminal *c-jun* fusion protein. The phosphorylated protein was then detected by Western blot analysis. Mesangial cell lysates were studied at baseline, 15, 30, and 60 minutes after the addition of 400 mM of sorbitol. SAPK activity correlated with the appearance of phosphorylated SAPK.

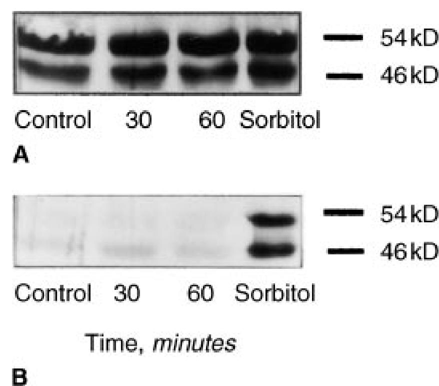

Fig. 2. Effect of 40 mM of glucose on SAPK (*A*) and SAPK phosphorylation (*B*) in primary cultured mesangial cells. (A) Western blot analysis of SAPK at baseline and 30 and 60 minutes after the addition of 40 mM of glucose and 60 minutes after the addition of 400 mM of sorbitol. (B) Western blot analysis of phosphorylated SAPK at baseline and 30 and 60 minutes after the addition of 40 mM of glucose and 60 minutes after the addition of 400 mM of sorbitol. Phosphorylated SAPK was detected 60 minutes after the addition of sorbitol, but not in the lysates from cells exposed to 40 mM of glucose.

p42/p44 and p38/HOG mitogen-activated protein kinase activity assays

Activity of p42/44 MAP kinase was determined in cells 30 and 60 minutes and 24 hours after exposure to 40 mM

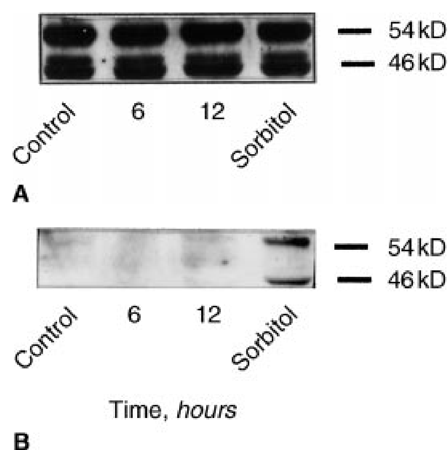

Fig. 3. Effect of 40 mM of glucose on SAPK (*A*) and SAPK phosphorylation (*B*) in primary cultured mesangial cells. (A) Western blot analysis of SAPK at baseline, 6, and 12 hours after the addition of 40 mM of glucose and at 60 minutes after the addition of 400 mM of sorbitol. (B) Western blot analysis of phosphorylated SAPK at baseline, 6, and 12 hours after the addition of 40 mM of glucose and at 60 minutes after the addition of 400 mM of sorbitol. Phosphorylated SAPK was detected 60 minutes after the addition of sorbitol, but not in the lysates from cells exposed to 40 mM of glucose.

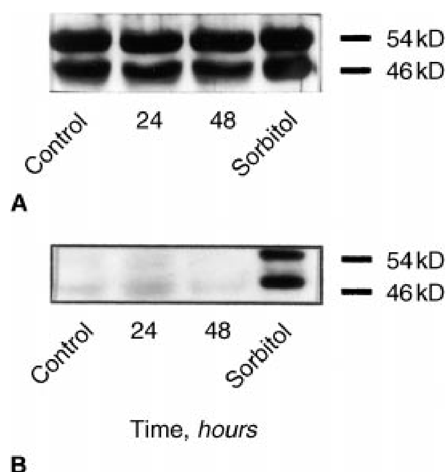

Fig. 4. Effect of 40 mM of glucose on SAPK (*A*) and SAPK phosphorylation (*B*) in primary cultured mesangial cells. (A) Western blot analysis of SAPK at baseline, 24, and 48 hours after the addition of 40 mM of glucose and at 60 minutes after the addition of 400 mM of sorbitol. (B) Western blot analysis of phosphorylated SAPK at baseline, 24, and 46 hours after the addition of 40 mM of glucose and at 60 minutes after the addition of 400 mM of sorbitol. Phosphorylated SAPK was detected 60 minutes after the addition of sorbitol, but not in the lysates from cells exposed to 40 mM glucose.

of glucose (New England Biolabs, Ltd.). The activity of p38 MAP kinase was also determined in cells 30 and 60 minutes after exposure to 40 mM glucose (New England Biolabs, Ltd.). After immunoprecipitation, pellets were washed twice with 0.5 ml kinase buffer (25 mM Tris, 5 mM β-glycerophosphate, 2 mM Na orthovanadate, 10 mM $MgCl_2$). p42/44 MAP kinase activity was then performed by suspending the pellet in 50 μl of 1 × kinase buffer, with 200 μmol ATP and 2 μg Elk-1 fusion protein, and p38 activity by using 2 μg of ATF-2 fusion protein as substrate. After incubation for 30 minutes at 30°C, the reaction was terminated with 25 μM 3 × sample buffer (187.5 mM Tris-HCl, pH 6.8, 6% wt/vol SDS, 30% glycerol, 150 mM DTT, 0.3% wt/vol bromophenol blue), boiled for five minutes, vortexed, and then microcentrifuged for two minutes. Twenty microliters of the sample were then run on an SDS-PAGE gel. After blotting to nitrocellulose, membranes were incubated for three hours at room temperature with 25 ml of blocking buffer (1 × TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk) and then overnight at 4°C with phosphospecific anti–Elk-1 (ser 383) antibody (p42/44 MAPK activity) or phospho-specific anti-ATF-2 (thr 71) antibody (p38 activity), 1:1000 in 10 ml of antibody dilution buffer (1 × TBS, 0.05% Tween-20 with 5% BSA). Gels were washed three times with TTBS and were then incubated with HRP-conjugated antirabbit secondary antibody (1:2000) for one hour at room temperature. After three further Tris-buffered saline washes, the membrane was incubated with LumiGlo reagent (KPL Inc.) and then exposed to x-ray film (X-OMAT; Kodak).

Analysis

For whole animal data, statistical significance of differences among values of individual parameters was evaluated by analysis of variance (ANOVA). Following ANOVA, Fisher's PLSD was used to assess the significance of differences between individual group means. Significance was defined as a *P* of less than 0.05. Values are expressed as mean ± SE.

RESULTS

In vitro studies

Stress-activated protein kinase was detected by Western blot analysis in our mesangial cells (Fig. 1A). Two isoforms of SAPK were present with molecular weights of 46 and 54 kDa, respectively. No phospho-SAPK was evident in immunoblots of total cell lysates in the unstimulated or control cell (Fig. 1B). The measurement of SAPK activity (Fig. 1C) showed that in accordance with our inability to detect phospho-SAPK, we could not detect phosphorylation of the c-jun protein by extracts from our control cells. An exposure of the mesangial cells to 400 mM of sorbitol was associated with the prompt appearance of phosphorylated SAPK after 15 minutes (Fig. 1B, lane 2). The appearance of phosphorylated SAPK correlated with our activity assay. Fifteen minutes after exposure to 400 mM sorbitol, we were able to dem-

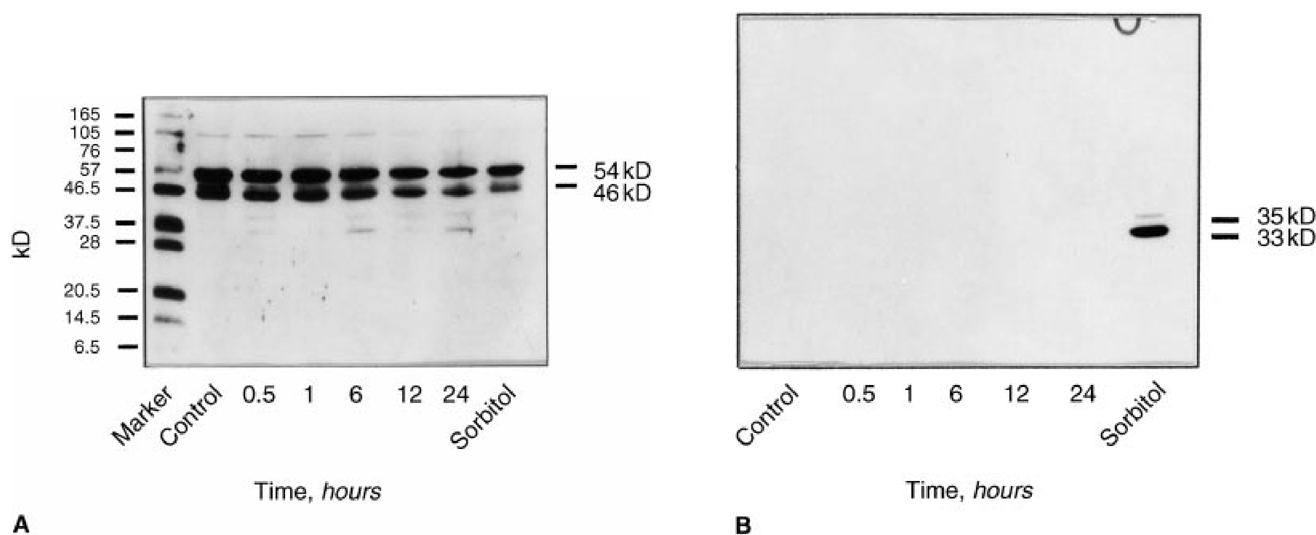

Fig. 5. Effect of 40 mM glucose on SAPK (*A*) and SAPK activity (*B*) in primary cultured mesangial cells. (A) Western blot analysis of SAPK at baseline, 30 minutes, and 1, 6, 12, and 24 hours after the addition of 40 mM of glucose. (B) Western blot analysis of a phosphorylated N-terminal *c-jun* fusion protein. SAPK activity was measured by determining the ability of cell lysates to phosphorylate an N-terminal *c-jun* fusion protein. The phosphorylated protein was then detected by Western blot analysis. Mesangial cell lysates were studied at baseline, 30 minutes, and 1, 6, 12, and 24 hours after the addition of 40 mM of glucose and at 60 minutes after the addition of 400 mM of sorbitol. SAPK was activated by sorbitol, as expected, but exposure to 40 mM of glucose did not increase SAPK activity.

Table 1. Stress activated protein kinase activity in isolated glomeruli after the onset of hyperglycemia

	Glucose *mM*	Body wt *g*	Kidney wt *g*	*Kidney wt/ body wt %*
Normal	*5.1 ± 0.1*	*304 ± 7*	*2.1 ± 0.03*	*0.69 ± 0.01*
Diabetic 1 day	*17.0 ± 0.2*[a]	*274 ± 2*	*2.1 ± 0.04*	*0.77 ± 0.01*[a]
Diabetic 1 week	*20.7 ± 1.3*[a]	*238 ± 11*[a]	*2.3 ± 0.21*	*0.96 ± 0.04*[a]
Diabetic 2 weeks	*19.5 ± 0.9*[a]	*235 ± 23*[a]	*2.7 ± 0.27*[a]	*1.14 ± 0.01*[a]

[a] $P < 0.05$ vs. normal

onstrate phosphorylation of the c-jun protein with our *in vitro* kinase assay (Fig. 1C, lane 2). Phosphorylated SAPK was present 30, 60, and 90 minutes after exposure to sorbitol, and at each time point, our *in vitro* kinase assay revealed that whole cell extracts were able to phosphorylate the c-jun protein. Based on these studies, we used the 60-minute time point after exposure to sorbitol as a positive control in all of the following *in vitro* and *in vivo* studies of SAPK.

We then studied the effect of high glucose (40 mM) on SAPK expression and phosphorylation at three time points. The results of short-term exposure to glucose (30 and 60 min) are illustrated in Figure 2. SAPK was present in the cell extracts at each time point. However, we could not detect increased phosphorylated SAPK 30 to 60 minutes after exposure to high glucose. Lane 4 (Fig. 2B) compares the effect of 400 mM sorbitol in the phosphorylation of SAPK.

We further examined the effect of high glucose on SAPK phosphorylation after 6, 12, and 24 hours. Again, SAPK was readily detected (Fig. 3A), but no phosphorylated SAPK was present. Finally, we examined the effect of high glucose on SAPK phosphorylation after 24 and 48 hours. We could not detect increased phosphorylation of SAPK by Western blot analysis (Fig. 4 A, B).

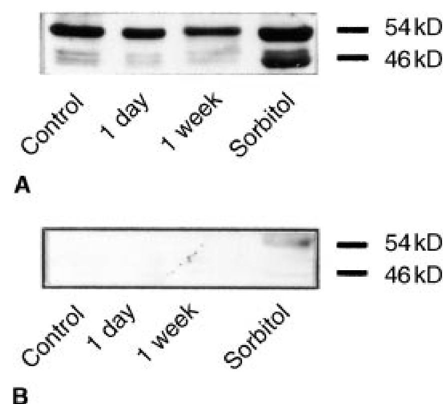

Fig. 6. Effect of experimental type I diabetes mellitus on SAPK (*A*) and SAPK phosphorylation (*B*) in isolated rat glomeruli. (A) Western blot analysis of SAPK in lysates of glomeruli from normal rats and glomeruli of rats hyperglycemic (blood glucose levels more than 15 mM) for one day and one week. Lane 4 is a western blot analysis of mesangial cell lysates 60 minutes after the addition of 400 mM of sorbitol. The 54 kDa isoform of SAPK was more abundant than the 46 kDa isoform. (B) Western blot analysis of phosphorylated SAPK in lysates of glomeruli from normal rats and glomeruli of rats hyperglycemic (blood glucose levels more than 15 mM) for one day and one week. Lane 4 is a Western blot analysis of mesangial cell lysates 60 minutes after the addition of 400 mM of sorbitol. Phosphorylated SAPK was in mesangial cell lysates 60 minutes after the addition of sorbitol, but not in the lysates of glomeruli from normal or diabetic rats.

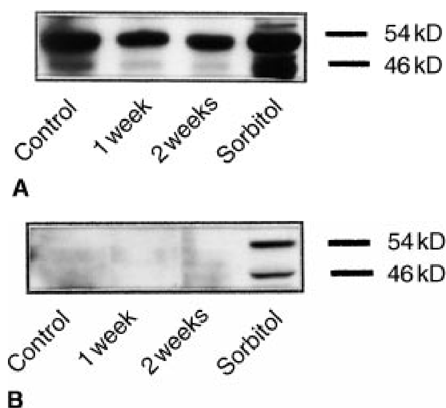

Fig. 7. Effect of experimental type I diabetes mellitus on SAPK (*A*) and SAPK phosphorylation (*B*) in isolated rat glomeruli. (A) Western blot analysis of SAPK in lysates of glomeruli from normal rats and glomeruli of rats hyperglycemic (blood glucose levels more than 15 mM) for one week and two weeks. Lane 4 is a Western blot analysis of mesangial cell lysates 60 minutes after the addition of 400 mM of sorbitol. The 54 kDa isoform of SAPK was more abundant than the 46 kDa isoform. (B) Western blot analysis of phosphorylated SAPK in lysates of glomeruli from normal rats and glomeruli of rats hyperglycemic (blood glucose levels more than 15 mM) for one week and two weeks. Lane 4 is a Western blot analysis of mesangial cell lysates 60 minutes after the addition of 400 mM of sorbitol. Phosphorylated SAPK was in mesangial cell lysates 60 minutes after the addition of sorbitol, but not in the lysates of glomeruli from normal or diabetic rats.

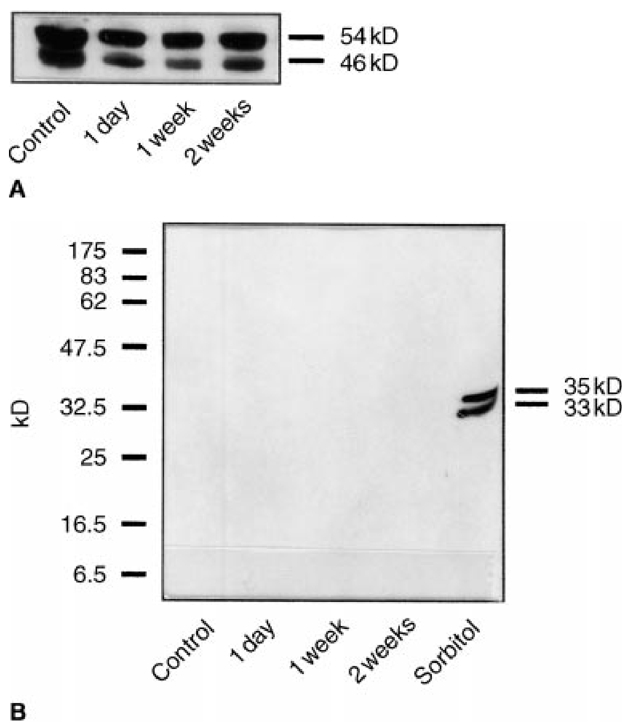

Fig. 8. Effect of experimental type I diabetes mellitus on SAPK (*A*) and SAPK Activity (*B*) in isolated rat glomeruli. (A) Western blot analysis of SAPK in lysates of glomeruli from normal rats and glomeruli of rats hyperglycemic (blood glucose levels more than 15 mM) for one day, one week, and two weeks. Lane 4 is a Western blot analysis of mesangial cell lysates 60 minutes after the addition of 400 mM of sorbitol. (B) Western blot analysis of a phosphorylated N-terminal *c-jun* fusion protein. SAPK activity was measured by determining the ability of lysates from isolated glomeruli to phosphorylate an N-terminal *c-jun* fusion protein. The phosphorylated protein was then detected by Western blot analysis. Isolated glomeruli were studied from normal rats and glomeruli of hyperglycemic rats (blood glucose levels more than 15 mM) for one day, one week, and two weeks. Lysates from mesangial cells were also studied 60 minutes after the addition of 400 mM of sorbitol. SAPK was activated by sorbitol, as expected, but SAPK activity was not detected in normal glomeruli or in the glomeruli of hyperglycemic rats.

In order to relate these observations to activity, SAPK activity was measured in cell lysates from mesangial cells 30 minutes and 1, 6, 12, and 24 hours after exposure to 40 mM of glucose. A 60-minute exposure to 400 mM of sorbitol was used as the control condition. SAPK was readily detectable by Western blot analysis of each cell lysate (Fig. 5A). SAPK activity was induced by sorbitol (Fig. 5B, lane 7), but glucose did not increase SAPK activity.

In vivo studies

Whole animal data for our experiments with normal and streptozotocin-induced diabetic rats are shown in Table 1. We studied hyperglycemic diabetic rats during the early phase of renal hypertrophy. SAPK was detected in protein extracts from isolated glomeruli (Fig. 6A), although unlike the *in vitro* studies, the 54 kDa SAPK was the most abundant isoform. We could not detect phosphorylated SAPK in isolated glomeruli from the normal or diabetic rats one day and one week after the onset of hyperglycemia (Fig. 6B). Figure 7 shows further experiments one and two weeks after the onset of hyperglycemia. Phosphorylated SAPK could not be detected in glomerular protein extracts 7 and 14 days after the onset of hyperglycemia.

To relate these observations to activity, SAPK activity was measured in cell lysates from isolated glomeruli one day, one week, and two weeks after the onset of hyperglycemia and was compared with normal glomeruli. A 60-minute exposure to 400 mM of sorbitol was used as the control condition (Fig. 8). SAPK was readily detectable by Western blot analysis of each cell lysate (Fig. 8A). As a positive control condition for the assay, we were able to detect SAPK activity after exposure to 400 mM of sorbitol (Fig. 8B, lane 5), but SAPK activity was not detected in isolated glomeruli from normal rats or diabetic rats one day, one week, and two weeks after the onset of hyperglycemia (Table 1).

p42/p44 mitogen-activated protein kinase activity assay

p42/44 MAPK was detected by Western blot analysis in our mesangial cells (Fig. 9A). There was no effect of short-term (30 and 60 min) incubation with 40 mM of glucose on p42/44 MAPK expression. In contrast to our observations with SAPK, the measurement of p42/44 MAPK activity (Fig. 9B) showed that we could detect

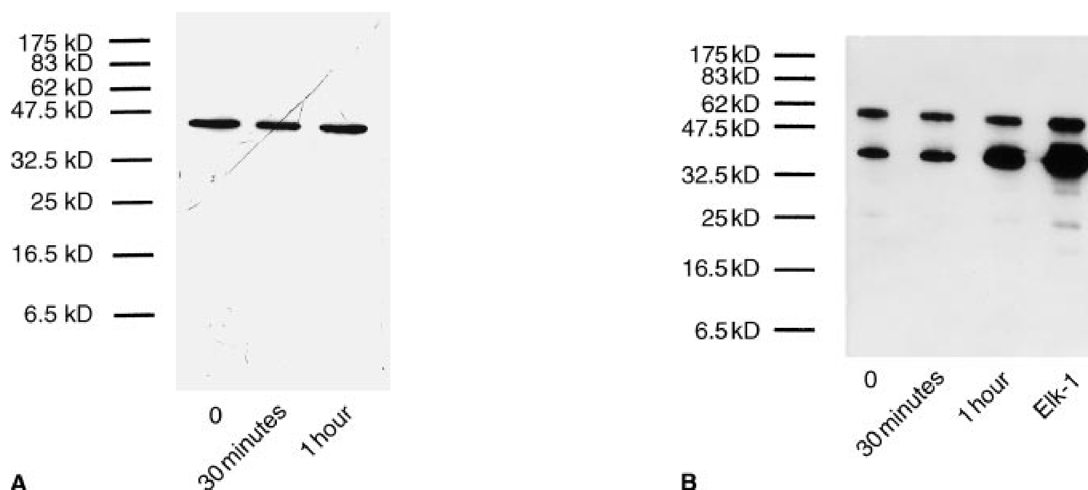

Fig. 9. Effect of 40 mM glucose on p42/44 MAPK (*A*) and p42/44 MAPK activity (*B*) in primary cultured mesangial cells. (A) Western blot analysis of p42/44 MAPK at baseline, 30 minutes, and1 hour after addition of 40 mM of glucose. (B) Western blot analysis of phosphorylated *Elk-1* fusion protein. p42/44 MAPK activity was measured by detecting the phosphorylation of an *Elk-1* fusion protein by mesangial cell immunoprecipitates, as described in the **Methods** section. The phosphorylated protein was then detected by Western blot analysis at baseline, 30 minutes, and 1 hour after the addition of 40 mM of glucose.

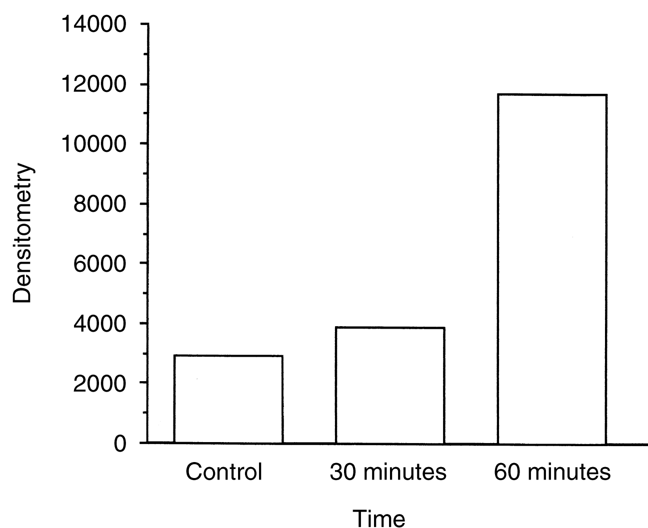

Fig. 10. p42/44 MAPK activity. Densitometry measures of Western blot analysis of phosphorylated *Elk-1* fusion protein. p42/44 MAPK activity was measuring by detecting the phosphorylation of an *Elk-1* fusion protein by mesangial cell immunoprecipitates, as described in the **Methods** section. The phosphorylated protein was detected by Western blot analysis at baseline, 30 minutes, and 1 hour after addition of 40 mM glucose.

basal phosphorylation of the Elk-1 protein in immunoprecipitates from our control cells. Exposure of the mesangial cells to 40 mM glucose led to a fourfold to fivefold increase in p42/44 MAPK activity at 60 minutes (Fig. 10).

p38/HOG mitogen-activated protein kinase activity assays

p38 MAPK was detected by Western blot analysis in our mesangial cells (Fig. 11A). There was no effect of short-term (30 and 60 min) incubation with 40 mM glucose on p38 MAPK expression. Basal activity of p38 MAPK was low in growth-arrested mesangial cells (Fig. 9B), although phosphorylation of the ATF-2 protein by immunoprecipitates from control cells was detected. In contrast to the effect of glucose on SAPK activity, marked increases in p38 MAPK activity were evident as early as 30 minutes after exposure to 40 mM glucose. p38 MAPK activity increased further after 60 minutes (Fig. 12).

Western blot analysis of mitogen-activated protein kinase phosphatase-1

Mitogen-activated protein kinase phosphatase 1 (MPK-1) was detected by Western blot analysis in mesangial cell lysates (Fig. 13). There was a marked increase in expression within one hour of exposure to 40 mM of glucose (Fig. 13A), and the increase was present, although of less magnitude, 24 hours after exposure to 40 mM of glucose (Fig. 13B). After 48 hours, MPK-1 protein expression declined to control levels. Mean values for densitometry are illustrated in Figure 14.

DISCUSSION

The rationale for this study was motivated by two experimental observations: (*1*) our report that *c-jun* expression was increased in isolated glomeruli from diabetic rats during the early phase of hyperglycemia-induced renal hypertrophy [19], and (*2*) by the recent report by Haneda et al that high glucose stimulated MAP kinase activity in mesangial cells *in vitro*, and in isolated glomeruli from diabetic rats [32].

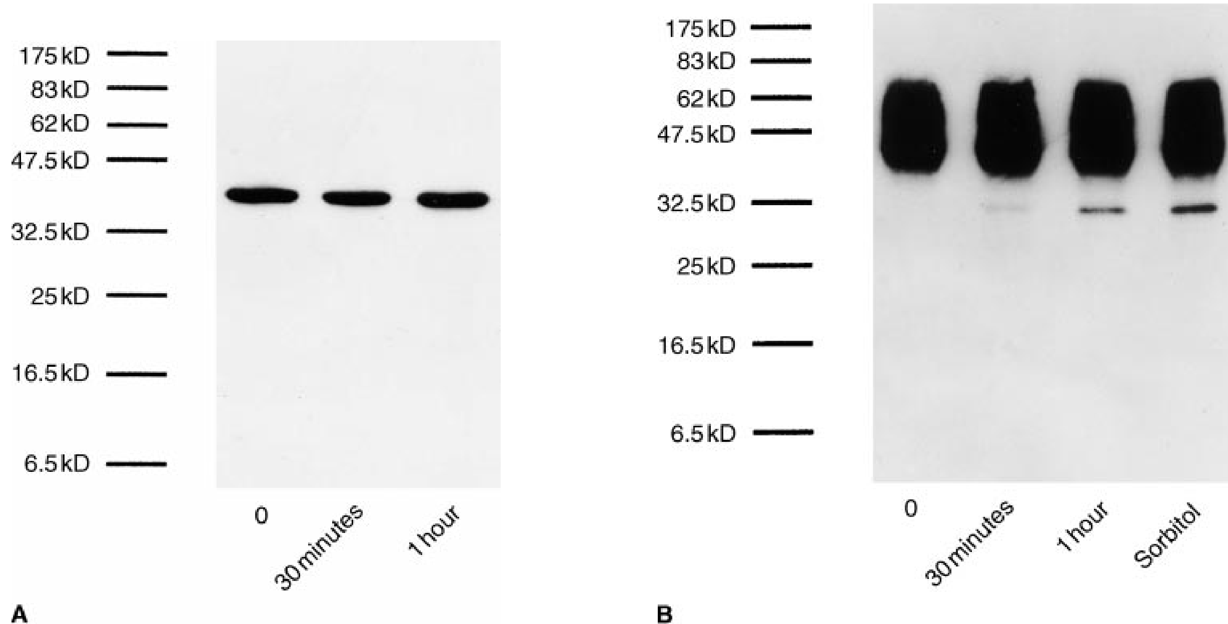

Fig. 11. Effect of 40 mM glucose on p38 MAPK (*A*) and p38 MAPK activity (*B*) in primary cultured mesangial cells. (A) Western blot analysis of p38 MAPK at baseline, 30 minutes, and 1 hour after addition of 40 mM glucose. (*B*) Western blot analysis of phosphorylated *ATF-2* fusion protein. p38 MAPK activity was measured by detecting the phosphorylation of an *ATF-2* fusion protein by mesangial cell immunoprecipitates, as described in the **Methods** section. The phosphorylated protein was then detected by Western blot analysis at baseline, 30 minutes, and 1 hour after the addition of 40 mM glucose.

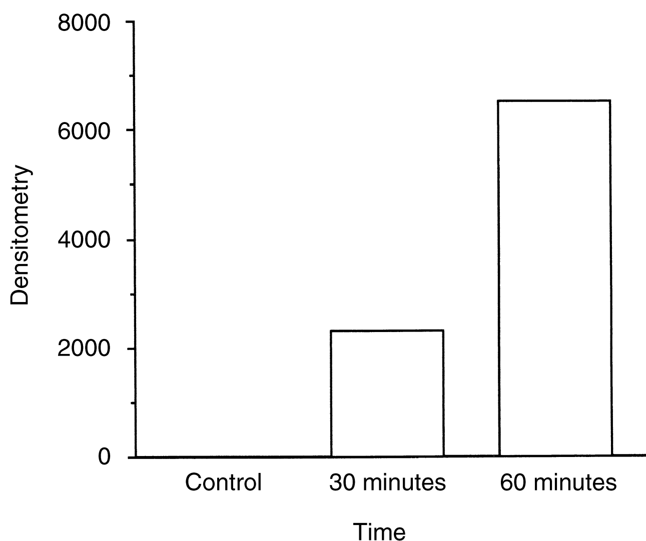

Fig. 12. p38 MAPK activity. Densitometry measures of Western blot analysis of phosphorylated *ATF-2* fusion protein. p42/44 MAPK activity was measured by detecting the phosphorylation of an *ATF-2* fusion protein by mesangial cell immunoprecipitates, as described in the **Methods** section. The phosphorylated protein was detected by Western blot analysis at baseline, 30 minutes, and 1 hour after the addition of 40 mM of glucose.

Growth-related protooncogenes such as *c-jun* encode proteins that regulate gene transcription and are typically expressed transiently by cells in response to a stimulus [13–17]. Post-translational modification of *c-jun* plays an important role in the regulation of the functional activity of the protein [21, 22]. Specifically, phosphorylation of two serine residues in the transactivation domain of *c-jun* (serine 63 and serine 73) enhance *c-jun* transcriptional activity [22, 33]. The SAPKs, also known as JNKs, are responsible for this phosphorylation of *c-jun* [22–26, 33–36].

Stress-activated protein kinases (SAPK/JNKs), extracellular signal-regulated kinases (ERKs or the p42/p44 MAPKs), and p38 kinase are members of the MAP kinase superfamily. These three kinase cascades constitute functionally distinct but structurally related transduction pathways by which extracellular signals are transmitted to the nucleus to regulate gene expression [37]. ERKs play an important role in cell proliferation [38] and cell hypertrophy [39], whereas SAPK has been implicated in the cellular response to environmental stresses such as extracellular osmolality [40], ultraviolet irradiation, heat shock, and inflammatory cytokines [23, 24, 27–29, 31–33, 44].

Stress-activated protein kinases must be phosphorylated at Thr and Tyr residues for activation, although the phosphorylation site motif present in SAPKs (Thr-Pro-Tyr) is distinct from that of ERKs (Thr-Glu-Tyr) [24, 45]. "Cross-talk" between the ERK and SAPK pathways has been reported [46–58]. Haneda et al have recently reported that MAPK activity is increased by high glucose *in vitro* and *in vivo* [32], but the effect of glucose on SAPK activity has not been defined.

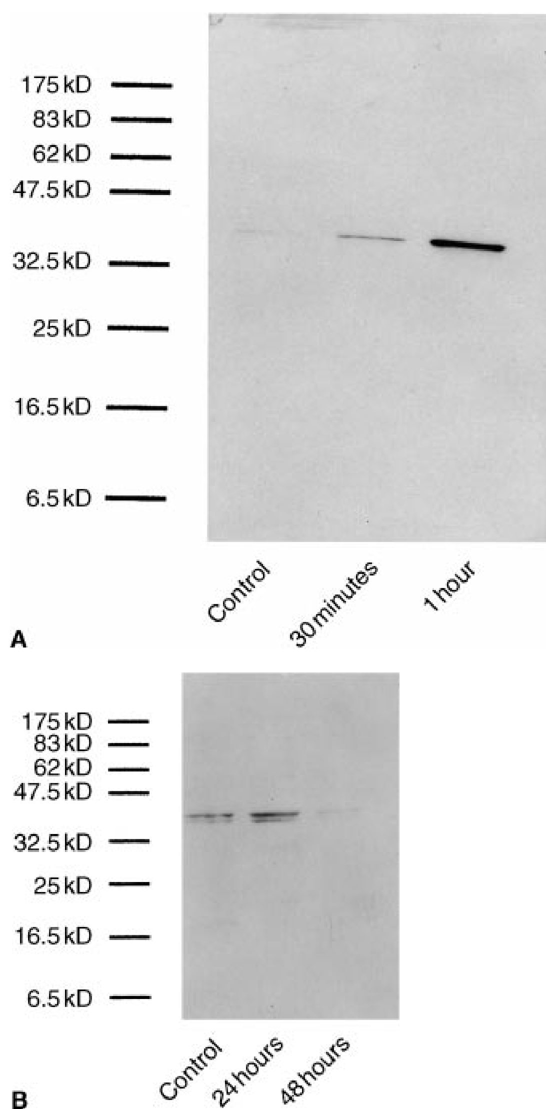

Fig. 13. Effect of 40 mM of glucose on MKP-1 expression in primary cultured mesangial cells. (*A*) Western blot analysis of MKP-1 at baseline, 30 minutes, and 1 hour after the addition of 40 mM glucose. (*B*) Western blot analysis of MKP-1 at baseline, 24, and 48 hours after the addition of 40 mM of glucose.

Accordingly, the aims of this study were first to determine if high glucose concentrations increased SAPK/JNK activity in primary cultured mesangial cells and second, to compare this response with the effect of hyperglycemia on SAPK/JNK activity in diabetic glomeruli during the early phase of hyperglycemia-induced renal hypertrophy.

Our first major observation was that although SAPK was expressed and readily activated by 400 mM of sorbitol, exposure to 40 mM of glucose did not lead to the phosphorylation and activation of SAPK in primary cultured mesangial cells. SAPK was detected by Western blot analysis in cell lysates from control mesangial cells and mesangial cells exposed to high glucose concentrations, and we detected two isoforms with molecular weights of 46 and 54 kDa, respectively. Activation of SAPK/JNK occurs via phosphorylation at two residues, Thr183 and Tyr185, by the dual-specificity enzyme, SAPK and ERK kinase-1 (SEKI), which has also been termed MKK4 [23, 24, 49]. Because dual phosphorylation of SAPK/JNK at Thr183/Tyr185 is essential for kinase activity, we used phosphorylation at this site as a marker of SAPK/JNK activity [23, 24, 49]. We could not detect phosphorylated SAPK in growth-arrested mesangial cells maintained in 5.6 mM glucose or in mesangial cells exposed to high glucose for up to 48 hours. In contrast, phosphorylated SAPK was present when cell lysates from mesangial cells exposed to 400 mM of sorbitol were studied. In accord with these Western blot analyses, SAPK activity was not detected in mesangial cells under control conditions or after exposure to high glucose *in vitro*, but SAPK activity correlated with the detection of phosphorylated SAPK in mesangial cells exposed to 400 mM of sorbitol.

Our second major observation was that *in vivo*, hyperglycemia did not lead to the phosphorylation and activation of SAPK during the phase of early diabetic renal hypertrophy. Both the 46 and 54 kDa SAPK isoforms were detected by Western blot analysis in protein lysates from glomeruli isolated from normal and diabetic rats. We could not detect phosphorylated SAPK by Western blot analysis or measure SAPK activity in lysates from isolated glomeruli of normal rats or diabetic rats, suggesting that although SAPK is present in glomerular cells, it is not activated under normal conditions or during the early phase of diabetic renal hypertrophy when c-jun expression is increased [19].

In vitro, SAPK is activated in glomerular mesangial cells incubated with endothelin-1 (10^{-8} M) [50], and following renal ischemia and reperfusion, SAPK is activated within 5 minutes and remains activated up to 60 and 90 minutes in the kidney [41]. The activation of SAPK by sorbitol, but not high glucose, raises the possibility that SAPK may be selectively dephosphorylated or down-regulated when glucose levels are high. This hypothesis is supported by our observations that both p42/44 MAPK and p38 MAPK were activated by high glucose concentration in our experimental conditions (Figs. 9–12) and by the recent report by Wilmer and Cosio that 30 mM glucose conditions for five days did not activate SAPK in cultured mesangial cells [51].

The mechanism(s) responsible for the differential activation of intracellular kinases by high glucose concentrations was not determined in this study, although we examined the effect of glucose on expression of MKP-1. MKP-1 is a dual-specificity phosphatase that dephosphorylates and inactivates MAPK *in vitro* [39] and *in vivo* [38]. Recent studies show that constitutive activation of MAPK cascade or sustained increase in protein kinase

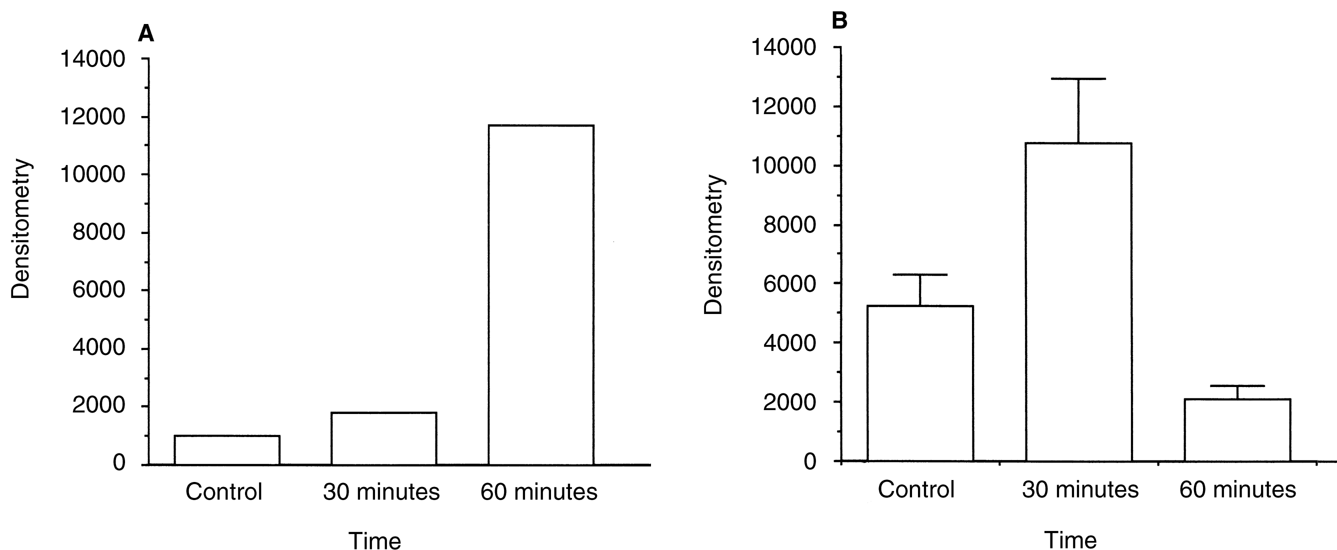

Fig. 14. Densitometry of Western blot analysis of the effect of 40 mM of glucose on MKP-1 expression in primary cultured mesangial cells. (*A*) Baseline, 30 minutes, and 1 hour after addition of 40 mM of glucose. (*B*) Baseline, 24, and 48 hours after the addition of 40 mM of glucose.

C induces expression of MKP-1 [52]. Overexpression of MKP-1 can inactivate both p42/44 MAPK (ERK) and SAPK [48], and in a rat model of glomerulonephritis in which MKP-1 was not expressed, SAPK was activated [53]. We observed that 40 mM of glucose led to an increase in MKP-1 expression in mesangial cells *in vitro* after 60 minutes. Similar increases in MKP-1 protein expression have been observed in mesangial cells treated with ANP for 120 minutes *in vitro* [54] and in the aortae of rats after 60 minutes of hypertension [55]. Increased MKP-1 might then serve to limit mesangial cell SAPK activation after exposure to 40 mM of glucose, but the increase was not sufficient to prevent early glucose-induced activation of both p42/44 MAPK and p38 MAPK.

Another suppressor of SAPK has been recently characterized, a cytoplasmic protein called JNK interacting protein-1 (JIP-1). JIP-1 is considered to be a cytoplasmic anchor that prevents SAPK translocation into the nucleus, the critical step for functional SAPK to reach transcription factors located in the nucleus. The cytoplasmic retention ultimately terminates signal transduction of the SAPK pathway [56]. JIP-1 is present in the kidney [56], but the effect of high glucose concentrations on JIP-1 expression is unknown. JIP-1 binds to SAPK and retains the kinase in the cytoplasm so that although it suppresses gene expression regulated by SAPK, it does not prevent SAPK phosphorylation [56, 57]. We studied both SAPK phosphorylation and SAPK activity in mesangial cells exposed to 40 mM of glucose. Our failure to detect phosphorylated SAPK argues against sequestration of phosphorylated SAPK in the cytoplasm by JIP-1 as a mechanism limiting SAPK-regulated gene expression in mesangial cells exposed to 40 mM glucose, although other studies are necessary to define the effects of high glucose on JIP-1 expression in mesangial cells and isolated glomeruli.

In summary, we have observed that SAPK is expressed and readily activated by 400 mM of sorbitol in primary cultured mesangial cells, but exposure of growth-arrested mesangial cells to 40 mM of glucose, for up to 48 hours, does not lead to the phosphorylation or activation of SAPK. In contrast, short-term exposure to glucose activates both p42/44 MAPK and p38 MAPK. The 46 and 54 kDa SAPK isoforms are both expressed by glomerular cells *in vivo*, but SAPK is not phosphorylated or activated in normal glomeruli or in glomeruli from diabetic rats during the early phase of diabetic renal hypertrophy.

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APPENDIX

Abbreviations used in this article are: ATP, adenosine 5′-triphosphate; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetatic acid; EGTA, egtazic acid; ERK, extracellular signal-regulated protein

kinase; HRP, horseradish peroxidase; JIP-1, JNK interacting protein-1; JNKs, c-*jun* NH2-terminal kinases; MAP, mitogen activated protein; MAPK, mitogen activated protein kinase; MPK-1, mitogen-activated protein kinase phosphatase-1; PBS, phosphate buffered saline; SAPKs, stress-activated protein kinases; SEKI, SAPK and ERK kinase-1 combined.

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