ERK and p38 mediate high-glucose-induced hypertrophy and TGF-β expression in renal tubular cells

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Fujita, Hisayo, Sayyu Omori, Kenji Ishikura, Mariko Hida, and Midori Awazu. ERK and p38 mediate high-glucose-induced hypertrophy and TGF-β expression in renal tubular cells. Am J Physiol Renal Physiol 286: F120–F126, 2004. First published September 2, 2003; 10.1152/ajprenal.00351.2002.—We investigated the expression of ERK, p38 mitogen-activated protein kinase (p38), and JNK in renal tubules of diabetic rats following 3 wk after streptozotocin injection (DM). Although the expression of ERK was not different between controls and DM, phosphorylated ERK was expressed more intensely in DM. p38 And phosphorylated p38 were detected only in the diabetic kidney and were localized in all tubular segments. JNK and phosphorylated JNK were expressed similarly in controls and DM. Transforming growth factor (TGF)-β was expressed in all tubular segments of DM, coinciding with the localization of p38. In LLC-PK1 cells, forming growth factor (TGF)-β/H9252 phosphorylated JNK were expressed similarly in controls and DM. Tubules of diabetic rats following 3 wk after streptozotocin injection served as controls. Two diabetic rats were treated with a daily injection of insulin (Novolin U, Novo Nordisk Pharmaceutical) starting 7 days after streptozotocin administration. Rats matched for age and weight at the time of streptozotocin injection were used as controls. Two diabetic rats were treated with a daily injection of insulin (Novolin U, Novo Nordisk Pharma). At death, blood was drawn from the abdominal aorta for determination of nonfasting plasma glucose by the glucose oxidase method. Kidneys were harvested and fixed with neutral buffered formalin. Induction of diabetes. Male Sprague-Dawley rats, weighing 180 g, were injected with 60 mg/kg streptozotocin (60 mg/ml in 0.01 M citric acid, 0.0% saline, pH 4) by tail vein. Rats were killed 3 wk after the injection. Rats matched for age and weight at the time of streptozotocin administration served as controls. Two diabetic rats were treated with a daily injection of insulin (Novolin U, Novo Nordisk Pharma). At death, blood was drawn from the abdominal aorta for determination of nonfasting plasma glucose by the glucose oxidase method. Kidneys were harvested and fixed with neutral buffered formalin. Immunohistochemistry. After fixation, kidneys were embedded in paraffin. Immunohistochemical staining was performed on serial sections 3-μm thick, using the enzyme-labeled antibody method. Paraffin sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by incubating sections in 0.3% H2O2/methanol for 15 min. To unmask antigens, slides were boiled at 100°C for 10 min in 10% citrate buffer (pH 6.0)/methanol. Sections were incubated with antibodies against ERK (dilution 1:200), p38 (1:100), JNK (1:20), P-ERK (1:200), P-p38 (1:20), P-JNK (1:200), TGF-β (1:10), or WT1 (1:50). The incubation time was 60 min at room temperature or overnight at 4°C. After being incubated with secondary antibody at a concentration of 1:100, immunoreaction products were developed with 3,3′-diaminobenzidine as the chromogen, with standardized
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Development times. Sections were then counterstained with methyl green. Positive controls (brain for ERK and JNK, and bone marrow for p38) were run simultaneously. Negative controls included adding saturating titers of antigen (for ERK), omitting the primary antibody or substituting of the primary antibody with rabbit serum.

Cell culture. LLC-PK1 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in medium 199 containing 0.5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin supplemented with 5.5 mM glucose (normal glucose) or 25 mM glucose (high glucose). In control experiments, mannitol was added to normal-glucose medium to bring total osmolality to values equivalent to high-glucose medium. In the experiments to inhibit ERK or p38, cells were incubated with high glucose containing 5 μM PD-98059 or 5 μM SB-203580. Media were replaced every day. The cultures were maintained at 37°C in a humidified atmosphere of 95% O2-5% CO2.

Immunoblot analysis. After 24 to 72 h of culture, cells were washed with cold PBS and lysed in solubilization buffer containing 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Insoluble material was removed by centrifugation (10,500 g, 10 min). The protein content in cell lysates was measured with a DC protein assay (Bio-Rad Laboratories, Tokyo, Japan). Lysates were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore, Bedford, MA). Nonspecific binding sites were blocked in TBS buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 5% skim milk overnight at 4°C. Antibodies were added to TBS containing 0.1% Tween 20 in saturating titers and incubated with mixing for 2 h at 25°C. Bound antibodies were detected using the ECL Western blotting system (Amersham, Arlington Heights, IL). Blots were scanned and quantitatively analyzed by NIH Image software.

[^3H]leucine incorporation. Cells were subcultured in a 24-well dish at a density of 1 x 10^5/4. After 24 h, cells were incubated with normal or high-glucose media in the presence or absence of SB-203580 or PD-98059 with daily exchange. After 24 h, cells were washed with ice-cold PBS and 5% trichloroacetic acid, solubilized in 0.5 N NaOH, and counted by a liquid scintillation counter.

Measurement of cell protein and cell number. Cells were cultured in a 24-well dish under normal- or high-glucose media in the presence or absence of SB-203580 or PD-98059 with daily exchange. After 24 h, cells were washed with ice-cold PBS and either trypsinized for counting cell number or solubilized in 0.5 N NaOH for protein content determination. Cell number was counted using a hemocytometer.

Measurement of cell protein and DNA. Cells were trypsinized and pelleted at 1,500 g for 5 min. Each pellet was resuspended in 1 ml buffer (50 mM Na2PO4, pH 7.4), and cells were lysed on ice by repeated passage through a 27-gauge needle. The lysate was aliquoted for protein and DNA determination. DNA was measured using the fluorescent compound bisbenzimide H-33258 fluorochrome.

Statistical analysis. The results are expressed as means ± SE. Statistical analysis was performed with ANOVA followed by multiple comparisons as appropriate. Statistical significance was determined as P < 0.05.

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Values are means ± SE, n, No. of rats. *P < 0.05 vs. control rats.

RESULTS

Immunohistochemical localization of MAPKs and TGF-β in kidneys from control, diabetic, and insulin-treated diabetic rats. Animal data are presented in Table 1. All streptozotocin-treated rats were diabetic with a mean plasma glucose of 594 mg/dl. Kidney weight corrected by body weight was significantly greater in diabetic rats than in control rats. Insulin treatment abrogated the increases in plasma glucose and kidney weight per body weight.

ERK and phosphorylated ERK. ERK was expressed in distal tubules and collecting ducts, and phosphorylated ERK was detected in occasional distal tubules and collecting ducts of control and diabetic kidneys (Fig. 1, A-E). Stronger and more frequent immunostaining for phosphorylated ERK was observed in diabetic kidneys compared with controls. Insulin treatment abrogated the upregulation of phosphorylated ERK. In glomeruli, podocytes of both control and diabetic kidneys stained positive for ERK. Phosphorylated ERK was not detected in glomeruli.

p38 and phosphorylated p38. No immunostaining for p38 or phosphorylated p38 was observed in control kidneys (Fig. 1, F-I). In the diabetic kidney, p38 and phosphorylated p38 were detected in distal tubules, collecting ducts, and occasional proximal tubules with weaker intensity. In glomeruli, p38 was detected in podocytes of diabetic kidneys, and phosphorylated p38 was also faintly positive. Again, insulin treatment inhibited the upregulation of p38 and phosphorylated p38.

JNK and phosphorylated JNK. JNK localized to distal tubules, collecting ducts, and occasional proximal tubules, and phosphorylated JNK was detected in distal tubules and collecting ducts of control and diabetic kidneys (Fig. 1, J-N). There was no difference in JNK or phosphorylated JNK expression between control and diabetic kidneys. Podocytes of both control and diabetic kidneys stained weakly positive for JNK but were negative for phosphorylated JNK.

TGF-β. TGF-β was not detected in control kidneys (Fig. 1, O and P). In the diabetic kidney, TGF-β was expressed strongly in distal tubules, collecting ducts, and moderately in proximal tubules. Thus the tubular localization of TGF-β coincided with that of p38 and phosphorylated p38. Insulin treatment completely inhibited the high-glucose-induced TGF-β expression.

Cell culture studies. We next investigated the role of activated ERK and p38 in renal tubular cells cultured under high-glucose conditions. Because proximal tubular cells are the major contributor to diabetic kidney enlargement, LLC-K1 cells were used. We cultured cells in normal- or high-glucose media for 24 to 72 h.

ERK and p38 are activated in LLC-PK1 cells cultured under high glucose. Activation of MAPKs was detected by Western blot analysis using phosho-specific antibodies. Phosphory-
lated ERK1 and ERK2 were elevated twofold in cells cultured under high glucose compared with control cells at 24 h (Fig. 2). Both ERK1 and ERK2 remained activated at 72 h. Of note, phosphorylated ERK was increased at 72 h under normal glucose compared with that at 24 h, probably due to autocrine/paracrine action of growth factors secreted by the cell. In a similar manner to ERK, p38 was phosphorylated at 24 h and high levels were sustained up to 72 h (Fig. 3). Total MAPK protein levels were unaltered by high glucose throughout the course.

Fig. 1. Immunohistochemical localization of MAPKs and transforming growth factor (TGF)-β in kidneys from control (CON), diabetic (DM) and insulin-treated diabetic rats (Ins; ×200 except for C and L). ERK (A, B, C) and phosphorylated ERK (D, E) expression is shown. ERK was expressed in distal tubules (A, B), collecting ducts (B), and podocytes (A, C) of control kidneys (CON). C (×400); podocyte-specific marker Wilms’ tumor 1 (WT1) and ERK were stained in serial sections. Phosphorylated ERK was detected in occasional distal tubules and collecting ducts of CON (D, E). In kidneys from DM rats at 3 wk after streptozotocin injection, ERK was detected in the same location as CON (A, B). Phosphorylated ERK was localized to distal tubules and collecting ducts with more frequency and stronger intensity (D, E). Insulin treatment abrogated upregulation of phosphorylated ERK (D, E), p38 (F, G, C) and phosphorylated p38 (H, I). CON was entirely negative for p38 and phosphorylated p38 (F, G, H, I). In DM, p38 and phosphorylated p38 were detected in distal tubules (F, G), collecting ducts (G), and occasional proximal tubules (F). In proximal tubules, staining was weaker and diffuse. p38 Was expressed in podocytes of DM (F, C), and phosphorylated p38 was also faintly positive (H). Insulin treatment inhibited upregulation of p38 and phosphorylated p38 (F, G, H, I). JNK (J, K, L) and phosphorylated JNK (M, N) expression is shown. JNK localized to distal tubules (J, K), collecting ducts (K), occasional proximal tubules (J), and podocytes (J) of CON (L; ×400) and DM. Phosphorylated JNK was detected in distal tubules of CON and DM (M, N). There was no difference in the expression of JNK and phosphorylated JNK between CON and DM. TGF-β was not detected in CON. In DM, intense staining was observed in distal tubules (O, P), collecting ducts (P), and weaker staining was detected in proximal tubules (O). Insulin treatment abrogated TGF-β expression. Q: negative control omitting primary antibody.
High glucose-induced activation of ERK and p38 is osmolality independent. Both ERK and p38 were phosphorylated at 48 h under high-glucose conditions confirming that their activation is sustained throughout the 72-h period (Fig. 4). To investigate whether high-glucose-induced activation of ERK and p38 in LLC-PK1 cells is induced by hyperosmolality, we examined the effect of mannitol with the same osmolality as high glucose. Although incubation of cells with high glucose for 48 h activated ERK and p38, incubation with mannitol had no effect on either of the MAPKs. These results demonstrate that activation of ERK and p38 by high glucose is not due to the osmotic effect.

ERK and p38 mediate high-glucose-induced increases in protein content, [3H] leucine incorporation, and the protein-to-DNA ratio. We next examined the role of activated ERK and p38 in high-glucose-induced hypertrophy in LLC-PK1 cells. In agreement with a previous report (4), treatment of LLC-PK1 cells with high glucose for 72 h caused a 1.9-fold increase in total protein content (P < 0.05; Fig. 5A). Coincubation with MEK inhibitor 5 μM PD-98059 or p38 inhibitor 5 μM SB-203580 completely reversed the high-glucose-induced increase in protein content. Similarly, treatment with high glucose for 72 h caused a 1.4-fold increase in [3H]leucine incorporation (P < 0.05; Fig. 5B). In the presence of either PD-98059 or SB-203580, the high-glucose-induced increase in [3H]leucine incorporation was abolished. Although cell number tended to be lower in high-glucose-treated cells compared with control...
cells, there was no significant difference (Fig. 5C). PD-98059 or SB-203580 did not affect the cell number of high-glucose-treated cells. Cell hypertrophy was assessed more specifically by measuring the protein-to-DNA ratio (Fig. 5D). PD-98059, SB-203580, or both had no effect on the baseline protein-to-DNA ratio. The protein-to-DNA ratio increased under high-glucose conditions, which was partially inhibited by PD-98059 or SB-203580. Coincubation with both PD-98059 and SB-203580 completely abolished the high-glucose-induced increase in the protein-to-DNA ratio. Altogether, these results demonstrate that the high-glucose-induced cellular hypertrophy is independently mediated by ERK and p38.

**ERK and p38 mediate high-glucose-induced TGF-β protein expression.** Because high-glucose-induced hypertrophy in proximal tubular cells has been suggested to be mediated by TGF-β (4), we next examined the role of ERK and p38 in high-glucose-induced TGF-β expression. TGF-β protein was detected predominantly as a 25-kDa dimer. As shown in Fig. 6, treatment with high glucose for 72 h increased TGF-β expression. The addition of PD-98059 or SB-203580 abolished the high-glucose-induced increase in TGF-β protein, indicating that high-glucose-induced TGF-β expression is ERK and p38 dependent.

**DISCUSSION**

The present study demonstrates that ERK and p38 are activated in renal tubules of the diabetic kidney. Although activation of ERK and p38 has been reported in glomeruli and mesangial cells under the diabetic state (1, 2, 5), studies on MAPKs in renal tubular cells have been scarce. Because tubulointerstitial changes are thought to predict renal survival...
in various glomerulopathies including diabetic nephropathy, our findings may have important clinical relevance. Our observation that insulin treatment abrogated the change in ERK and p38 expression supports this possibility. The activation of ERK and p38 is reproduced in LLC-PK1 cells cultured under high-glucose conditions. Both in vivo and in vitro results suggest that ERK and p38 may be involved in high-glucose-induced cellular hypertrophy and TGF-β expression, critical events leading to tubular injury in diabetic nephropathy.

Tubules of streptozotocin-induced diabetic rats showed increased staining for p38, phosphorylated p38, and phosphorylated ERK. Most remarkably, p38 and phosphorylated p38 were detected only in the diabetic kidney. The absence of p38 expression in the control kidney is in accord with our previous study (14). Thus p38 was detected predominantly in the fetal kidney, suggesting its role in kidney development. In the diabetic kidney, p38 and phosphorylated p38 were found in all segments of the tubules. ERK, on the other hand, was expressed by distal tubules and collecting ducts of both control and diabetic kidneys. Phosphorylated ERK was detected in distal tubules and collecting ducts and stained more intensely in diabetic kidneys than controls. TGF-β protein was detected in distal tubules, collecting ducts, and to a lesser extent in proximal tubules of the diabetic kidney. Thus the localization of TGF-β coincides with that of p38, suggesting a role of p38 in the induction of TGF-β.

We further investigated the role of activated ERK and p38 using cultured cells. We studied a proximal tubular cell line (LLC-PK1) because proximal tubular cells contribute a large part of the kidney enlargement seen in diabetes. Also, the in vitro effect of high glucose has been well characterized in proximal tubular cells. Although the activation of MAPKs by high glucose has previously been demonstrated in various cell types, the functional role of activated MAPKs in the diabetic milieu is not completely understood. We demonstrated that both ERK and p38 are necessary for high-glucose-induced cellular hypertrophy and TGF-β expression in LLC-PK1 cells. Tubular cells cultured under high-glucose conditions have been shown to undergo hypertrophy and to increase collagen production (27). These changes are thought to be mediated by TGF-β. Thus expression of TGF-β mRNA and bioactivity were reported to be increased by high glucose, and neutralizing anti-TGF-β antibodies attenuated the effect of high glucose (17). Our data demonstrated that the inhibition of ERK or p38 abolished the high-glucose-induced hypertrophy, as defined by increased protein content, stimulation of protein synthesis, and the protein-to-DNA ratio. Notably, the effects of MEK inhibitor PD-98059 and p38 inhibitor SB-203580 on cellular hypertrophy were additive, indicating that ERK and p38 mediate the effect of high glucose through parallel pathways. We further demonstrated that PD-98059 or SB-203580 inhibited high-glucose-induced TGF-β expression. Thus ERK or p38 may mediate cellular hypertrophy by inducing TGF-β expression. In agreement with our results, Isono et al. (9) demonstrated that ERK mediated TGF-β expression in mesangial cells. Also, Weigert et al. (22) recently showed that high-glucose-induced activation of TGF-β promoter was mediated by activating protein-1 through p38 in mesangial cells. An alternative, not mutually exclusive, explanation points to the possibility that PD-98059 or SB-203580 may inhibit TGF-β signaling. TGF-β has been suggested to activate both ERK and p38. Thus Hayashida et al. (6) demonstrated that TGF-β activated ERK and that ERK mediated collagen expression in mesangial cells. In LLC-PK1 cells, TGF-β-activating kinase-1 (TAK1), a downstream kinase of TGF-β, has been shown to activate p38, and TAK1/p38 pathway mediated the growth-inhibitory effects of TGF-β (19). Of note, Zhang et al. (26) recently reported that high-glucose-stimulated angiotensinogen gene expression was mediated by p38. Because high-glucose-induced hypertrophy in renal tubule cells was blocked by angiotensin-converting enzyme inhibitors (4), p38 might mediate hypertrophy, in part, by inducing angiotensinogen.

The upregulation of p38, phosphorylated p38, phosphorylated ERK, and TGF-β was most intense in distal tubules and collecting ducts. Previous studies demonstrated that high glucose along with TGF-β caused hypertrophy in a distal tubular cell line, Mardin-Darby canine kidney cells (25). Furthermore, a study by Rasch et al. (13, 16) demonstrated that distal tubules also resulted in cellular hypertrophy and hyperplasia in streptozotocin-induced diabetes. Thus activated ERK or p38 may also play a role in diabetic alteration of distal tubules.

The mechanism of cellular hypertrophy has recently been elucidated at the level of the cell cycle. Cells undergo hypertrophy when they arrest at the late G1 phase (15, 23). High-glucose-induced hypertrophy is considered to be caused by mitogen-induced movement of cells into the G1 phase followed by arrest at the G1/S (7). Recent studies demonstrated functional interactions between Ras/Raf/ERK cascade and G1 phase cell-cycle progression and suggested that ERK is essential for the stimulation of cyclin D1 (11). Activated ERK in the diabetic state, therefore, may stimulate cells to enter the G1 phase. Failure of cells to progress into S phase occurs when cyclin E kinase is not fully activated. Huang and Preisig (7) suggested that the inhibition of cell cycle progression in proximal tubular cells harvested from diabetic rats might be mediated by TGF-β. Previous studies showed that anti-TGF-β-neutralizing antibody attenuated the glucose-induced inhibition of cell proliferation (17). Collectively, ERK and p38 may be involved in high-glucose-induced arrest at G1/S boundary by inducing TGF-β and/or mediating TGF-β signaling.

Previous studies by Ishida et al. (8) and Gruden et al. (3) demonstrated that stretch-induced fibronectin and TGF-β production in mesangial cells were mediated by ERK and p38, respectively. Mechanical stretch is an in vitro model for glomerular hypertension seen in diabetes. Their results underscore the importance of ERK and p38 in diabetic kidney disease. Of note, the present study demonstrated that p38 and ERK were immunohistochemically detected in podocytes. Although currently no in vitro data are available, our observation may suggest a possible role of podocytes in the pathogenesis of diabetic nephropathy.

In conclusion, the present study in renal tubular cells, together with previous results in glomeruli and mesangial cells, suggests that ERK and p38 may play pivotal roles in the development and progression of diabetic nephropathy. These molecules may be a potential target for the treatment of the disease.

GRANTS

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