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BMK1 is activated in glomeruli of diabetic rats and in mesangial cells by high glucose conditions

YUKI SUZAKI, MASANORI YOSHIZUMI, SHOJI KAGAMI, AKIRA NISHIYAMA, YUICHI OZAWA, MOE KYAW, YUKI IZAWA, YASUHISA KANEMATSU, KOICHIRO TSUCHIYA, and TOSHIAKI TAMAKI

Department of Pharmacology and Pediatrics, The University of Tokushima School of Medicine, Tokushima; and Department of Pharmacology, Faculty of Medicine, Kagawa University, Kagawa, Japan

BMK1 is activated in glomeruli of diabetic rats and in mesangial cells by high glucose conditions.

Background. High glucose causes renal cell injury through various signal transduction pathways, including mitogen-activated protein (MAP) kinases cascades. Big MAP kinase 1 (BMK1), also known as extracellular signal-regulated kinase 5 (ERK5), is a recently identified MAP kinase family member and was reported to be sensitive to osmotic and oxidative stress. However, the role of BMK1 in diabetic nephropathy has not been elucidated yet.

Methods. We investigated whether BMK1 is activated in the glomeruli of Otsuka Long Evans Tokushima Fatty (OLETF) rats, a model of type 2 diabetes mellitus in comparison with the control Long Evans Tokushima Otsuka (LETO) rats. We also examined the effect of high glucose on BMK1 activity in cultured rat mesangial cells.

Results. BMK1 and ERK1/2 but not p38 were activated in the glomeruli of OLETF rats, which showed diabetic nephropathy at 52 weeks of age. High glucose, in addition to a high concentration of raffinose, caused rapid and significant activation of BMK1 in rat mesangial cells. MAP kinase/ERK kinase (MEK) inhibitors, U0126 and PD98059, both inhibited BMK1 activation by high glucose in a concentration-dependent manner. Protein kinase C (PKC) inhibition by GF109203X and PKC down-regulation with long-time phorbol myristate acetate (PMA) treatment both inhibited BMK1 and Src kinase activation. Src kinase inhibitors, herbimycin A and PP2, also inhibited high glucose-induced BMK1 activation. PKC inhibitors, Src inhibitors and MEK inhibitors, all inhibited cell proliferation by high glucose. Finally, transfection of dominant-negative MEK5, which is an upstream regulator of BMK1, abolished the BMK1-mediated rat mesangial cell proliferation stimulated by high glucose.

Conclusion. In the present study, we demonstrated that high glucose activates BMK1 both in vivo and in vitro. It was suggested that high glucose induces PKC- and c-Src-dependent BMK1 activation. It could not be denied that BMK1 activation is induced through an osmotic stress-sensitive mechanism. BMK1-mediated mesangial cell growth may be involved in the pathogenesis of diabetic nephropathy.

Key words: c-Src, protein kinase C, proliferation, diabetic nephropathy.

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Diabetic nephropathy is the most common cause of end-stage renal failure in patients starting dialysis in developed countries [1]. Clinical trials have demonstrated that high glucose is the principal cause of renal damage in both type 1 [2] and type 2 diabetes [3]. Although the underlying genetic predisposition to this microvascular complication remains elusive, an investigation of the cellular and molecular mechanisms was performed. Many studies reported that various renal cell types cultured in high glucose medium or stimulated by high glucose exhibit the typical features of cellular hypertrophy, cell proliferation, and excessive production of extracellular matrix (ECM) that are characteristic of diabetic nephropathy. Altered mesangial cell function in high glucose exposure plays a central role in the pathogenesis of progressive diabetic glomerulopathy. Activation of protein kinase C (PKC) is one of the major mechanisms involved in high glucose-induced glomerular injury [4] and produces reactive oxygen species (ROS) and subsequent lipid peroxidation [5–8]. High glucose generates ROS as a result of glucose auto-oxidation, metabolism, and formation of advanced glycosylation end products [8]. All these signaling molecules involved into mitogen-activated protein (MAP) kinase signaling pathways in glomerular cells [9]. High glucose-induced diabetic complications have been implicated, in part, to the activation of MAP kinases [9].

Four subfamilies of MAP kinases that are activated by high glucose have been identified as follows: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase (JNK), p38 kinase, and big MAP kinase 1 (BMK1 or ERK5) [10, 11]. Each subfamily may be regulated via different signal transduction pathways and modulate specific cell functions [12]. BMK1 was recently identified as a MAP kinase family member with a large COOH terminal and a unique loop-12 sequence that

shares the TEY activation motif with ERK1/2 but is activated by MAP kinase/ERK kinase 5 (MEK5) [13, 14]. BMK1 was reported to be activated by serum, epidermal growth factor (EGF), and nerve growth factor, oxidative stress, and osmotic stress in various cells. However, the role of BMK1 in diabetic nephropathy and high glucose-induced BMK1 activation in mesangial cells have not been reported yet. Since high glucose causes osmotic stress and produces ROS in mesangial cells, BMK1 may be involved in diabetic nephropathy. Therefore, we hypothesized that BMK1 may be activated by high glucose in mesangial cells which may play a role in diabetic nephropathy.

In the present study, we first investigated whether BMK1 is activated in the glomeruli of Otsuka Long Evans Tokushima Fatty (OLETF) rats, a model of type 2 diabetes mellitus, compared to its age-matched control Long Evans Tokushima Otsuka (LETO) rats. Thereafter, we examined the effect of high glucose on the change in BMK1 activity in cultured rat mesangial cells. Furthermore, we investigated the signaling pathways that are involved in BMK1 activation and their pathophysiologic implications in high glucose-induced cellular injury.

METHODS

Animals and reagents

All procedures were in accordance with institutional guidelines for animal research. Age-matched male OLETF rats and control male LETO rats were provided by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). They were fed standard laboratory chow and given tap water ad libitum. Kidney samples were harvested at the end of 52 weeks of age in both OLETF and LETO rats. The left kidney was perfused with chilled saline solution and snap-frozen in liquid nitrogen and stored at −80°C until processed for cortex protein extraction and analysis for immunofluorescence staining with phospho-BMK1/ERK5 antibody. The right kidney was perfused with chilled saline solution and fixed in 10% buffered paraformaldehyde for histologic examination. All materials were purchased from Sigma except where indicated. Herbimicyn A and PP2 were purchased from Calbiochem (San Diego, CA, USA). U0126 was from Promega (Madison, WI, USA). PD98059, GF109203X, and phorbol myristate acetate (PMA) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-ERK5 and anti-ERK1/2 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-BMK1/ERK5 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Anti-p38 antibody, anti-phospho-p38 antibody and anti-phospho-ERK1/2 antibody were from Cell Signaling Technology (Beverly, MA, USA). Anti-Src antibody was from Upstate Biotechnology. Anti-Src phospho-specific antibody (Tyr418) was from Biosource (Camarillo, CA, USA). All other materials were commercial products of reagent grade.

Histology and immunohistochemistry

Kidney tissues from both OLETF and LETO rats were processed and examined by light microscopy and immunofluorescence staining as previously described [15]. For evaluation of mesangial matrix accumulation and glomerular hypercellularity, the kidneys were fixed with 10% buffered paraformaldehyde (pH 7.4), embedded in paraffin, sectioned into 3 μm slices, and stained with periodic acid-Schiff (PAS) reagent. At least 30 glomeruli were observed to evaluate the severity of glomerular injury. For immunofluorescence staining with phospho-BMK1/ERK5 antibody, 3 μm slices of frozen sections were fixed in acetone. They were then incubated with the sheep anti-BMK1/EKR5 antibody for 1 hour and subsequently with fluorescein isothiocyanate (FITC)-coupled donkey antisheep IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). As a control, kidney sections were incubated with nonimmune sheep serum or unrelated goat IgG antibody, followed by FITC-coupled secondary antibody. These controls gave entirely negative results. Immunofluorescence was visualized using a fluorescent microscope (Olympus, Tokyo, Japan). For semiquantitative evaluation of glomerular hypercellularity and mesangial matrix accumulation, all 3 μm PAS-stained sections were coded and read by a blinded observer. At least 30 glomeruli were selected at random, cell nuclei were counted, and the degree of glomerular matrix expansion was determined using a published method [15]. The percentage of each glomerulus occupied by mesangial matrix was estimated and assigned a code as follows: 0, no localized increase of glomerular mesangial matrix; 1, segmental increase of mesangial matrix with less than 25% of glomerular tuft; 2, segmental increase of mesangial matrix with 25% to 50% of glomerular tuft; 3, increase of mesangial matrix with 50% to 75% of glomerular tuft; and 4, increase of mesangial matrix with more than 75% of glomerular tuft.

Cell culture and transfection

Rat mesangial cells were obtained from intact glomeruli of 4- to 6-week-old Sprague-Dawley rats and characterized according to published methods [16]. Rat mesangial cells were used between passages 5 and 15 and were maintained in 199 medium supplemented with 18% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in tissue culture flasks. In this medium, the glucose concentration was 5.5 mmol/L. The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The cells were then removed from the flasks with 0.05% trypsin plus 0.01% ethylenediaminetetraacetic acid (EDTA) and

seeded onto 35 mm or 100 mm dishes. Cells at 70% to 80% confluence were growth arrested by incubation in serum-free 199 medium for 24 to 48 hours prior to use. All experiments except methyl-thiazolyl tertrazolium (MTT) assay were performed with growth-arrested cells to minimize basal BMK1 activity. For transfection, the constitutive-active MEK5 (CA-MEK5) or the dominant-negative MEK5 (DN-MEK5) forms of MEK5 was used in 30% to 50% confluent rat mesangial cells. Each plasmid DNA was provided by Dr. Eisuke Nishida of Kyoto University [17]. CA-MEK5 and DN-MEK5 were subcloned into a plasmid pcDL-SRα. For transient expression experiments, cells were transfected with SuperFector (B-Bridge International, Inc., San Jose, CA, USA) in reduced serum OPTI-minimal essential medium I (OPTI-MEM I) (Invitrogen Corporation, Carlsbad, CA, USA) for 5 hours. After transfection, cells were replaced with 199 medium supplemented with 18% FBS and antibiotics. After 24 hours of incubation with or without the addition of 10 mmol/L glucose (final concentration was 15.5 mmol/L), cells were used for experiments of MTT assay.

Protein extraction

For in vivo experiments, renal cortex of both OLETF and LETO rats were excised and homogenized in lysis buffer [20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L ethyleneglycol tetraacetate (EGTA), 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. For in vitro experiments, subconfluent rat mesangial cells in 100 mm culture dishes were made quiescent by placing them in serum-free medium. Rat mesangial cells in the serum-free medium were treated with or without stimulation: each indicated time or dose-point. After stimulation, the cells were treated with 0.5 mL of lysis buffer and flash frozen on liquid nitrogen. After allowing the cells to thaw, cells were scraped off the dish and both the samples of rat mesangial cells protein extract and renal cortex were sonicated (Sonifier 250; Branson Ultra-Sonics Co., Danbury, UK) on ice for 1 minute. Then, the samples were centrifuged at 14,000*g* (4°C for 30 minutes) and the protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) and stored at −80°C until the protein kinase assay.

Immunoprecipitation kinase assay of BMK1

For immunoprecipitation, protein extraction samples were incubated with 10 μg of anti-ERK5 antibody (Santa Cruz Biotechnology) overnight and then incubated with protein A agarose beads for 3 hours on a roller system at 4°C. After immunoprecipitation, samples were evaluated with Western blot analysis using phospho-specific antibody for BMK1, ERK5 as described previously [18]. For Western blot analysis, samples were boiled for 5 minutes in Laemmli sample buffer and were subjected to 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were then transferred to a nitrocellulose membrane (Hybond™-ECL; Amersham Pharmacia Biotech, Buckinghamshire, England). Complete protein transfer to the membrane was verified by staining the gel with Coommasie blue. The membrane was blocked for 1 hour at room temperature with 5% bovine serum albumin (BSA) in phosphate-buffered saline-Tween 20 (PBS-T). The blots were incubated overnight at 4°C with anti-BMK1/ERK5 phospho-specific antibody (Upstate Biotechnology), followed by incubation for 1 hour with antisheep secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) with ECL reagent (Amersham Pharmacia Biotech) treatment and exposure to Hyperfilm-ECL. The intensity of the bands was measured using a Macintosh computer (Apple Computer, Inc.) with an optical scanner (Epson GT-8000; Seiko, Tokyo, Japan), using the public domain NIH Image program.

Western blot analysis for ERK1/2, p38, and Src kinase activations

ERK1/2, p38, and Src kinase activities were measured with Western blot analysis as described previously [18]. ERK1/2 and p38 kinase activities were measured in the protein of the renal cortex of both OLETF and LETO rats. Src kinase was measured in the protein of rat mesangial cells. For evaluation of activated ERK1/2, p38, and c-Src, phospho-specific ERK1/2 antibody, phospho-specific p38 antibody (Cell Signaling Technology) and phospho-specific Src antibody at Tyr418 (Biosource), were used, respectively. For Western blot analysis, the renal cortex or protein extract (30 μg) of rat mesangial cells was boiled for 5 minutes in Laemmli sample buffer and then subjected to SDS-PAGE, and the protein extracts were transferred to a nitrocellulose membrane (Hybond™-ECL; Amersham Pharmacia Biotech). The membrane was blocked for 1 hour at room temperature with 5% BSA in PBS-T. The blots were incubated overnight at 4°C with phospho-ERK1/2, phospho-p38 or phospho-Src antibody, followed by incubation for 1 hour with secondary antibody (horseradish peroxidase-conjugated). Visualization of immunoreactive bands and measurement of band intensity were performed by the methods for immunoprecipitation kinase assay of BMK1.

PKC activity assay

PKC activity was measured with the Pep Tag assay system (Promega), in which the change in

charge of a fluorescent-tagged PKC-specific substrate (PLSRTLSVAAK), which occurs with phosphorylation, is detected by separation on agarose gel electrophoresis at a natural pH [19]. At the end of the incubation period, the rat mesangial cells were washed with Hanks' balanced salt solution containing 1 mmol/L PMSF and 1 μmol/L leupeptin, placed in the same buffer, and then frozen-thawed for lysis. Aliquots were taken for the assay, which was performed according to the manufacturer's instructions.

Cell proliferation determination with MTT assay

The MTT assay measures the functional activity of mitochondrial dehydrogenase [20]. MTT is a yellow-colored tetrazolium salt which is reduced to purple formazan corresponding to the reduction reaction products with concomitant oxidation of nicotinamide adenine dinucleotide (NADH) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Measurements of cellular MTT reduction were carried out as described previously [21]. Rat mesangial cells were cultured at 30% to 50% confluency. After serum starvation with serum-free 199 medium, cells were incubated in a serum- and antibiotic-free OPTI-MEM I (Invitrogen Corporation) for transfection experiments. Following the indicated incubation time with glucose, MTT was added to a final concentration of 0.5 mg/mL, and after a further 1 hour incubation rat mesangial cells were lysed with isopropanol that contained 0.04 N HCl. MTT reduction was read at 550 nm using a spectrophotometer.

Statistics

Values are presented as mean ± SD for five separate experiments. One-way analysis of variance (ANOVA) was used to determine significance among groups, after which the modified *t* test with the Bonferroni correction was used for comparison between individual groups. A value at $P < 0.05$ was considered to be significant.

RESULTS

Body weight, plasma glucose, and urinary protein excretion of OLETF and LETO rats

Functional findings of body weight, plasma glucose, and urinary protein excretion of OLETF and LETO rats at 52 weeks of age are shown in Table 1. The body weights of OLETF rats were significantly higher than those of LETO rats. In addition, plasma levels of glucose in OLETF rats were 2.3-fold higher than those of LETO rats. Urinary protein excretion was significantly increased in OLETF rats compared to LETO rats. These results are consistent with previous findings [22, 23] and OLETF rats at 52 weeks of age were observed to be characteristic of diabetic nephropathy.

Table 1. Characteristics of Long Evans Tokushima Otsuka (LETO) and Otsuka Long Evans Tokushima Fatty (OLETF) rats at 52 weeks of age

	LETO ($N = 12$)	OLETF ($N = 11$)
Body weight *g*	534 ± 9	614 ± 18[a]
Blood glucose *mg/dL*	66 ± 9	152 ± 26[a]
Urinary protein excretion μg*/day*	7.0 ± 1.4	67.5 ± 15.2[a]

Data are mean ± SD. [a] $P < 0.01$ vs. LETO rats.

Activation of BMK1 in the glomeruli of OLETF rats

As shown in Figure 1A (a and b), immunofluorescence staining of the glomeruli revealed that BMK1 was activated in OLETF rats compared to the control LETO rats. Histologic examination with PAS staining also showed a marked mesangial cell proliferation and ECM accumulation in the glomeruli of OLETF rats [Fig. 1A (c and d)]. There was a significant increase of glomerular hypercellularity and matrix score in the glomeruli of OLETF rats compared with those of LETO rats (68 ± 2.2 cells/glomerulus vs. 47 ± 0.4 cells/glomerulus; 1.8 ± 0.07 cells/glomerulus vs. 0.32 ± 0.07 cells/glomerulus, $P < 0.05$, respectively) (Fig. 1B and C). BMK1 activity of the renal cortex was measured by immunoprecipitation kinase assay and ERK1/2 and p38 activities of the renal cortex were measured by Western blot analysis as described in the **Methods** section (Fig. 2A). BMK1 and ERK1/2 activity of the renal cortex was higher in OLETF rats than LETO rats. However, no activation of p38 was observed in the renal cortex of OLETF rats, in contrast to LETO rats. Consistent with the results of immunofluorescence staining, BMK1 activity was increased in the renal cortex of OLETF rats (Fig. 2).

High glucose-induced BMK1 activation in rat mesangial cells

It was reported that BMK1 activation was induced by high concentrations of sorbitol in rat vascular smooth muscle cells and bovine aortic endothelial cells [24, 25]. No studies have been published regarding the changes in BMK1 activity in response to high glucose stimulation in rat mesangial cells. To confirm the above in vivo findings, we next examined whether BMK1 is activated by high glucose in rat mesangial cells. Growth-arrested rat mesangial cells were treated for various times and with different concentrations of glucose. For comparison, the effects of various concentrations of raffinose were also examined. Raffinose is a trisaccharide that, unlike glucose, is unable to enter the cell and thus exerts an osmotic effect [26]. In the examined concentration range (5.5 to 35.5 mmol/L glucose), the BMK1 activity increased with a bell-shaped response curve, and maximal activation occurred at a glucose concentration of 15.5 mmol/L in the medium (Fig. 3).

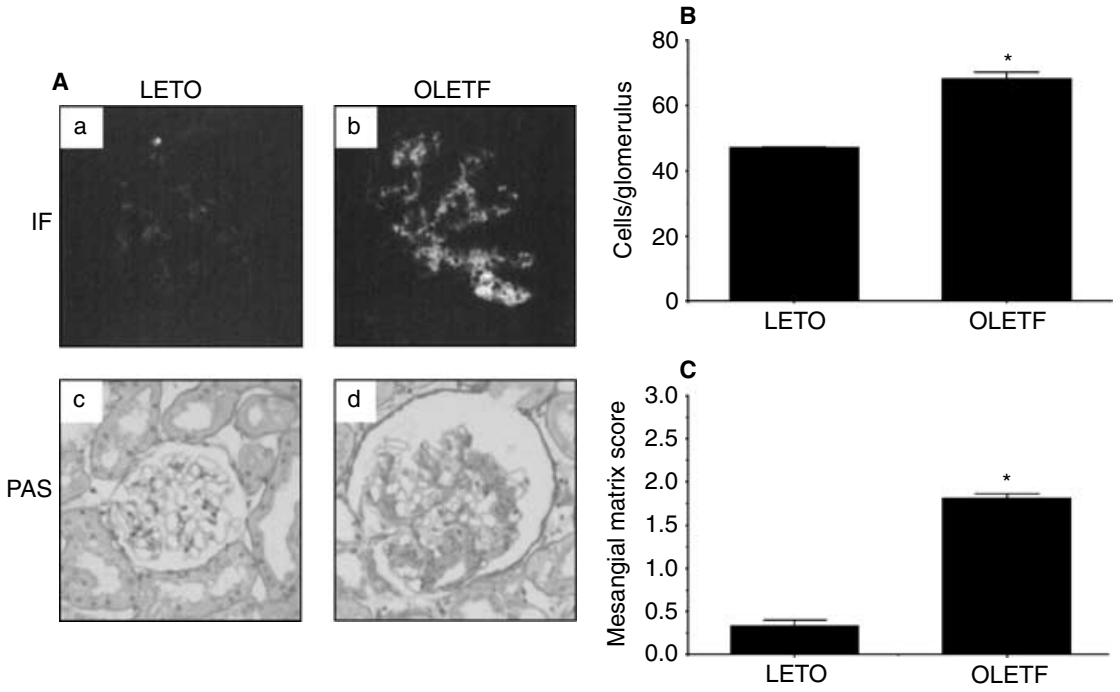

Fig. 1. Big mitogen-activated protein (MAP) kinase 1 (BMK1) is activated in the glomeruli of Otsuka Long Evans Tokushimas Fatty (OLETF) rats. Immunofluorescence staining (IF) for phospho-BMK1/extracellular signal-regulated kinase (ERK) 5 in kidney section from Long Evans Tokushima Otsuka (LETO) rats (*A*, a) and OLETF rats (A, b) (original magnification, ×200). Periodic acid-Schiff (PAS) staining of glomeruli from LETO rats (A, c) and OLETF rats (A, d) (original magnification, ×200). Quantitation of glomerular hypercellularity (*B*) and mesangial matrix accumulation (*C*). Data are shown as means ± SD The asterisks indicate significant differences compared to the value of LETO rats. $^{*}P < 0.05$.

The 20 mmol/L raffinose incubation with 5.5 mmol/L glucose caused maximal activation of BMK1 (Fig. 3). The 15.5 mmol/L glucose treatment of cells resulted in a maximal activation of BMK1 at 30 minutes (Fig. 4). The effect of 20 mmol/L raffinose on BMK1 activity peaked at 20 minutes and this activation was transient (Fig. 4).

Involvement of MEK5 in BMK1 activation by high glucose exposure

Recently, MEK1/2 inhibitors, U0126 and PD98059, were reported to inhibit MEK5, which is known as an upstream regulator of BMK1 in various cells [17, 27]. We also reported that H_2O_2-induced BMK1 activation was inhibited by preincubation with U0126 and PD98059 in PC 12 cells [21]. Thus, we examined whether U0126 and PD98059 are able to inhibit BMK1 activation induced by high glucose in rat mesangial cells. Pretreatment of rat mesangial cells with U0126 inhibited BMK1 activation in a concentration-dependent manner (Fig. 5A). Similar results were obtained in cells treated with another MEK1/2 inhibitor PD98059, which also inhibited BMK1 activation by high glucose to a similar extent as U0126 (Fig. 5B).

Involvement of PKC in BMK1 activation by high glucose

Numerous studies have suggested that high glucose induces activation of PKC and MAP kinase in mesangial cells [28]. However, involvement of PKC in BMK1 activation in rat mesangial cells has not been elucidated. Therefore, we investigated the role of PKC in high glucose-mediated BMK1 activation. To determine the participation of PKC in BMK1 activation by high glucose, cells were pretreated for 60 minutes with the specific PKC inhibitor, GF109203X (Fig. 6A). For the down-regulation of PKC, rat mesangial cells were treated with the phorbol ester PMA for 24 hours. GF109203X showed significant inhibition of BMK1 activation in a concentration-dependent manner (Fig. 6A). The down-regulation of PKC by PMA treatment for 24 hours also effectively abolished the BMK1 activation by high glucose in rat mesangial cells (Fig. 6B).

Involvement of c-Src tyrosine kinase in BMK1 activation by high glucose exposure

Previous studies reported that c-Src is involved in BMK1 activation by oxidative stress [21, 29]. Thus, we examined whether c-Src mediates high glucose-induced BMK1 activation in rat mesangial cells. As shown in Figure 7, pretreatment with a tyrosine kinase inhibitor, herbimycin A (Fig. 7A) or Src kinase inhibitor, PP2 (Fig. 7B), both inhibited high glucose-induced BMK1 activation in a concentration-dependent manner.

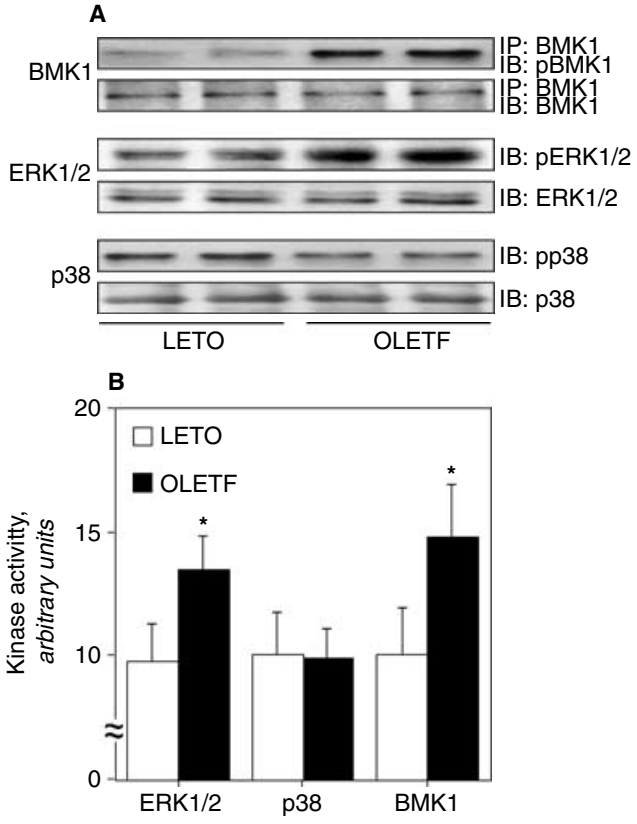

Fig. 2. Big mitogen-activated protein (MAP) kinase 1 (BMK1) is activated in the renal cortex of Otsuka Long Evans Tokushima Fatty (OLETF) rats. (*A*) The activities of BMK1, extracellular signal-regulated kinase (ERK) 1/2 and p38 in the renal cortex of Long Evans Tokushima Otsuka (LETO) rats and OLETF rats. BMK1 activity was measured by immunoprecipitation (IP) kinase assay as described in the **Methods** section. ERK1/2 and p38 activities were measured by Western blot [immunoblot (IB)] analysis in the **Methods** section. pBMK1, pERK1/2, and pp38 represent phosphorylated form of BMK1, ERK1/2, and p38, respectively. BMK1, ERK1/2 and p38 represent the total (phosphorylated and unphosphorylated) BMK1, ERK1/2, and p38, respectively. (*B*) Densitometric analysis of phosphorylated BMK1, ERK1/2, and p38. Bars are the means ± SD from five different specimen of renal cortex. The asterisks indicate significant differences compared to the value of LETO rats. $^{*}P < 0.05$.

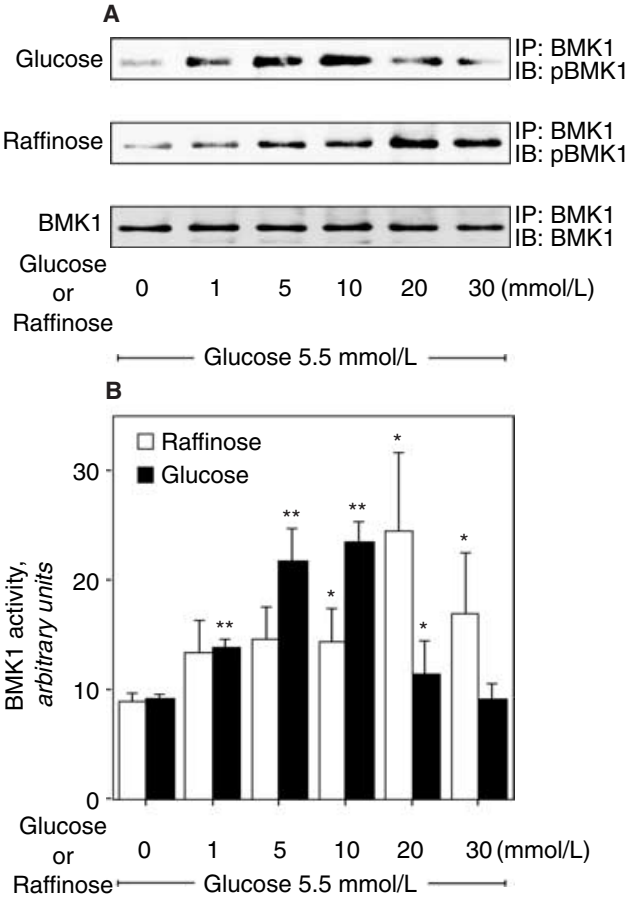

Fig. 3. Concentration-response curves of glucose- or raffinose-induced big mitogen-activated protein (MAP) kinase 1 (BMK1) activation in rat mesangial cells. Cells were stimulated with the indicated concentrations of glucose for 30 minutes or raffinose for 20 minutes in addition to the normal glucose (5.5 mmol/L) media for BMK1 activation. The activity of BMK1 was measured by immunoprecipitation (IP) kinase assay or immunoblot (IB) as described in the **Methods** section. BMK1 is total BMK1 that contains phosphorylated BMK1 (pBMKI), and unphosphorylated BMK1. No significant differences in the amount of BMK1 were observed in samples by Western blot analysis with anti-extracellular signal-regulated kinase (ERK) 5 antibody. (*A*) Representative blots are shown. (*B*) Densitometric analysis of BMK1. Bars are the means ± SD from five independent experiments. The asterisks indicate significant differences compared to the value of glucose stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

PKC exists upstream of c-Src tyrosine kinase for BMK1 activation

To investigate whether PKC or c-Src exists upstream of the other, we examined the effects of PKC inhibitors on Src activation and the effects of Src inhibitors on PKC activation. High glucose treatment of cells resulted in a maximal activation of PKC at 1 minute (2.97- ± 0.22-fold from basal activity) and Src kinase at 2 minutes (3.81- ± 0.45-fold from basal activity). As shown in Figure 8, both GF109203X and 24 hours PMA treatment inhibited high glucose-induced Src activation in a concentration-dependent manner. However, PKC activation by high glucose was not affected by pretreatment with Src inhibitors, herbimycin A and PP2 (data not shown). These results suggest that PKC is upstream of c-Src for BMK1 activation in rat mesangial cells.

Role of BMK1 activation in rat mesangial cell proliferation induced by high glucose exposure

It was reported that mesangial cell proliferation was augmented by high glucose exposure [30]. Since BMK1 was activated by high glucose exposure, we investigated the role of BMK1 activation in high glucose-induced rat mesangial cell proliferation. We examined the effects of GF109203X, PMA, herbimycin A, PP2, U0126, and PD98059 on rat mesangial cell proliferation induced

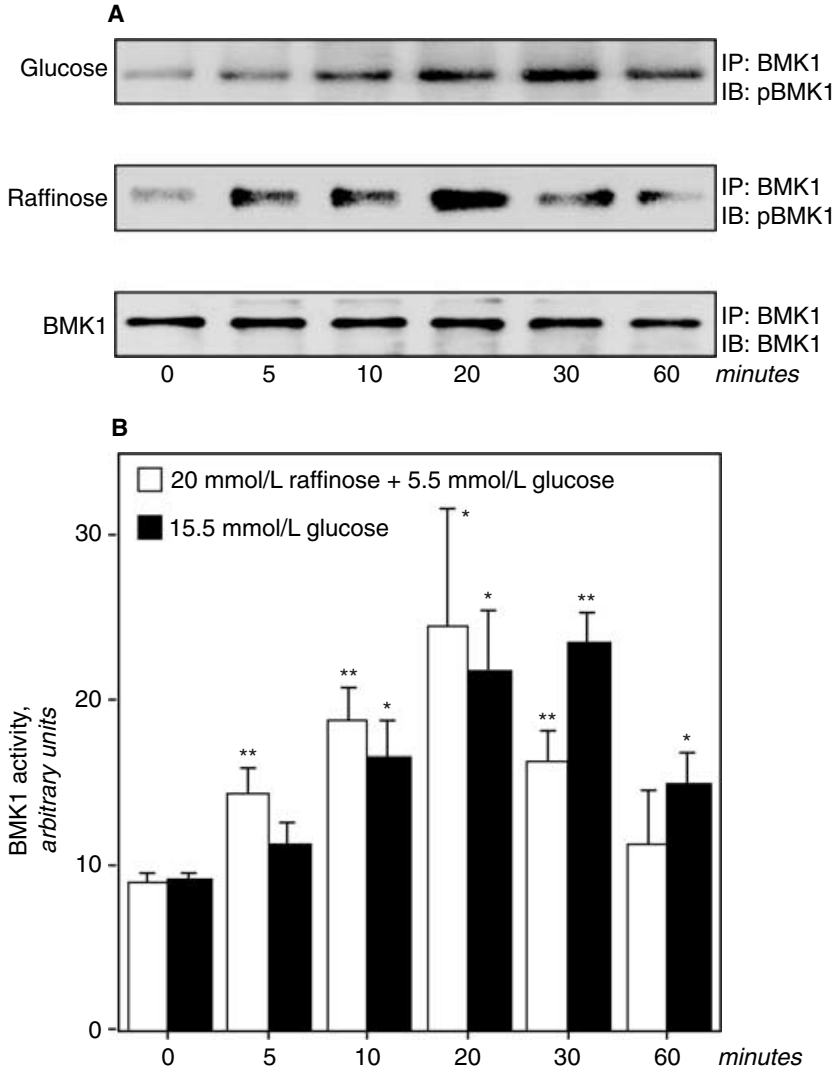

Fig. 4. Time course of high concentrations of glucose- and raffinose-induced big mitogen-activated protein (MAP) kinase 1 (BMK1) activation in rat mesangial cells. Cells were stimulated with 10 mmol/L glucose or 20 mmol/L raffinose in addition to the normal glucose (5.5 mmol/L) media for the indicated periods. The activity of BMK1 was measured by immunoprecipitation (IP) kinase assay or immunoblotting (IB) as described in the **Methods** section. BMK1 is total BMK1 that contains phosphorylated (pBMK1) and unphosphorylated BMK1. No significant differences in the amount of BMK1 were observed in samples by Western blot analysis with anti-extracellular signal-regulated kinase (ERK) 5 antibody. (*A*) Representative blots are shown. (*B*) Densitometric analysis of BMK1. Bars are the means ± SD from five independent experiments. The asterisks indicate significant differences compared with the value of glucose stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

by high glucose. Using MTT assay, cell proliferation was evaluated as described in 'METHODS'. As shown in Figure 9, all these inhibitors showed concentration-dependent inhibition of rat mesangial cell proliferation induced by high glucose exposure.

Although we demonstrated that U0126 and PD98059 inhibited the MEK5-BMK1 pathway in rat mesangial cells as shown in Figure 5, these drugs also inhibit MEK1/2 in various cells [31–33]. Since mesangial cell proliferation is also facilitated by the activation of ERK1/2 [28], which is a downstream MAP kinase of MEK1/2, involvement of ERK1/2 in high glucose-induced rat mesangial cell proliferation cannot be excluded. Thus, we utilized transfected rat mesangial cells with CA-MEK5 and DN-MEK5 constructs in this set of experiments. As shown in Figure 10, transfection with DN-MEK5 inhibited BMK1 activation similar to rat mesangial cell proliferation caused by high glucose. In addition, transfection with CA-MEK5 increased both BMK1 activity and cell proliferation even though cells are in the normal glucose condition.

DISCUSSION

Previous studies showed that ERK1/2 and p38 were activated in mesangial cells exposed to high glucose and in rat glomeruli of type 1 diabetes [34, 35]. In a type 2 diabetic model, it was reported that ERK activity was activated in the renal cortex of *db/db* mice as compared to nondiabetic mice [36]. However, it is not yet documented whether BMK1 is activated in diabetic nephropathy. BMK1 was originally shown to be activated by serum or growth factors in CHO-K1 cells, NIH-3T3 cells, and Hela cells [37, 38]. Recent studies have reported that other stimuli, such as oxidative or osmotic stress, can also activate BMK1 in various cell types. It was reported that high osmotic stress with sorbitol activated BMK1 in

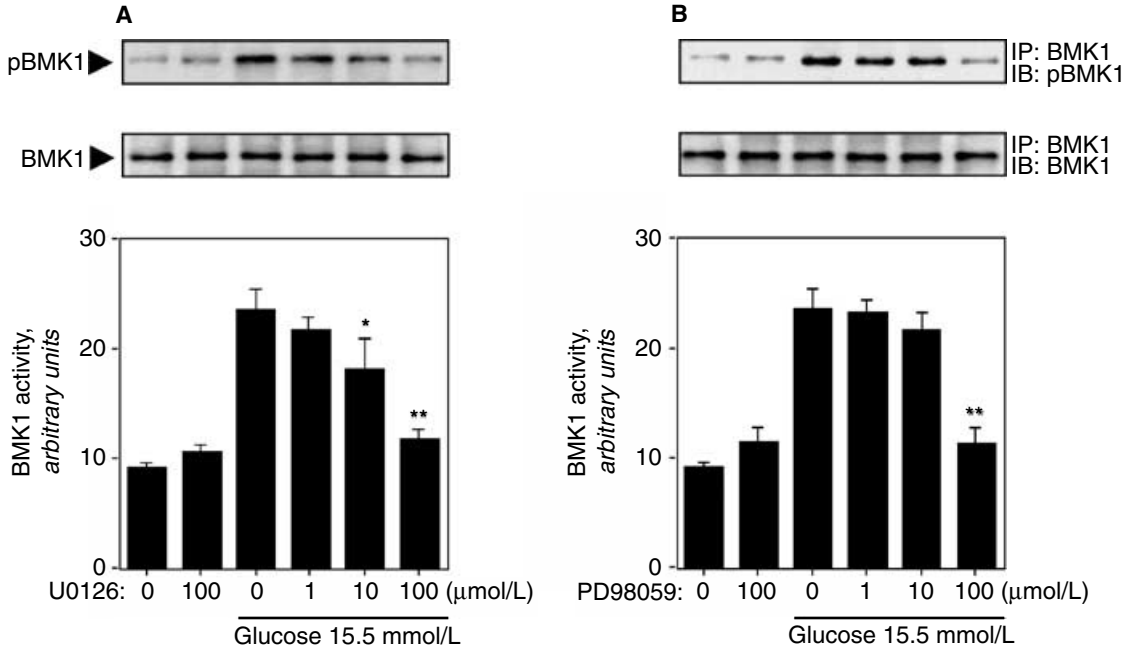

Fig. 5. Effects of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitors, U0126 and PD98059, on big MAP kinase 1 (BMK1) activation by high glucose in rat mesangial cells. Cells were pretreated with U0126 (*A*) and PD98059 (*B*) at the indicated concentrations for 30 minutes. Then, the cells were stimulated with 15.5 mmol/L glucose at the final concentration for 30 minutes. The activity of BMK1 was measured using immunoprecipitation (IP) kinase or immunoblot (IB) assay as described in the **Methods** section. BMK1 is total BMK1 that contains phosphorylated (pBMK1) and unphosphorylated BMK1. No significant differences in the amount of BMK1 were observed in samples by Western blot analysis with anti-extracellar signal-regulated kinase (ERK) 5 antibody (A and B, middle). Bars are the means ± SD from five independent experiments. The asterisks indicate significant differences compared with the value of glucose stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

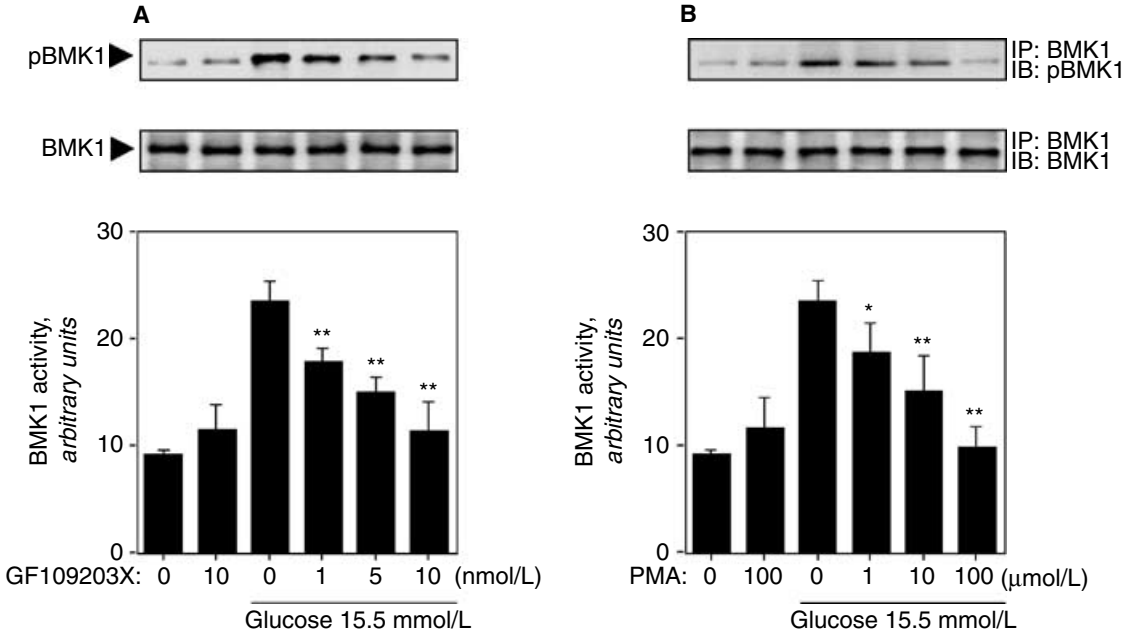

Fig. 6. Effects of protein kinase C (PKC) inhibitor, GF109203X, and down-regulation of PKC with phorbol myristate acetate (PMA) on big mitogen-activated protein (MAP) kinase 1 (BMK1) activation by high glucose in rat mesangial cells. (*A*) Cells were pretreated with GF109203X at the indicated concentrations for 60 minutes. (*B*) Cells were pretreated with PMA at the indicated concentrations for 24 hours. Then the cells were stimulated with 15.5 mmol/L glucose at the final concentration for 30 minutes. The activity of BMK1 was measured by immunoprecipitation (IP) kinase or immunblot (IB) assay as described in the **Methods** section. BMK1 is total BMK1 that contains phosphorylated (pBMK1) and unphosphorylated BMK1. No significant differences in the amount of BMK1 were observed in samples by Western blot analysis with anti-extracellular signal-regulated kinase (ERK) 5 antibody (A and B, middle). Bars are the means ± SD from five independent experiments. The asterisks indicate significant differences compared with the value of 15.5 mmol/L glucose stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

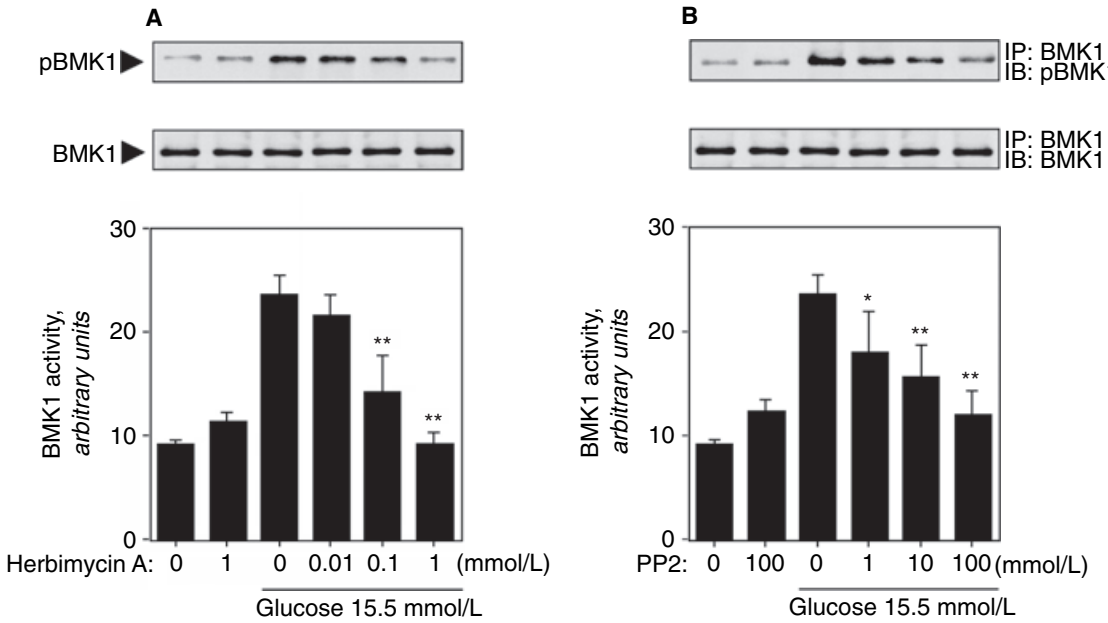

Fig. 7. Effects of Src inhibitors, herbimycin A and PP2, on big mitogen-activated protein (MAP) kinase 1 (BMK1) activation by high glucose in rat mesangial cells. Cells were pretreated with herbimycin A for 16 hours (*A*) and PP2 for 15 minutes (*B*) at the indicated concentrations. Then, the cells were stimulated with 15.5 mmol/L glucose at the final concentration for 30 minutes. The activity of BMK1 was measured by immunoprecipitation (IP) kinase or immunoblot (IB) assay as described in the **Methods** section. BMK1 is total BMK1 that contains phosphorylated (pBMK1) and unphosphorylated BMK1. No significant differences in the amount of BMK1 were observed in samples by Western blot analysis with anti-extracellular signal-regulated kinase (ERK) 5 antibody (A and B, middle). Bars are the means ± SD from five independent experiments. The asterisks indicate significant differences compared with the value of 15.5 mmol/L glucose stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

vascular endothelial cells [25]. It was also reported that sorbitol activated BMK1 in vascular smooth muscle cells [39]. Therefore, we hypothesized that BMK1 may be activated in the glomeruli of diabetic rat model. OLETF rats are a newly developed model of human type 2 diabetes mellitus and show a character of diabetic nephropathy in elderly [40]. As shown in Figure 1, we observed that BMK1 was activated in the glomeruli of 52 weeks age of OLETF rats, whereas no significant activation of BMK1 was observed in the control LETO rats. Quantification of mesangial hypecellularity and matrix score revealed the glomerular injury in OLETF rats (Fig. 1B and C). Western blot analysis of renal cortex protein showed an increase in BMK1 and ERK1/2 activities but not p38 in OLETF rats (Fig. 2A and B).

To confirm this further, we examined the effect of high glucose on BMK1 activity in cultured rat mesangial cells. As a result, we found for the first time that endogenous BMK1 was rapidly and significantly activated by high glucose levels in intact rat mesangial cells (Figs. 3 and 4). The maximal concentration-response was observed at 15.5 mmol/L glucose, which was suggested to be observed in patients with severe diabetes mellitus. These results are consistent with others which reported that 25 mmol/L glucose caused activation of BMK1 in vascular endothelial cells [26]. In addition, a trisaccharide raffinose also activated BMK1 in rat mesangial cells (Figs. 3 and 4). Since raffinose, unlike glucose, cannot enter the cells, the ability of glucose to induce BMK1 activation may be attributable to the osmotic effect. However, since the time course and the concentration-response were not similar between glucose and raffinose, involvement of mechanisms other than osmotic stress cannot be denied in high glucose-induced BMK1 activation.

The intracellular signaling mechanisms that lead to BMK1 activation have been investigated. Using the yeast two-hybrid system, MEK5 was identified by Zhou, Bao, and Dixon [13] as the molecule responsible for regulating BMK1 activity. Kato et al [37] reported that MEK5 specifically activated BMK1 but not other mammalian MAP kinases in vivo [37]. Therefore, we examined whether MEK5 is involved in the BMK1 activation by high glucose in rat mesangial cells. We utilized MEK1/2 inhibitors, U0126 and PD98059, which were also reported to inhibit MEK5 in various cells [17, 41]. Although it was reported that higher concentration of these inhibitors is needed to suppress MEK5 than to inhibit MEK1/2, pretreatment of the cells with both U0126 and PD98059 resulted in an inhibition of BMK1 activation by high glucose in a concentration-dependent manner (Fig. 5). These results suggest that MEK5 is responsible for high glucose-induced BMK1 activation.

In the glomeruli of diabetic nephropathy, numerous studies have reported the importance of PKC [28]. It was reported that MEK5 is a critical target of atypical PKC in mitogenic signaling [42]. It was also reported that

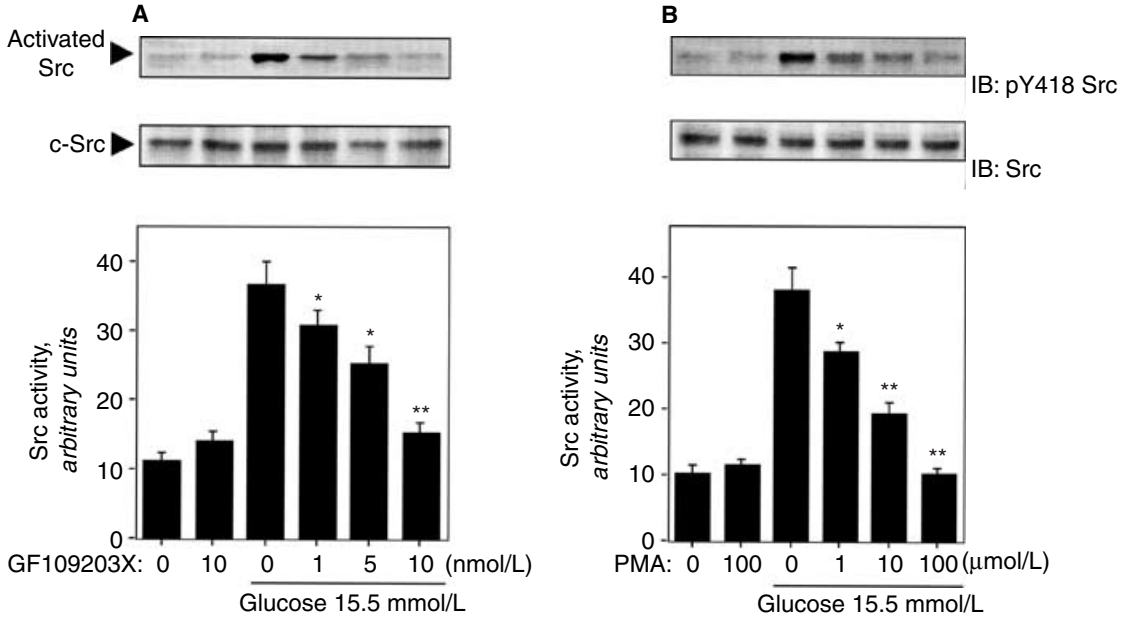

Fig. 8. Effects of protein kinase C (PKC) inhibitor, GF109203X, and down-regulation of PKC with phorbol myristate acetate (PMA) on c-Src activation by high glucose in rat mesangial cells. (*A*) Cells were pretreated with GF109203X at the indicated concentrations for 30 minutes. (*B*) Cells were pretreated with PMA at the indicated concentrations for 24 hours. Then the cells were stimulated with 15.5 mmol/L glucose at the final concentration for 2 minutes. The activity of c-Src was measured by Western blot analysis [immunoblot (IB)] with phospho-Src antibody as described in the **Methods** section. pY418 Src is phosphorylated Src. c-Src is total Src that contains phosphorylated and unphosphorylated Src. No significant differences in the amount of Src were observed in samples by Western blot analysis with anti-Src antibody (A and B, middle). Bars are the means ± SD from five independent experiments. The asterisks indicate significant differences compared with the value of 15.5 mmol/L glucose stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

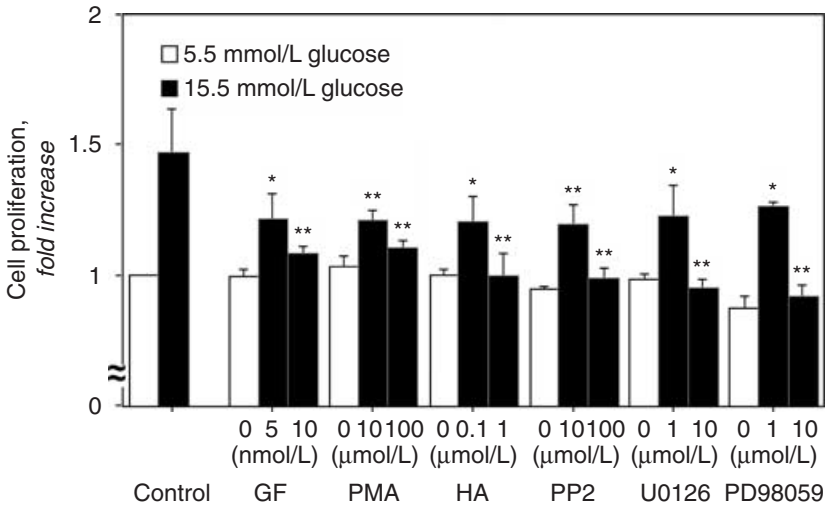

Fig. 9. Effects of protein kinase C (PKC) inhibitors, Src inhibitors, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitors on rat mesangial cell proliferation by high glucose. Cells were pretreated with phorbol myristate acetate (PMA) at the indicated concentration for 24 hours. Cells were pretreated with GF109203X (GF), U0126 and PD98059 for 30 minutes, herbimycin A (HA) for 16 hours, and PP2 for 15 minutes at the indicated concentrations. After the addition of these inhibitors, cells were treated with 5.5 mmol/L glucose or 15.5 mmol/L glucose for 48 hours. Cell proliferation was evaluated by methyl-thiazolyl tertrazolium (MTT) reduction as described in the **Methods** section. Values are the means ± SD of five experiments performed in triplicate. The asterisks indicate significant differences compared with the value of 15.5 mmol/L glucose stimulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

granulocyte colony-stimulating factor-induced BMK1 activation is regulated by PKC [43]. We found in the present study that PKC inhibition by GF109203X or PKC depletion by PMA exposure for 24 hours both inhibited high glucose-induced BMK1 activation (Fig. 6). Since PKC-dependent ERK1/2 activation in mesangial cells is well documented [34], BMK1 activation may also be dependent on PKC. Although the involvement of PKC in BMK1 activation was suggested, the molecule(s) responsible for PKC activation are still unknown. Since PKC was shown to be sensitive to ROS [44], ROS may be involved in this process.

In addition to PKC, c-Src tyrosine kinase may also be involved in BMK1 activation and c-Src was reported to be sensitive to ROS. c-Src-mediated BMK1 activation in response to oxidative stress in fibroblasts was also reported [29]. In addition, we previously observed that H_2O_2-induced BMK1 activation was c-Src-dependent

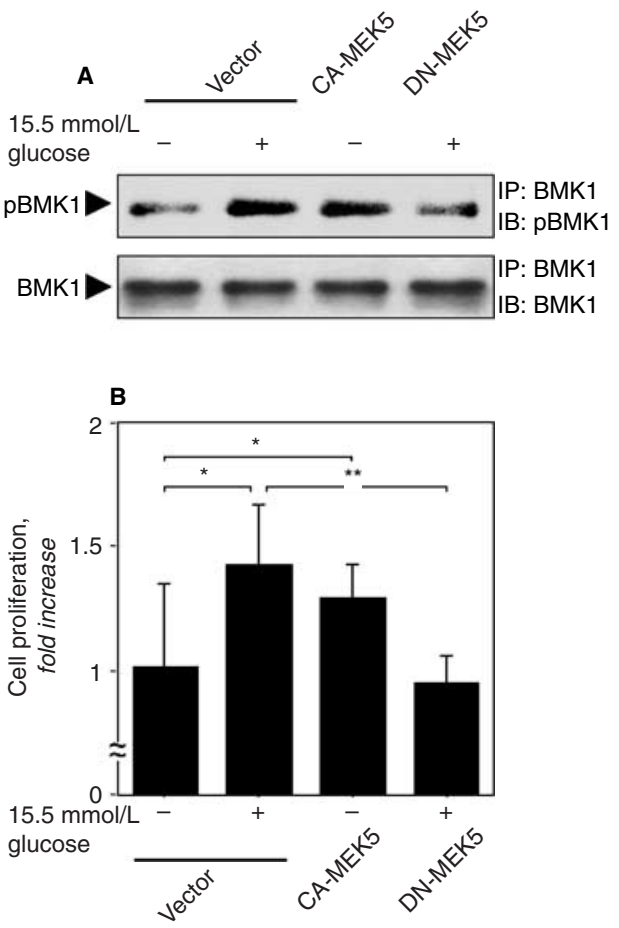

Fig. 10. Transfection with dominant-negative mitogen-activated protein kinase (MAP)/extracellular signal-regulated kinase kinase 5 (DN-MEK5) abolished the big MAP kinase 1 (BMK1) activation and proliferation of rat mesangial cells, whereas constitutive-active MEK5 (CA-MEK5) transfection increased BMK1 activation and cell proliferation even though cells were in the normal glucose condition. Cells were transfected with empty vector (pcDL-SRα), DN-MEK5, or CA-MEK5 and 5 hours later washed and maintained with normal medium. The activity of BMK1 was measured by immunoprecipitation (IP) kinase or immunoblot (IB) assay as described in the **Methods** section. BMK1 is total BMK1 that contains phosphorylated (pBMK1) and unphosphorylated BMK1. (*A*) Representative blots of BMK1 activation are shown. After 24 hours, cells were moved to serum-free media then the cells were stimulated with 15.5 mmol/L glucose at the final concentration for 30 minutes. (*B*) Results of rat mesangial cells proliferation are shown. After transfection, cells were treated with 5.5 mmol/L glucose or 15.5 mmol/L glucose for 48 hours. Cell proliferation was evaluated by methyl-thiazolyl tertrazolium (MTT) reduction as described in the **Methods** section. Values are the means ± SD of five experiments performed in triplicate. The asterisks indicate significant differences compared with the value of the cells transfected with the empty vector alone. $^{*}P < 0.05$; $^{**}P < 0.01$.

in PC12 cells [21]. Therefore, in the present study, we examined whether or not c-Src mediates high glucose-induced BMK1 activation. As shown in Figure 7, specific inhibitors of Src family tyrosine kinases, herbimycin A and PP2, both inhibited high glucose-induced BMK1 activation in a concentration-dependent manner. Consistent with the present findings, tyrosine kinase-dependent BMK1 activation was also reported [43]. However, since we did not determine whether c-Src directly regulates MEK5 activity in this study, further studies are required to define the precise nature of Src kinases in BMK1 activation. In addition, we investigated which molecule is upstream. Although Src inhibitors failed to inhibit PKC activation by high glucose, PKC inhibitors significantly inhibited high glucose-induced Src activation in rat mesangial cells (Fig. 8). Therefore, it was suggested that PKC exists upstream of c-Src for BMK1 activation in rat mesangial cells.

The pathophysiologic role of BMK1 in the kidney has not been reported. A critical role of BMK1 for S-phase entry in the cell cycle has been reported [38]. It was also reported that BMK1 is important for vascular smooth muscle proliferation [39] and skeletal muscle differentiation [45]. Thus, we investigated the involvement of BMK1 activation in rat mesangial cell proliferation induced by high glucose levels. All inhibitors examined, such as PKC inhibitors, Src inhibitors and MEK inhibitors, abolished rat mesangial cell proliferation induced by high glucose (Fig. 9). In addition, transfection with DN-MEK5 inhibited BMK1 activation similar to rat mesangial cell proliferation caused by high glucose levels, though transfection with CA-MEK5 increased both BMK1 activity and cell proliferation even though the condition was a normal glucose concentration (Fig. 10). In addition to the results of immunohistochemistry in the glomeruli of OLETF rats, these results suggest that BMK1 has a role in the proliferation of rat mesangial cells induced by high glucose levels. Since accumulating evidence suggests that mesangial cell proliferation is one of the causative factors for progression of diabetes nephropathy [9, 28], BMK1 may be another possible candidate that is involved in these processes.

CONCLUSION

We showed for the first time that BMK1 is activated by high glucose levels both in in vivo and in vitro. As signaling molecules, PKC and c-Src were suggested to be involved in high glucose-induced BMK1 activation. Although the pathophysiologic role of BMK1 in diabetic nephropathy still remains to be elucidated, it may be an alternative candidate for its cause.

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Reprint requests to Masanori Yoshizumi, M.D., Ph.D., Department of Pharmacology, The University of Tokushima School of Medicine, Kuramoto-cho, Tokushima 770-8503, Japan.
E-mail: yoshizu@basic.med.tokushima-u.ac.jp

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