Statin Attenuates High Glucose-Induced and Angiotensin II-Induced MAP Kinase Activity Through Inhibition of NAD(P)H Oxidase Activity in Cultured Mesangial Cells

H.Y. Yu, T. Inoguchi*, M. Nakayama, H. Tsubouchi, N. Sato, N. Sonoda, S. Sasaki, K. Kobayashi and H. Nawata

Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University

Abstract: An increased oxidative stress may contribute to the development of diabetic nephropathy. We have recently reported that high glucose level stimulated superoxide production through protein kinase C (PKC)-dependent activation of NAD(P)H oxidase in cultured vascular cells. Here we show that 3-hydroxy-3-methylglutaryl CoA reductase inhibitor (statin) attenuates both high glucose level-induced and angiotensin II (Ang II)-induced activation of p42/44 mitogen-activated kinase (MAP kinase) in cultured human mesangial cells through inhibition of NAD(P)H oxidase activity. The intracellular oxidative stress in cultured mesangial cells was evaluated by electron spin resonance (ESR) measurement. MAP kinase activity was evaluated by western blot analysis using anti phospho-specific MAP kinase antibody and anti-ERK-1 antibody. Exposure of the cells to high glucose level (450mg/dl) for 72 hrs significantly increased MAP kinase activity as compared to normal glucose level (100mg/dl). This increase was completely blocked by the treatment of pitavastatin ($5x10^{-7}$M) as well as a NAD(P)H oxidase inhibitor (diphenylene iodonium, 10^{-5}M) in parallel with the attenuation of oxidative stress. Ang II-induced activation of MAP kinase was also completely blocked by pitavastatin as well as a diphenylene iodonium in parallel with the attenuation of oxidative stress. In conclusion, pitavastatin attenuated high glucose-induced and Ang II- induced MAP kinase activity in mesangial cells through inhibition of NAD(P)H oxidase. Thus, statins may have a potential as a therapeutic tool for early diabetic nephropathy.

Key Words: Diabetes, nephropathy, MAP kinase, NAD(P)H oxidase, mesangial cell, oxidative stress.

INTRODUCTION

Oxidative stress may contribute to the onset or the development of diabetic nephropathy. Several potential sources for increased reactive oxygen species (ROS) production in diabetes have been postulated, such as enhanced formation of advanced glycation end products [1], altered polyol pathway activity [2], increased xanthine oxidase activity [3], increased superoxide release from mitochondria [4] and activation of vascular NAD(P)H oxidase [5]. Recently, attention is increasingly focused on vascular NAD(P)H oxidase as the most important source of ROS production in diabetic vascular tissues [6-7]. We reported that high glucose level stimulated ROS production through a protein kinase C (PKC)-dependent activation of NAD(P)H oxidase in vascular cells [5]. Recent reports including ours have further supported the evidence that NAD(P)H oxidase may be a major source of increased ROS production in diabetic kidney [8, 9] as well as diabetic vascular tissues [10-13]. The glomerular mesangium expansion is an important feature of diabetic nephropathy. Since mitogen-activated protein kinase (MAP kinase) functions as a key regulator of cell proliferation and protein synthesis, altered MAP kinase activity may contribute to the mesangium expansion. The first objective of the present study is to show that NAD(P)H oxidase-derived ROS production is linked to both high glucose level-induced and angiotensin II-induced MAP kinase activation in cultured mesangial cells.

Recent reports have shown that the 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins) inhibit angiotensin II-induced superoxide production in vascular cells *via* inhibition of NAD(P)H oxidase activity [14, 15]. Recently, we also have shown that statin inhibited high glucose level-induced superoxide production in vascular cells *via* inhibition of NAD(P)H oxidase activity [16]. Therefore, the second objective of this study is to show that statin inhibits both high glucose level-induced and angiotensin II-induced MAP kinase activation in cultured mesangial cells. Thus, these findings may provide a new insight into the therapeutical use of statins for preventing diabetic nephropathy.

MATERIALS AND METHODS

Cell Culture

Human mesangial cells were obtained from Clonetics (East Rutherford, N.J., USA). The cells were maintained in a Mesangial Cell Growth Medium (MsGM) (Clonetics) containing 5% fetal calf serum in atmosphere of 95% O2/5%Co2 at 37 C. The cells used in these experiments were from 3rd to 6th passages.

Assay of ROS Production by ESR

Acetoxymethyl-2, 2, 6, 6-tetramethylpiperidine-1-oxyl-3-carboxylate (CxT-AM) was synthesized as previously described [5] for *in vitro* electron spin resonance (ESR) measurement. As for *in vitro* ESR measurement, a

*Address correspondence to this author at the Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan; Tel: +91-92-642-5284; FAX: +91-92-642-5287; E-mail: toyoshi@intmed3.med.kyushu-u.ac.jp

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conventional X-band spectrometer (JOEL RE-1X) was used at 9.4 GHz of microwave frequency. For the experiments, the cells were allowed to reach confluence in 100 mm dishes, and then the medium was changed to an MsGM supplemented with 0.5% serum and high glucose level (400mg/dl) or normal glucose level (100 mg/dl). *In vitro* ESR measurement was performed as previously described [5]. Briefly, the cells were removed from dishes by treatment with 0.25% trypsin, centrifuged at *800g* for 5 min, and suspended in the medium without serum at a concentration of $1x10^7$ cells/ml. The nitroxide radical, CxT-AM, was added to the cell suspension at the final concentration of 20μmol/l, and the samples were mixed quickly but gently. Then the samples were immediately drawn into gas-permeable Teflon tubes (Zeus Industries, Raritan, NJ) and inserted into a quartz ESR tube open at each end. Experiments were performed at 37C and the microwave power was set at 10mW. The field modulation width was 0.2mT and the magnetic field range was swept at a scan rate of 5mT/min.

Assay of MAP Kinase Activity

For the assay of MAP KINASE activity, the cells were cultured on 35 mm dishes. The cells were scraped and then homogenized on ice with 20 strokes of a Dounce homogenizer in a buffer containing 10 mmol/l TRIS, 150 mmol/l NaCl, 2 mmol/l EGTA, 2 mm/l DTT, 1 mmol/l orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4. The homogenates were centrifuged at 10,000 g x 20 min to precipitate cell debris. For western blot analysis, the samples were denatured by boiling in Laemmli sample buffer, subjected to SDS-PAGE, and immunoblotted with phospho-specific MAP kinase antibody (New England Biolbs, Beverly, MA, USA). The blots were then incubated with horseradish peroxidase-linked second antibody followed by chemiluminescence detection, according to the manufacture's instructions (Pierce, Rockford, IL, USA). And the same time, blots were immunoblotted with anti-Erk-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Video-densitometry analysis of the blots was carried out and the ratio of phospho-MAP kinase to total MAP kinase was calculated. Results were expressed as a mean percentage of control±SE from 4 independent experiments.

Statistical Analysis

Statistical analysis was done by analysis of variance (ANOVA) followed by Fisher's comparison test.

RESULTS

Exposure of the human mesangial cells to high glucose level (400 mg/dl) for 72 hrs induced a significant increase in ROS production compared with normal glucose level (100 mg/dl), as evaluated by ESR measurement. Treatment of the cells with angiotensin II (10^{-7}M) for 20 min also induced a significant increase in ROS production. Both effects of high glucose level and angiotensin II were significantly inhibited by the treatment of NAD(P)H oxidase inhibitor, diphenylene iodonium (10^{-5}M), and PKC inhibitor, calphostin C (5x 10^{-7}M) (Fig **1**). In addition, treatment of the cells with pitavastatin (5x 10^{-7}M) for 36 hrs significantly inhibited both high glucose level-induced and angiotensin II-induced ROS production (Fig. **2**).

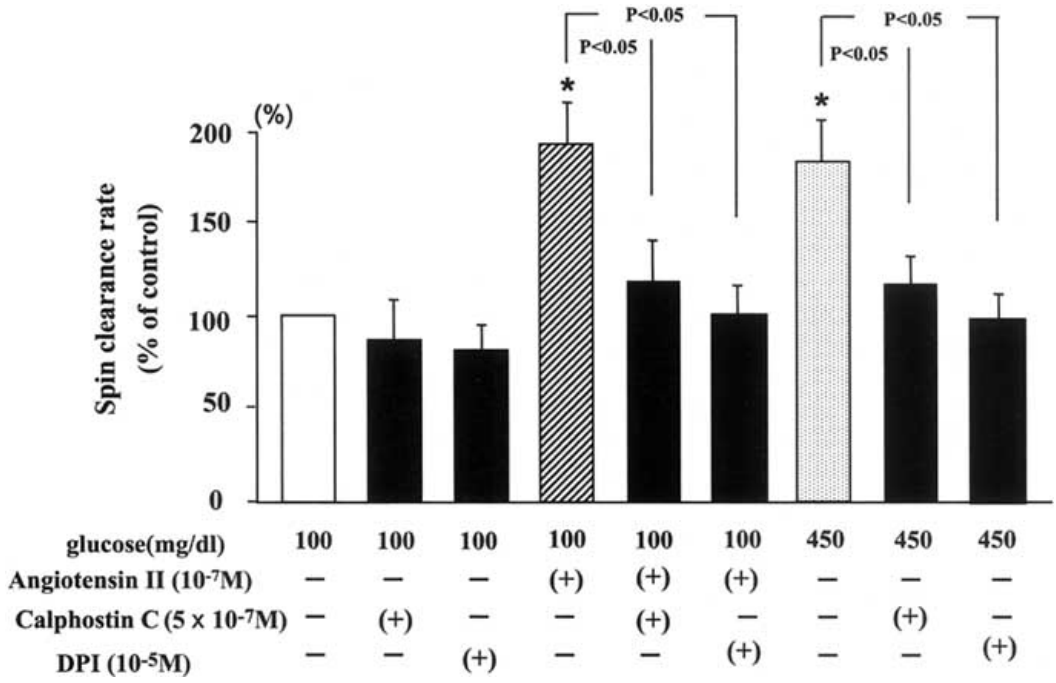


Fig. (1). The effect of high glucose level and angiotensin II on reactive oxygen species (ROS) production in cultured human mesangial cells evaluated by electron spin resonance (ESR) method. The confluent cells were incubated with the test media containing 1% serum and 100 mg/dl or 400 mg/dl for 72 hrs. For the last 2 hrs of incubation, calphostin C or diphenylene iodonium (DPI) was added. For angiotensin II treatment, angiotensin II was added to the test media containing 100 mg/dl glucose for 20 min. ROS was measured by ESR method as described in Methods. Results are expressed as mean±SE from 4 independent experiments. *P<0.01 *vs* control.

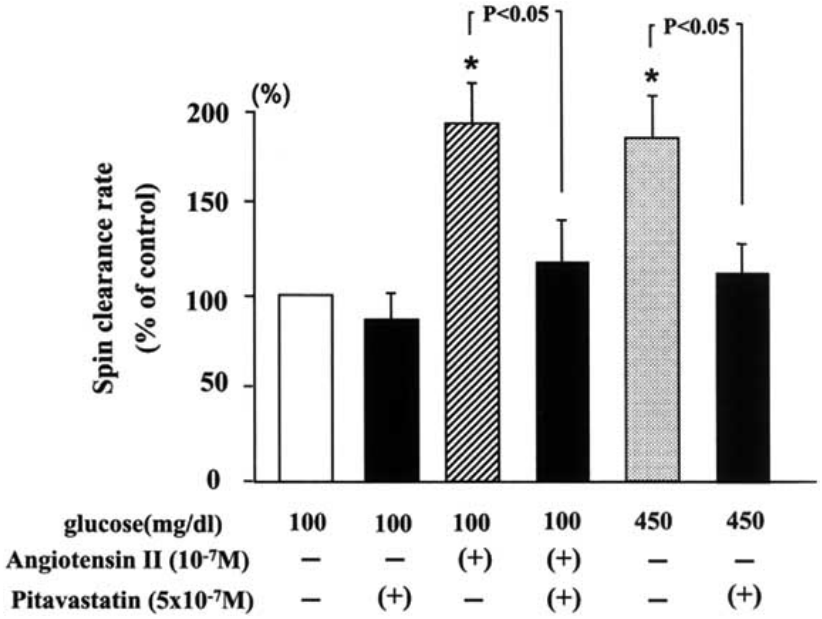
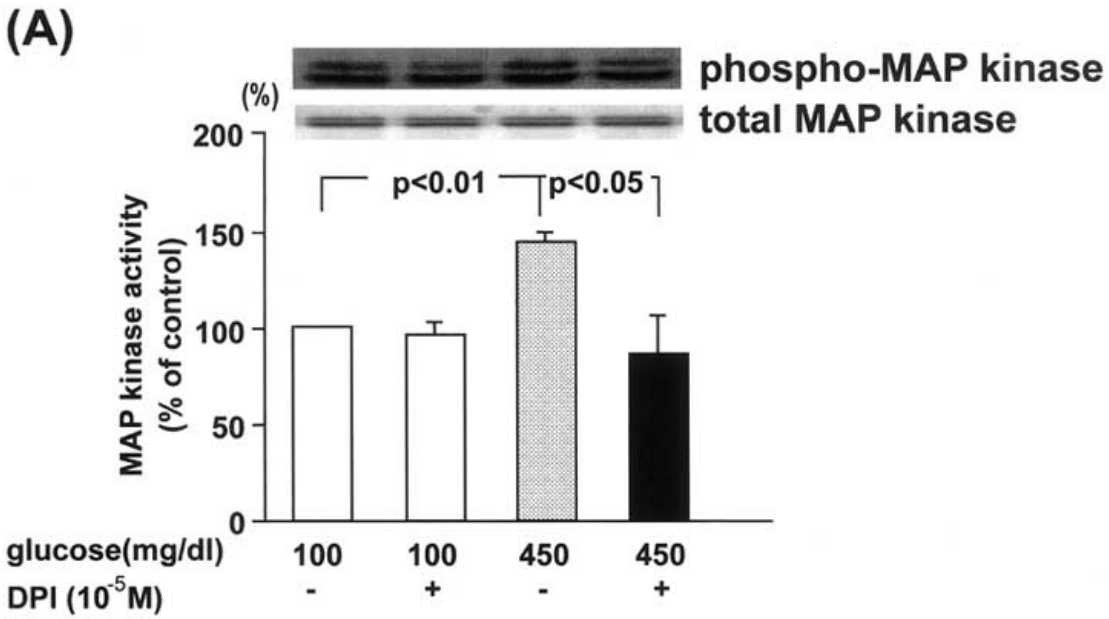


Fig. (2). The effect of pitavastatin on high glucose level-induced and angiotensin II-induced reactive oxygen species production in cultured mesangial cells. The confluent cells were incubated with the test media containing 1% serum and 100 mg/dl or 400 mg/dl for 72 hrs. For the last 36 hrs of incubation, pitavastatin was added. For angiotensin II treatment, angiotensin II was added to the test media containing 100 mg/dl glucose for 20 min. ROS was measured by ESR method as described in Methods. Results are expressed as mean±SE from 4 independent experiments. *P<0.01 *vs* control.

Western blot analysis using phospho-specific MAP kinase antibody showed the high glucose level-induced excessive phosphorylation of both p42/p44 MAP kinase, and the total MAP kinase protein level was not altered by high glucose level, as assessed by a polyclonal anti-Erk-1 antibody. As a result, high glucose level significantly stimulated the ratio of phospho-MAP kinase to total MAP kinase (Fig **3A**), suggesting the high glucose-induced activation of MAP kinase. This effect was significantly inhibited by treatment with diphenylene iodonium (Fig. **3A**) and pitavastatin (Fig. **3B**). Treatment of the cells with angiotensin II also significantly stimulated the ratio of phospho-MAP kinase to total MAP kinase (Fig. **4A**).This effect was again significantly inhibited by diphenylene iodonium (Fig. **4A**) and pitavastatin (Fig. **4B**).

DISCUSSION

The present study showed that both high glucose level and angiotensin II stimulated p42/44 MAP KINASE activity in renal mesangial cells, and this effect was blocked by



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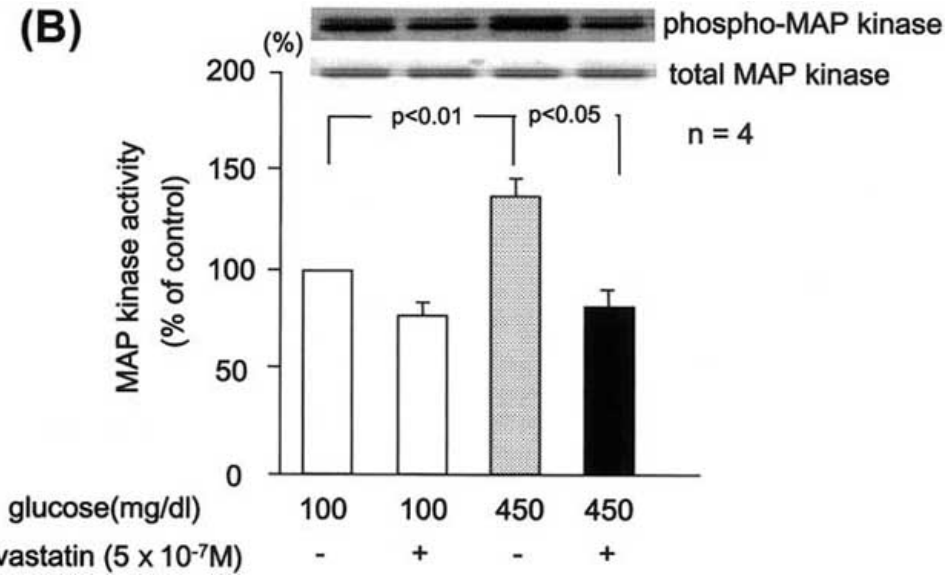

Fig. (3). The effect of diphenylene iodonium (A) and pitavastatin (B) on high glucose level-induced activation of MAP kinase in cultured mesangial cells.

The extracted proteins (20 μg) from the cells were subjected to SDS-PAGE and immunoblotted with phospho-specific MAP kinase antibody (upper panel). At the same time, the extracted proteins (10 μg) were subjected to SDS-PAGE and immunoblotted with anti-Erk-1 antibody (upper panel). Videodensitometry analysis of the ratio of phospho-MAP kinase to total MAP kinase (lower panel). Results were expressed as a mean percentage of control±SE from 4 independent experiments. DPI, diphenylene iodonium chloride.

NAD(P)H oxidase inhibitor, diphenylene iodonium, suggesting the role of NAD(P)H oxidase. Previously, Haneda *et al.* reported that high glucose level stimulated p42/44 MAP kinase through PKC-dependent mechanism in mesangial cells [17]. We reported that high glucose level stimulated ROS production through PKC-dependent activation of NAD(P)H oxidase in cultured aortic endothelial cells and smooth muscle cells [5]. In combination, these results suggest that PKC-dependent activation of MAP kinase induced by high glucose level may be mediated by NAD(P)H oxidase-generated ROS. In vascular smooth muscle cells, recent report showed that angiotensin II stimulated p38 MAP kinase, JUNK and ERK5 through NAD(P)H oxidase-generated ROS, but not p42/44 MAP kinase [18]. In contrast, the present results suggested that angiotensin II also stimulated p42/44 MAP KINASE in mesangial cells. Since angiotensin II activates PKC in mesangial cells, the present results suggest that angiotensin II-dependent activation of p42/44 MAP kinase may also be mediated by NAD(P)H oxidase-generated ROS.

The glomerular mesangium expansion is an important feature of diabetic nephropathy and is caused by an excessive accumulation of extracellular matrix proteins. Since p42/44 MAP kinase functions as a key regulator of cell proliferation and protein synthesis [19. 20], high glucose level-induced and angiotensin II-induced MAP kinase activation may contribute to the onset or the development of diabetic nephropathy. Such mechanisms may be a therapeutic target preventing the development of diabetic nephropathy. There have been a number of clinical findings that blockade of angiotensin II with either angiotensin-converting enzyme inhibitor (ACEI) or an ARB can prevent or delay the progression of renal injury associated with diabetes [21-24]. The effect of inhibition of the RAS is in addition to, but independent of blood pressure control. The beneficial effect of ARB or ACEI may be at least in part attributed to the inhibitory effect on p42/44 MAP kinase in mesangial cells.

The present results showed that pitavastatin significantly inhibited both high glucose-induced and angiotensin II-induced activation of MAP kinase in mesangial cells in parallel with inhibition of ROS production. These results suggest that pitavastatin may also inhibit activated p42/44 MAP kinase activity in mesangial cells of diabetic kidneys *via* inhibition of NAD(P)H oxidase. We recently reported that pitavastatin inhibited NAD(P)H oxidase activity by inhibition of small GTPase Rac-1 activity which was a key regulator of NAD(P)H oxidase [16]. In addition, previous studies have shown that statins inhibit superoxide production *via* inhibition of angiotensin II-induced NAD(P)H oxidase activation [14, 15]. Complete overexpression of dominant negative Rac-1 was also shown to inhibit angiotensin II-induced intracellular oxidation in cardiac myocytes [25]. Taken together, these results suggest that the inhibitory effect of statins on both high glucose-induced and angiotensin II-induced activation of NAD(P)H oxidase may be related to their inhibitory effect on Rac-1 activity. Considering its mechanism, this effect of pitavastatin appeared to be a class effect for these agents, although whether other agents in this class have a similar effect needs to be determined in future studies. Several clinical studies have suggested the beneficial effect of statins on the progression of early stages of diabetic nephropathy [26-28] although this evidence remains to be

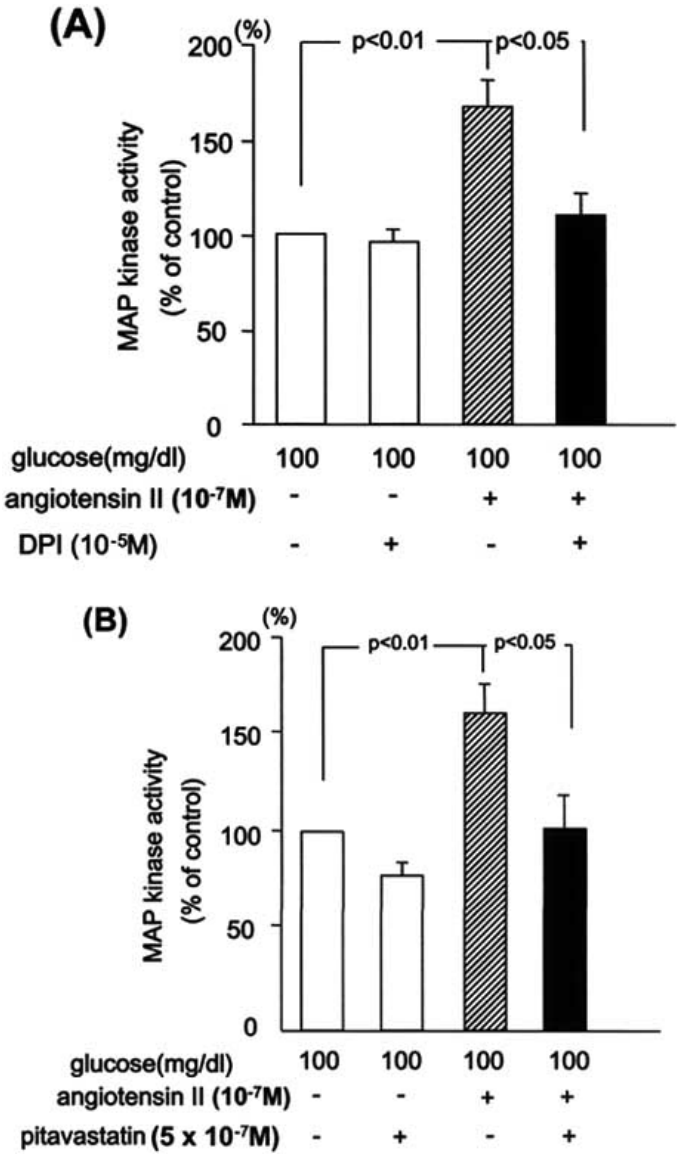

Fig. (4). The effect of diphenylene iodonium (A) and pitavastatin (B) on angiotensin II-induced activation of MAP kinase in cultured mesangial cells.The extracted proteins (20 μg) from the cells were subjected to SDS-PAGE and immunoblotted with phospho-specific MAP kinase antibody. At the same time, the extracted proteins (10 μg) were subjected to SDS-PAGE and immunoblotted with anti-Erk-1 antibody. Videodensitometry analysis of the ratio of phospho-MAP kinase to total MAP kinase. Results were expressed as a mean percentage of control +SE from 4 independent experiments. DPI, diphenylene iodonium chloride.

established in the future studies. Thus, the present findings may provide a new insight into the therapeutic use of statins for preventing diabetic nephropathy.

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