Mechanism of preventive effect of HMG-CoA reductase inhibitor on diabetic nephropathy

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Background. Previously, we have found that pravastatin prevents diabetic nephropathy in streptozotocin-induced diabetic rats independently of serum lipid levels. The aim of this study was to clarify the impact of pravastatin on the mesangial cells exposed to high glucose.

Methods. Rat mesangial cells were cultured in DMEM containing low glucose (5 mM glucose), high glucose (25 mM glucose) and 25 mM glucose with 500 µM pravastatin for 48 hours, respectively. After harvesting, we examined membrane-associated Ras with immunoblot analysis, activity of mitogen-activated protein kinase (MAPK) in the cytosol fraction with in-gel kinase assay and expressions of TGF-β mRNA with Northern blot analysis.

Results. Membrane-associated Ras, activity of MAP kinase, and expression of transforming growth factor-β (TGF-β) mRNA were increased in the mesangial cells cultured exposed to high glucose compared to low glucose. Pravastatin suppressed all these changes in membrane-associated Ras, MAP kinase and TGF-β mRNA in high glucose.

Conclusions. This study suggests that pravastatin suppresses the activity of Ras-MAP kinase cascade and induction of TGF-β in the mesangial cells that have been exposed to high glucose.

Mesangial expansion plays a crucial role in the development of diabetic nephropathy. Additionally, alterations in the synthesis or action of various cytokines and growth factors have all been hypothesized to be involved in the pathogenesis of diabetic nephropathy. Recent investigations have revealed that transforming growth factor-beta (TGF-β) is an important cytokine in the pathogenesis of diabetic nephropathy, up-regulating extracellular matrix proteins synthesis in the mesangial cells. High glucose causes an increase in expression of TGF-β in the glomeruli of streptozotocin (STZ)-diabetic rats [1]; however, the precise mechanism whereby ambient glucose stimulates the synthesis of TGF-β remains unknown.

Mitogen-activated protein kinase (MAPK) cascade is one of the important kinase cascades that transmit extracellular stimuli to various cellular functions [2]. MAPK is activated via Ras-dependent intracellular signaling pathway associated with tyrosine-kinase-coupled receptors [3]. Recent studies indicate that MAPK is activated in the mesangial cells cultured under high glucose condition via activation of protein kinase C [4].

Previously, we have found that 3-hydroxy-3-methylglutaryl (HMG-CoA) reductase inhibitor, pravastatin, has a preventive effect on the development of diabetic nephropathy in STZ-diabetic rats independently of serum lipid levels [5]. HMG-CoA reductase inhibitor suppresses Ras-MAPK cascade by an inhibitory effect on prenylation of Ras. Hence, we speculate that the beneficial effect of pravastatin on diabetic nephropathy can be attributed to suppression of Ras-MAPK cascade by pravastatin in the glomeruli of the diabetic rats. To clarify the impact of pravastatin on Ras-MAPK cascade in diabetic milieu, we examined the effect of pravastatin on membrane-associated Ras, MAPK activity and expression of TGF-β mRNA in the rat mesangial cells cultured in high glucose medium.

METHODS

Mesangial cells were isolated from glomeruli of male Wistar rats (Crea, Japan) and maintained in 10 cm² Petri dishes in Dulbecco’s modified Eagle medium (DMEM; Nissui, Japan) supplemented with 10% fetal bovine serum, 50 µg/ml piperacillin Na, 100 µg/ml streptomycin sulfate, 5 µg/ml insulin, and 2 µg/ml amphotericin B. Cultures were kept at 37°C with 5% CO₂. For passage confluent cells were washed with HEPES buffer, removed with trypsin/EDTA (Clonetics, USA), and plated in DMEM. The experiments included in this study were performed on the cells between the 5th and 7th passages. For experiments the medium was exchanged to fresh DMEM plus 0.5% fetal bovine serum containing either low glucose (5.6 mM of D-glucose) or high glucose (25

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mm of D-glucose). We added 350 mg/dl D-mannitol to DMEM containing low glucose to achieve an equal final medium osmolality as that containing high glucose. As an additional experiment, the cells were incubated with high glucose medium in the presence of 500 μM pravastatin purchased from Sankyo, Japan. After 48 hours, the mesangial cells were harvested in ice-cold lysis buffer (50 mmol/liter Tris-HCl, pH 7.5, 1% Nonidet P40, 140 mmol/liter NaCl, 1 mmol/liter sodium orthovanadate, 50 mmol/liter NaF, 1 mmol/liter egtazic acid (EGTA), 1 mmol/liter PMSF, and 1 μg/ml leupeptin) and sonicated at 4°C for 30 seconds. Homogenates were centrifuged at 12,000 g at 4°C for 30 minutes and supernatants were used for the kinase assay. The concentrations of proteins in the supernatants were determined using modified Lowry assay kit (Pierce, USA). For the in-gel kinase assay lysates were applied to 10% SDS-polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP) as the substrate. After electrophoresis, the gels were washed to remove SDS with the buffer A (50 mmol/liter HEPES, pH 7.4, and 5 mmol/liter mercaptoethanol) containing 20% 2-propanol. The proteins in the gel were denatured in the buffer A containing 6 mol/liter guanidinium thiocyanate at room temperature for one hour and renatured in the buffer A containing 0.04% Tween-40 at 4°C for 16 hours. For kinase reaction the gels were incubated at 30°C for one hour in the buffer A containing 50 μmol/liter ATP and 250 μCi γ-[32P] adenosine 5’-triphosphate (ATP). The gels were washed with 5% trichloroacetic acid and 10 mmol/liter sodium pyrophosphate and followed by densitometric analysis with Imaging Analyzer BAS2000 (Fuji Photo Film, Japan). For Northern blot analysis, the cells were lysed with 4 mol/liter guanidinium thiocyanate containing 25 mmol/liter sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 mmol/liter 2-β-mercaptoethanol. Total RNA was isolated by the single-step method, using phenol and chloroform/isoamyl alcohol [6]. Electrophoresis of 20 μg of total RNA was carried out in 1% agarose gel with 2.2 mmol/liter formaldehyde. The RNA was then transferred overnight onto a nylon membrane (Amersham, Japan). The cDNA probes used in this study were v-H-ras (Takara Shuzo, Japan), rat TGF-β1 (gift of Dr. H. Kurata), and the rat GAPDH cDNA (Oncogene Research Product, Japan). The cDNAs were labeled with α-[32P]dCTP (3000 Ci/mmol, NEN, Japan) using random primer extension kit (Takara Shuzo, Japan) or γ-[32P]ATP (7000 Ci/mmol; NEN, Japan) using 5’-end labeling procedure (Takara Shuzo, Japan). Prehybridization and hybridization were performed overnight at 42°C in the buffer containing 50% formamide, 5 × SSPE, 5 × Denhardt solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. Then, the blots were washed five times in 2 × SSC, 0.1% SDS at room temperature and washed with 1 × SSC, 0.1% SDS at 65°C for an additional 30 minutes. After they dried, the membranes were exposed to Imaging Analyzer BAS2000 (Fuji Photo Film, Japan) and each band that hybridized with the probes was detected and quantitated as a relative ratio.

RESULTS

There was no difference in the levels of H-ras mRNA between the mesangial cells cultured in low and high glucose medium. Furthermore, addition of pravastatin did not affect the levels of H-ras mRNA in the mesangial cells (Fig. 1A). In contrast, the amount of Ras protein in the membrane fractions detected by immunoblot analysis was increased 1.6-fold in the mesangial cells cultured in high glucose compared to low glucose. Addition of pravastatin suppressed this increment of membrane-associated Ras in the mesangial cells cultured in high glucose (Fig. 1B).

The activity of MAPK was increased in the mesangial cells cultured in high glucose compared to low glucose. Addition of pravastatin suppressed the increment of the activity of MAPK in the mesangial cells cultured in high glucose medium (Fig. 2A).

The expression of TGF-β mRNA was elevated in the mesangial cells cultured in high glucose compared to low glucose. Addition of pravastatin suppressed the induction of TGF-β mRNA in the mesangial cells cultured in high glucose medium (Fig. 2B).

DISCUSSION

This study has revealed that ambient high glucose up-regulates the translocation of Ras to the cell membrane from the cytosol without influence on transcription of Ras gene and that pravastatin suppresses the exaggerated translocation of Ras in high glucose medium. Ras receives post-translational modification, prenylation, covalently binding farnesyl or geranylgeranyl group, both of which are intermediate metabolites of cholesterol synthesis. Prenylated Ras attaches to the cell membrane via these hydrophobic groups, which make Ras capable of mediating signal transduction associated with tyrosine-kinase-coupled receptor [7]. HMG-CoA reductase inhibitor may suppress the activity of Ras by its inhibitory effect on prenylation. O’Donnell et al reported that
HMG-CoA reductase inhibitor, lovastatin, suppresses the proliferation of cultured mesangial cells stimulated by fresh serum inhibiting farnesylation of Ras [8]. The present experiment also suggests that the effect of pravastatin is involved in prenylation of Ras, which may be accelerated in the mesangial cells cultured in high glucose medium.

In the present experiment, the activity of MAPK and the expression of TGF-β mRNA were simultaneously enhanced in the mesangial cells cultured in high glucose compared to low glucose. Pravastatin significantly suppressed the activity of MAPK and the expression of TGF-β mRNA induced in the mesangial cells cultured in high glucose medium. These data suggest that activity of MAPK plays a crucial role in the transcriptional regulation of TGF-β gene in the mesangial cells, and that pravastatin suppresses Ras-MAPK cascade that is enhanced by ambient high glucose.

What factor(s) or receptor(s) promotes the Ras-MAPK cascade in mesangial cells exposed to high glucose remains to be determined. Recent investigations suggest that downstream of platelet-derived growth factor (PDGF) the β receptor may involve translocation of Grb2/Sos complex leading to an activation of Ras [9]. To prove the possibility of PDGF-BB as a candidate of triggering Ras-MAPK cascade, we examined the influence of high glucose on the expression of PDGF-B mRNA in cultured rat mesangial cells [10]. We found that the expression of PDGF-B mRNA is increased in the mesangial cells cultured in high glucose compared to low glucose. Therefore, it is possible that PDGF-BB activates Ras in an autocrine manner via the PDGF-β receptor in the mesangial cells exposed to high glucose; however, further examination is required to prove this hypothesis.

On the basis of these data, we conclude that activation of Ras-MAPK cascade is responsible for induction of transcription of TGF-β in the mesangial cells cultured in high glucose medium, and that HMG-CoA reductase inhibitor, pravastatin, suppresses the expression of TGF-β through its inhibitory effect on Ras-MAPK cascade, which results in the prevention of diabetic nephropathy.
Fig. 2. Influence of high glucose and pravastatin on the activity of MAPK (A) and the expression of TGF-β mRNA (B) in cultured rat mesangial cells. Cells were treated as described in the Methods section. The culture media were replaced with low glucose (5 mM) media (a), high glucose (25 mM) media (b), high glucose media containing 500 μM pravastatin (p). The cells were harvested at 48 hours after exchange of culture media. The activity of MAPK was determined by the in-gel kinase assay. Total cellular RNA was extracted for Northern blot analysis performed with 32P-labeled TGF-β and GAPDH cDNAs. The relative kinase activities of MAPK and the relative intensities of TGF-β mRNA bands are shown. Values are expressed as mean ± sd of three experiments (*P < 0.05 versus low glucose).

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