

The Involvement of MAPK in Rat Peritoneal Mesothelial Cells under High Glucose Condition

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Peritoneal fibrosis is a serious complication in long-term continuous ambulatory peritoneal dialysis (CAPD) patients, but the underlying mechanism is not well understood. Since high glucose activates the p38 mitogen-activated protein kinase (MAPK) pathway in various kinds of cells, and because mesothelial cells always exposed to high glucose dialysate, we investigated the involvement of MAPK in rat peritoneal mesothelial cells (PMCs) under high glucose conditions. Rat PMCs were grown in Dulbecco's modified Eagle's medium with 0.5 % FBS in 24 hours and then exposed to 4% glucose or 4% mannitol. Cell viability was assessed by using WST-1 assay. Fibronectin (FN) accumulation in the supernatant was determined by Western blot. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were performed to determine mRNA and protein expression, respectively. High glucose and mannitol caused time-dependently increase in FN accumulation and FN mRNA in rat PMCs. High glucose also activated the extracellular signal-regulated kinase (ERK) and p38 MAPK. These increase in FN accumulation were not inhibited with anti-transforming growth factor (TGF)- β neutralizing antibody. Moreover, high glucose does not promote TGF- β accumulation in the culture media. Therefore, MAPK pathway may play an important role in high glucose-induced FN accumulation via TGF- β -independent mechanism. It is considered that high glucose acts as osmotic stimulation in the regulation of FN production in these cells. Taken together, these findings suggest that the activation of MAPK signaling pathways in rat PMCs by high glucose may contribute to the pathogenesis of peritoneal fibrosis in CAPD therapy.

Key words: peritoneal fibrosis, erk, p38, high-glucose, rat peritoneal mesothelial cells

Introduction

It is well known that long-term technique survival on continuous ambulatory peritoneal dialysis (CAPD) is poor, and estimated to be between 50-70% at 5 years in most published series^{1,2)}. The cause of this high technique loss is multifactorial, but loss of peritoneal membrane function is discontinuous CAPD. In recent study, loss of ultrafiltration was shown to be responsible for 51% of withdrawals from CAPD in patients who have been treated with the technique for more than 6 years³⁾. The technique of CAPD results in many morphological changes to the peritoneum. Peritoneal mesothelial

cells (PMCs) have been shown to denude gradually from the surface of peritoneum with time on dialysis, and be replaced by fibrous tissue⁴⁾.

Peritoneal fibrosis is one of the most serious complications after long-term CAPD. It is considered that high glucose, its degradation products and acidic pH of the conventional PD fluid are accelerating factors of peritoneal fibrosis. Especially, hyperosmolar glucose (4.25%) in PD solution is inhibitory to many resident peritoneal cells, and has been shown in many studies to inhibit phagocytic activity by leukocytes and production of cytokines by PMCs⁵⁾. Prior studies have postulated that high glu-

cose concentrations suppress the growth and regeneration of PMCs⁶, enhance transforming growth factor (TGF)- β gene expression⁷⁻¹⁰, and stimulate fibronectin synthesis⁹⁻¹¹, all of which may contribute to peritoneal fibrosis, but various glucose concentrations were used in the *in vitro* studies investigating the effects of glucose on extracellular matrix (ECM) accumulation. Usually, 1.5% or 2.5% glucose was used as an osmotic agent in the previous studies using cultured PMCs. Most highest concentration of glucose was 4% in PD solution and the precise cellular mechanisms modulating the expression of cytokine and ECM induced by high glucose are not fully understood.

The aim of the present study was to investigate whether mitogen activated protein kinase (MAPK) might be involved in the regulation of ECM accumulation in cultured rat PMCs under 4% (g/dl) glucose condition.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and other culture reagents were obtained from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum was obtained from ICN Bio-medical (Costa Mesa, CA, USA). Anti-TGF- β antibody was obtained from R & D Systems, Inc. (Minneapolis, MN, USA). WST-1 cell-counting kit was Wako Chemical (Tokyo, Japan). Rabbit anti-rat fibronectin (FN) antibody was from LSL Co. (Tokyo, Japan). Anti-active MAPK polyclonal antibody and anti-active p38 polyclonal antibody which were used for the detection of phosphorylated forms of ERK1/2 and p38 were from Promega (Madison, WI, USA). Pan p38 MAPK antibody was also purchased from Promega. FN primer for RT-PCR was obtained from Japan Bio Service (Tokyo, Japan). The inhibitors against extracellular signal-regulated kinase (ERK) (PD98059) and p38 MAPK (SB203580) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell preparation and characterization

Rat PMCs were isolated by enzymatic digestion of the parietal peritoneal wall. Cells detached from the peritoneal surface by trypsinization. Within

1 week of initiation, such cultures yield a phenotypically pure population of cells that displayed a cobblestone appearance at confluence. When grown in DMEM supplemented with 10% fetal bovine serum (FBS), these cells exhibited a doubling time of approximately 24 h and reached a saturation density of 1×10^5 cells/cm².

To confirm their mesothelial identity, cell isolated from the parietal peritoneal surface were subjected to immunofluorescence staining with a series of antibodies to a variety of cytoskeletal, cell-surface and cytoplasmic proteins. These cells stained positively for a number of mesothelial cell-specific cytokeratins (ie types 7, 8, 18 and 19) (data not shown). These cells also stained positively for vimentin, but were negative for desmin as well as for a rat endothelial cell-specific antigen. These results indicate a pattern surface in agreement with the pattern previously reported for both rat peritoneal and pleural mesothelial cells¹².

Experimental protocol

All experiments were carried out on quiescent, confluent, passage third to fifth rat PMCs. Cells for reverse transcription-polymerase chain reaction (RT-PCR) were grown in 60 mm² culture dish. For Western blotting, Rat PMCs were grown in 10 cm² culture dish. Near confluent rat PMCs grown in culture dish were incubated for 24 h in serum-depletion media (DMEM containing 0.5% FBS) to arrest and synchronize the cell growth. And then cells were treated with 4% of D-glucose.

Cell proliferation assay

The effect of glucose on cell proliferation was determined by using WST-1 cell counting kit. This method is based on the colorimetric conversion of a tetrazolium compound to formazon. The resulting absorbance is directly proportional to the number of living cells, and the method is well correlated with the [³H]thymidine incorporation method. Cells were rendered quiescent by serum deprivation for 24 h before experiments. Quiescent cells grown in 24-well palates were incubated for 48 h in 0.5% FBS medium with 4% glucose or 4% mannitol.

Measurement of FN and TGF- β accumulation

Quiescent cells grown in 6-well plates were incu-

bated for 24 and 48 h in 0.5% FBS medium with 4% glucose or 4% mannitol in the presence or absence of anti-human TGF- β antibody (10 μ g/ml). The equal amounts of protein in supernatant (10 μ l) were separated on 10% polyacrylamide gels using SDS-PAGE and transferred to PVDF membranes. The membrane were blocked for 1 h with phosphate-buffered saline (PBS; pH 7.4) containing 2.5% nonfat dry milk and then incubated for 1 h with rabbit anti-rat FN antibody (1 : 2000). After being washed with PBS, the membranes were incubated with secondary antibody (1 : 2000) for 1 h.

Finally, FN proteins on the membranes were detected by ECL kit (Amersham Pharmacia Biotech, Tokyo, Japan). The TGF- β concentration in the supernatant was assessed by an enzyme linked immunoadsorbent assay using anti-human TGF- β antibody as a primary antibody. Moreover, Quiescent cells grown in 6-well plates were incubated for 24 and 48 h in 0.5% FBS medium with 4% glucose or 4% mannitol in the presence or absence of MAPK inhibitors (10⁻⁷ M SB203580 or 10⁻⁵ M PD98059).

Determination of FN mRNA

FN mRNA was determined by reverse RT-PCR technique. Quiescent cells grown in 60 mm² dishes were incubated for 24 and 48 h in medium containing 0.5% FBS with 4% glucose or 4% mannitol. Total RNA was extracted by Isogen RNA isolation kit (Nippon Gene, Tokyo, Japan). RNA (200 μ g) was transcribed to cDNA by incubation with reverse transcription mixture (10 \times TaqMan RT buffer, 25 mM MgCl, deoxy NTPs mixture, random hexamer, RNase inhibitor, multiscribe reverse transcriptase) at 25 $^{\circ}$ C for 10 min and 48 $^{\circ}$ C for 30 min and 95 $^{\circ}$ C for 5 min.

PCR analysis was used for PCR mixture (10 \times Syber PCR buffer, 25 mM MgCl, deoxy NTPs mixture, sense primer, antisense primer, amplitaq gold, amperase UNG). The FN primer was used for rat FN 410-antisense: 5'-TGATCAAAA-CATTTCTCAGC TATTGG-3' and rat FN 410-sense 5'-TTTTGACAA CGGGAAGCATTATCAG-ATAA-3'. Amplifications were done for 40 cycles at 95 $^{\circ}$ C for 15 min and 60 $^{\circ}$ C for 60 min. The DNA products from PCR were analyzed by ABI7700.

Detection of ERK1/2 and p38MAPK by Western blotting

Subconfluent cells grown in 10 cm² dishes were incubated for 0, 30, 60 and 120 min in medium containing 0.5% FBS with 4% glucose or 4% mannitol. After stimulation with high glucose or high mannitol, cells were washed with PBS and lysed with lysis buffer (pH 7.4; 1 M Tris-HCl, 1% Triton X-100, sodium deoxycholate, 10% SDS). Solubilized proteins were centrifuged at 10,000 \times g in 4 $^{\circ}$ C for 30 min.

Extracted proteins were quantified by bicinchoninic acid protein assay. The equal proteins were separated on 10% polyacrylamide gels using SDS-PAGE and transferred to PVDF membranes. The membranes were blocked overnight with PBS containing 2.5% nonfat dry milk and then incubated overnight with primary antibodies (anti-active MAPK polyclonal antibody at 1 : 1000 and anti-active p38 at 1:500) as previously described¹³. After being washed with PBS, the membranes were incubated with secondary antibody were detected by ECL kit (Amersham) as previously described.

Statistical analysis

The results are expressed as mean \pm SEM, unless otherwise stated. These statistical analysis were carried out using Stat View IV on personal computer. Statistical significance ($p < 0.05$) was evaluated using the Student's t-test for normally distributed parameters and the Mann-Whitney U-test followed by Tukey-test for non-normally distributed data obtained from more than two groups. Statistical analyses were performed using the Stat View statistical software package (Stat View 5; SAS Institute, Cary, NC).

Results

Effect of high glucose on cell proliferation

Human PMCs exposed to high glucose showed the previously described antiproliferative and hypertrophic response. To assess the effect of high glucose on DNA synthesis in cultured rat PMCs, we performed a WST-1 cell counting assay. Subconfluent cells grown in 96-well plates were incubated for 48 h in 0.5% FBS medium with high glucose. As shown in Fig. 1, high glucose did not significantly decrease cell proliferation. The osmotic control in-

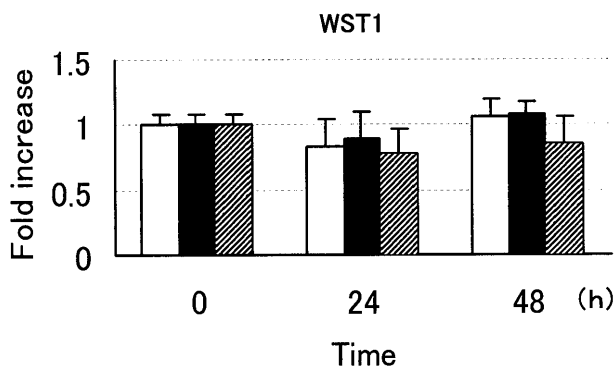


Fig. 1 Time-dependent effects of 4% glucose (■), 4% mannitol (□) and control (DMEM medium + 0.5% FBS, ▨) on cell proliferation detected by WST-1 assay in cultured rat peritoneal mesothelial cells (n=4)

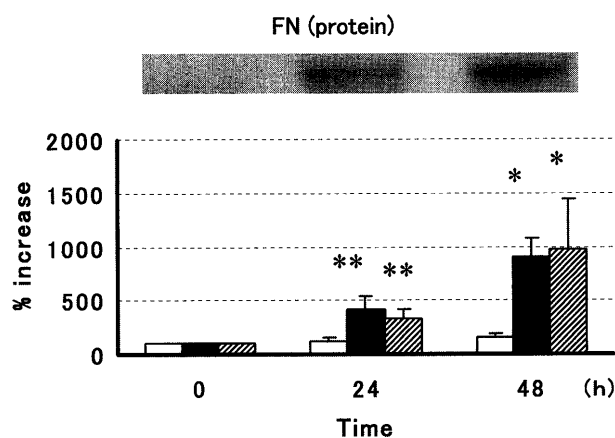


Fig. 2 Time-dependent effects of 4% glucose (■) and 4% mannitol (□), control (DMEM medium + 0.5% FBS, ▨) on fibronectin (FN) production in cultured rat peritoneal mesothelial cells (n=4)
*: $p < 0.05$, **: $p < 0.01$ vs basal levels.

duced a similar but decrease cell proliferation at 24 h.

High glucose increases FN production

The effects of high glucose on the expression of FN proteins were analyzed in rat PMCs. Rat PMCs were treated for 48 h with high glucose. FN protein in the supernatant was quantitated by immunoblotting.

Figure 2 shows that high glucose induced time-dependent increases in FN accumulation. To investigate whether TGF- β is involved in the stimulatory effects of high glucose, the TGF- β concentration in the supernatant was assessed by Western blot.

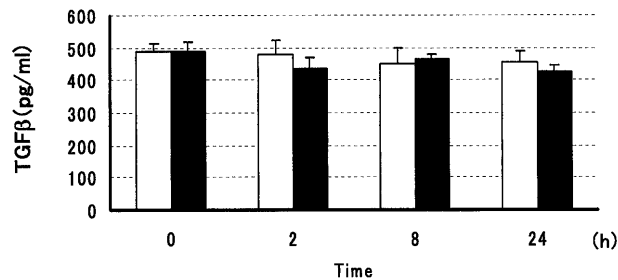


Fig. 3 The TGF- β concentration in the supernatant after stimulation with 4% glucose (■) and control (DMEM medium + 0.5% FBS, □) (n=3)

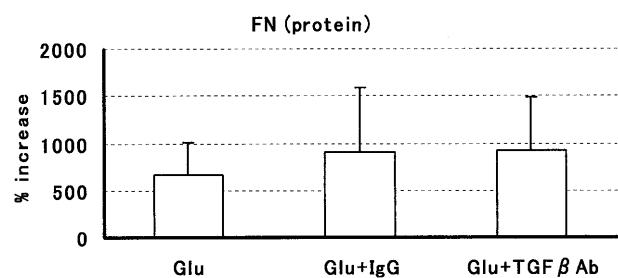


Fig. 4 Effect of anti-TGF- β antibody on fibronectin (FN) production in cultured rat peritoneal mesothelial cells (n=3)

Figure 3 clearly demonstrates that high glucose does not promote TGF- β accumulation in the culture media.

The effect of anti-TGF- β antibody on FN production in rat PMCs in response to 4% glucose is shown in Fig. 4. Anti-TGF- β antibody had no effect on FN levels when compared with FN levels stimulated with control rabbit IgG and medium only (control).

High glucose increases FN mRNA expression

Next, we examined whether high glucose could also lead to FN expression at the mRNA level in rat PMCs. Cells were stimulated for 48 h with high glucose. FN mRNA expression was determined by relative multiplex RT-PCR. As shown in Fig. 5, high glucose resulted in 2-fold increment of FN mRNA expression in rat PMCs. The osmotic control of mannitol induced a similar response, whereas medium only (control) did not.

High glucose activates ERK and p38 MAPK

Initially, we investigated whether exposure to high concentration of glucose altered the p38

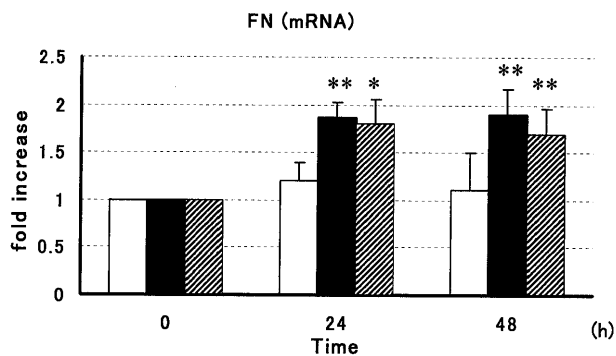


Fig. 5 Time-dependent effects of 4% glucose (■), 4% mannitol (□) and control (DMEM medium + 0.5% FBS, ▨) on fibronectin (FN) mRNA in cultured rat peritoneal mesothelial cells (n = 4)
*: p < 0.05, **: p < 0.01 vs basal levels.

MAPK and ERK, the phosphorylated isoforms, and whether these effects were attributable to an osmotic effect of high glucose. We tested these kinase activities using antibodies specific for the phosphorylated forms of p38 MAPK or ERK.

The immunoblotting of whole-cell extracts showed that significant activation of ERK was detected within 30 min of high glucose stimulation and thus marked activation was observed at 30 min in rat PMCs (Fig. 6A). The level of the maximal activation was about 5-fold compared to basal levels. High glucose condition also induced a time-dependent p38 MAPK activation with a maximal increase after 30 min stimulation.

Moreover, the exposure to high concentration of mannitol had increased ERK activation (Fig. 6B). The level of the maximal activation was about 6-fold compared to basal levels. The increase in phosphorylated MAPKs was associated with the increased in p38 MAPK detected by pan p38 antibody and the ratio of phosphorylated p38/pan p38 was about 0.8. High mannitol condition also induced a time-dependent p38 MAPK activation with a maximal increase after 30 min stimulation.

Finally, we tested whether MAPK inhibitors affect FN mRNA levels induced by high glucose. Both SB203580 (10%) and PD98059 (15%) significantly inhibited high glucose-induced increases in FN mRNA, suggesting that ERK/p38 MAPK induction is directly responsible for FN accumulation (data

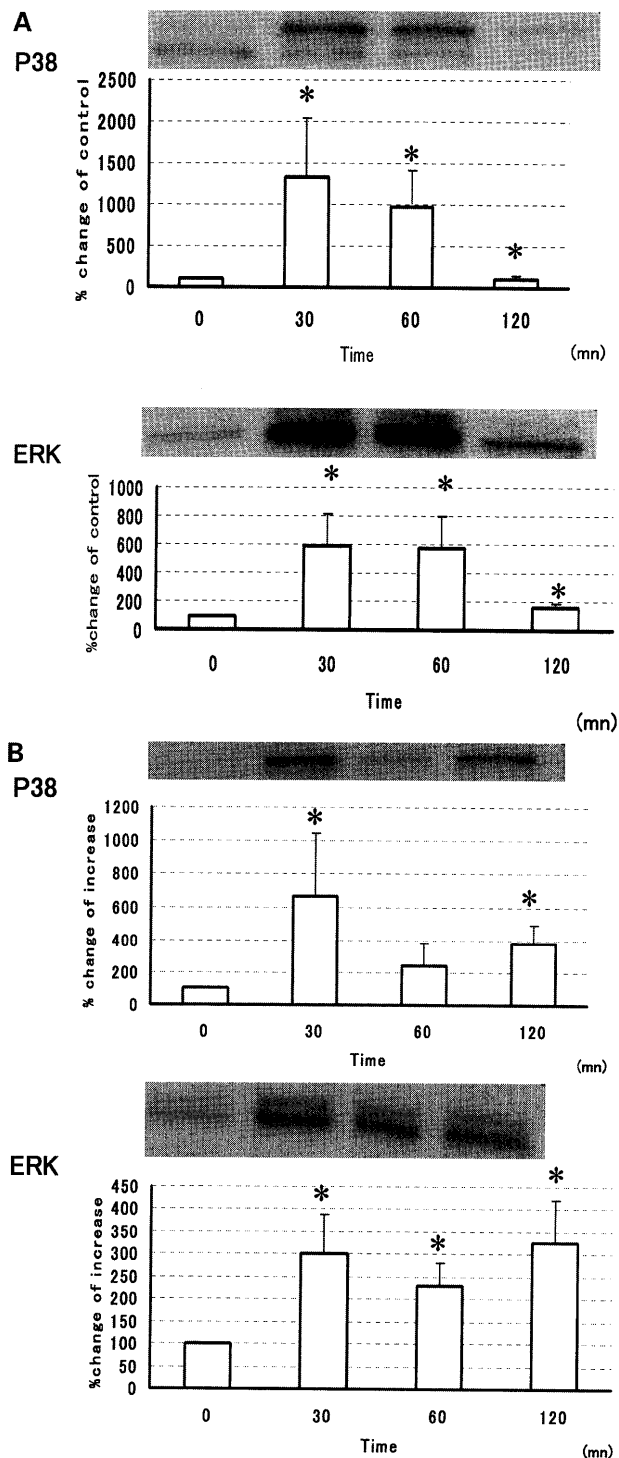


Fig. 6 A: Time-dependent effects of 4% glucose on p38 mitogen-activated protein kinase and extracellular signal-regulated kinase (ERK) in cultured rat peritoneal mesothelial cells

B: Time-dependent effects of 4% mannitol on p38 mitogen-activated protein kinase and ERK in cultured rat peritoneal mesothelial cells (n = 3)

*: p < 0.05 vs basal levels

not shown).

Discussion

Peritoneal fibrosis is one of the most serious complications of long-term CAPD and leads to ultrafiltration failure. It is characterized by a denudation of PMCs from the peritoneum and deposition of ECM proteins^{14)~16)}. PMCs *in vitro* have the capacity to produce a variety of matrix proteins¹⁷⁾¹⁸⁾. Hyperosmolar 4% glucose based dialysate is the most widely used dialysis solution for patients on CAPD, and hence it is important to understand the effects of this solution on the resident peritoneal cells. Several studies have shown that high glucose concentrations enhance FN gene expression in cultured human PMCs^{9)~11)}. Usually, 1.5% or 2.5% glucose is used as an osmotic agent in CAPD therapy. Most highest concentration of glucose was 4% in PD solution and the precise cellular mechanisms modulating the expression of cytokine and ECM induced by high glucose are not fully understood.

The present study has shown that high concentrations of glucose and mannitol caused time-dependently increase in FN accumulation and FN mRNA in cultured rat PMCs. High concentrations of glucose and mannitol also activated the p38 MAPK and ERK in association with the increase in FN protein accumulation. SB203580 (10%) and PD 98059 (15%) significantly inhibited high glucose-induced increases in FN mRNA. These data suggest that p38 MAPK and ERK pathways may play an important role in high glucose-induced FN accumulation. It is considered that high glucose acts as osmotic stimulation in these cells.

TGF- β is thought to be associated with the pathogenesis of peritoneal fibrosis. FN accumulation in human PMCs is through TGF- β secretion from these cells¹⁹⁾²⁰⁾. However, the increase in FN accumulation was not inhibited with anti-TGF- β neutralizing antibody in the present study. In addition, high glucose did not promote TGF- β accumulation in the culture media. These results suggest that FN accumulation induced by glucose is mediated with TGF- β -dependent or TGF- β -independent mechanism when PMC are cultured in various concentration of glucose. Moreover, PMCs obtained from vari-

ous species may show different response to high glucose conditions.

Medcalf et al¹⁹⁾ reported previously that moderate glucose concentration (40 mmol/l) stimulated FN accumulation and FN mRNA in human peritoneal mesothelial cells. This effect was independent of the increase in osmolality of the dialysis solution. The increase in FN accumulation in response to glucose was significantly reduced by anti-TGF- β antibody in these cells. Therefore, they concluded that the pro-fibrotic effect of glucose dialysate on HPMC was mediated through stimulation of TGF- β , which promoted FN gene expression and protein production. However, the cell damage rate assessed by lactate dehydrogenase release from human PMCs was increased in association with FN accumulation, suggesting the increment of TGF- β release from the damaged from human PMCs. The role of TGF- β in the regulation of ECM accumulation in cultured PMCs could be partly different according to experimental condition.

Increased p38 MAPK activity has been demonstrated in various cells in culture under high glucose conditions^{21)~24)}. P38 MAPK can be induced by stress signals such as proinflammatory cytokines²⁵⁾, oxidants²⁶⁾, and hyperosmolarity²⁷⁾. Igarashi et al²¹⁾ have demonstrated that moderate (22 mmol/l) hyperglycemia can activate p38 MAPK by a protein kinase C (PKC)-delta isoform-dependent pathway, but glucose at extremely elevated levels can also activate p38 MAPK by hyperosmolarity via a PKC-independent pathway in cultured rat aorta smooth muscle cells. Moreover, Nakagami et al²⁴⁾ have shown that glucose (25 mmol/l) induces apoptosis in human endothelial cells though phosphorylation of p38 MAPK.

A recent report has shown that p38 MAPK mRNA and activity are increased in diabetic glomeruli, which may contribute to extracellular matrix synthesis²⁸⁾. Since PMCs have the capacity to produce a variety of matrix proteins, MAPK activity might be increased in PMCs by high glucose. Xu et al²⁹⁾ have recently reported that p38 MAPK activity was increased in human PMCs exposed to high glucose, in parallel with increased MAPK

kinase 3/6 activity and decreased MAPK phosphatase-1 expression, resulting in cAMP-responsive element binding protein activation. Therefore, the activated p38 MAPK and ERK pathways might play important role in the pathogenesis of peritoneal fibrosis.

In conclusion, FN production was increased in rat PMCs under 4% glucose conditions, and this increase was associated with an increment in p38 MAPK and ERK activities. Both SB203580 (10%) and PD98059 (15%) significantly inhibited high glucose-induced increases in FN mRNA. The increase in FN accumulation was not inhibited with anti-TGF- β neutralizing antibody. In addition, high glucose did not promote TGF- β accumulation in the culture media. Taken together, these findings suggest that the activation of MAPK signaling pathways in rat PMCs by high glucose may contribute to the pathogenesis of peritoneal fibrosis.

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高糖条件下でのラット腹膜中皮細胞における MAPK の動態

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腹膜硬化症は腹膜透析の重大な合併症であり、誘因の一つとして長期にわたる腹膜への高糖暴露が報告されているが、その発症機序に関しては不明な点が多い。腹膜中皮細胞は透析液による暴露を最も受ける細胞である。今回我々は、培養腹膜中皮細胞に高糖刺激を行い、mitogen-activated protein kinase (MAPK) の動態を評価した。ラット腹膜中皮細胞は 0.5% FCS 含有 DMEM 培地で 24 時間培養した後、4% グルコースと 4% マニトール刺激を行った。細胞増殖能は WST-1 法を用いて測定し、上清中の fibronectin (FN) および MAPK の変化は Western blotting で半定量化した。FN mRNA の変化は RT-PCR で判定した。4% グルコースと 4% マニトール刺激は、いずれも経時的に FN mRNA と FN 蛋白を増加させた。また、4% グルコースと 4% マニトール刺激は、いずれも MAPK のうち extracellular signal-regulated kinase (ERK) と p38 MAPK を活性化し、MAPK 阻害剤で 4% グルコースによる FN mRNA 増加は抑制された。最後に、4% グルコース刺激による FN の増加は、transforming growth factor (TGF)- β 中和抗体の添加で抑制されず、培養上清中への TGF- β の分泌増加を伴わなかった。これらの結果より、高糖刺激による FN の増加には、TGF- β を介さない MAPK の活性化が関与していると考えられた。同濃度のマニトール刺激でも、グルコースと同様に MAPK の活性化や FN の増加が見られたことから、高糖刺激は浸透圧刺激として作用したものと思われた。したがって、高糖刺激により MAPK が活性化された結果として、腹膜中皮細胞による FN の産生が亢進し、腹膜線維化を惹起していることが示唆された。