Diabetologia (1998) 41: 134–140

Diabetologia

Effect of high glucose concentrations on prostacyclin-stimulating factor mRNA expression in cultured aortic smooth muscle cells

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Summary Prostacyclin (PGI_2) is a potent vasoactive prostanoid regulating vascular tone. We recently purified and cloned a PGI_2-stimulating factor (PSF), which stimulates PGI_2 production by vascular endothelial cells (ECs). Previous study demonstrated that PSF is predominantly located in vascular smooth muscle cells (SMCs) and present in serum. PSF may act on vascular ECs to regulate PGI_2 synthesis for maintaining vessel wall homeostasis. Decreased PSF production in the vessel wall may result in an imbalance of prostanoid synthesis, leading to the development of vascular lesions such as diabetic angiopathy. In the present study, to investigate the regulatory mechanisms of PSF gene expression, we examined the effect of high glucose concentrations on PSF mRNA expression in cultured bovine aortic SMCs. Expression of PSF mRNA was significantly decreased to 66 ± 6 % of control value ($p < 0.01$), when the glucose level was raised from 5.5 to 27.8 mmol/l. We also examined the effect of osmolarity on PSF mRNA expression by addition of an appropriate dose of mannitol to the culture medium. We confirmed that high glucose concentration itself reduced the expression of PSF mRNA and glucose had much more effect than the osmolarity control. The expression of PSF mRNA was significantly decreased to 72 ± 5 % of control value ($p < 0.05$) by a protein kinase C (PKC) activator, phorbol-12-myristate-13-acetate (PMA). The decreased expression of PSF mRNA in the presence of high glucose or PMA was restored by co-incubation with a PKC-specific inhibitor (GF109203X). These results suggest that PSF gene expression in vascular SMCs may be decreased via a specific effect of high glucose concentrations. High glucose-induced activation of PKC is suggested to participate partly in the regulation of PSF gene expression. [Diabetologia (1998) 41: 134–140]

Keywords Prostacyclin-stimulating factor, aortic smooth muscle cells, high glucose, protein kinase C.

Diabetic angiopathy is clinically important because it influences the prognosis and quality of life in patients with diabetes mellitus. The precise pathogenesis of diabetic angiopathy is still controversial, but hyperglycaemia is generally accepted as a significant factor in the development of both diabetic microangiopathy and macroangiopathy [1, 2]. The Diabetes Control and Complications Trial (DCCT) [3] recently demonstrated the clinical significance of hyperglycaemia in the development of diabetic angiopathy. Elevated glucose levels may cause a variety of metabolic abnormalities in the vascular cells and tissues of diabetic patients, including activation of the polyol pathway [4], myoinositol depletion [4], reduced Na^+-K^+-ATPase activity [4], formation of advanced glycation end-products [5], generation of superoxide and lipid peroxides [6, 7], alteration of the cellular redox balance [6], increased eicosanoid production [8], and activation of protein kinase C (PKC) [9–16].

Received: 22 July 1997 and in revised form: 19 September 1997

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Abbreviations: SMC, Smooth muscle cell; EC, endothelial cell; PDS, plasma-derived serum; PGI_2, prostacyclin; PSA, PGI_2-stimulating activity; PSF, PGI_2-stimulating factor; PG, prostaglandin; PKC, protein kinase C; PMA, phorbol-2 myristate-13-acetate; GFX, GF109203X.

Abnormalities of vasoactive substances have also been implicated in the pathogenesis of diabetic angiopathy [17, 18]. Prostacyclin (PGI_2) is mainly produced by vascular endothelial cells (ECs) and is a potent vasoactive prostanoid which acts on vascular smooth muscle cells (SMCs) and regulates vascular tone. PGI_2 has a vasodilatory effect and also inhibits platelet aggregation, contributing to the maintenance of vessel wall homeostasis [19–23]. PGI_2 production by vascular ECs has been reported to be decreased in sera from diabetic animals [24]. Our previous studies [24, 25] demonstrated that plasma-derived serum (PDS) contains a PGI_2-stimulating activity (PSA) and that this activity is significantly decreased in the PDS from experimental diabetic animals and patients with non-insulin-dependent diabetes mellitus. Thus, decreased PGI_2 production in diabetic patients may be due partly to a decreased level of PSA.

Recently, we purified and cloned a PGI_2-stimulating factor (PSF) corresponding to PSA from the conditioned medium of cultured human fibroblasts [26, 27]. PSF is a novel peptide with a molecular weight of about 31 000 daltons, which stimulates PGI_2 production by both cultured bovine and human vascular ECs [26–28]. PSF mRNA is extensively expressed in tissues with abundant vascularity such as lung and kidney, which are known to be sites of high prostaglandin (PG) synthesis [29]. Our previous immunohistochemical study also demonstrated the dominant staining for PSF in arterial SMCs [30]. Furthermore, Northern blot analysis demonstrated that PSF mRNA expression was observed dominantly in cultured bovine aortic SMCs [29]. Therefore, it appears that PSF produced mainly by vascular SMCs may act on vascular ECs to regulate PGI_2 synthesis. Decreased PSF production may result in an imbalance of prostanoid synthesis in the vessel wall leading to the development of vascular lesions such as diabetic angiopathy.

The present study was conducted to elucidate the effect of a high glucose concentration on PSF mRNA expression in cultured bovine aortic SMCs. Furthermore, the regulatory mechanisms of PSF mRNA expression by glucose concentration was evaluated in relation to the activation of the diacylglycerol-PKC pathway by hyperglycaemia.

Materials and methods

Culture of smooth muscle cells. Bovine aortic SMCs were obtained and subcultured according to the method described previously [31, 32]. In brief, the intima-media segments of bovine thoracic aorta were cut into small explants (1 mm^2) under sterile conditions. Approximately 20 explants were placed in a culture dish 100 mm in diameter (Falcon 3003; Becton Dickinson, Lincoln Park, N.J., USA) and a cover glass was placed on top. The cells attaching to the dish were cultured in growth medium [Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, N.Y., USA) containing 10 % heat-inactivated fetal calf serum (FCS; Gibco), 100 μU/ml penicillin, and 100 μg/ml streptomycin (Gibco)] in an atmosphere of 95 % air and 5 % CO_2 at 37 °C. The initial outgrowth of SMCs was detected by phase-contrast microscopy after 1–2 weeks of culture. The medium was replaced with fresh medium twice a week. When the cells became confluent, the explants were carefully removed from the dishes with sterilized tweezers. The cells were then routinely subcultured by trypsinization with 0.02 % (w/v) EDTA (Katayama Chemical, Osaka, Japan) containing 0.05 % (w/v) trypsin (Katayama) until sufficient cells for the experiments had been produced. The cultured SMCs showed features that were consistent with the morphological characteristics described by Ross [31]. SMCs from the 10th subculture were used in the present study.

When the cells became almost (80–90 %) confluent in the culture dishes, the medium was exchanged for FCS-free DMEM. After serum-free culture for 24 h, the cells were exposed to a high glucose concentration (FCS-free DMEM with 11.1 or 27.8 mmol/l glucose) or a normal glucose concentration (FCS-free DMEM with 5.5 mmol/l glucose) for 1–4 days with daily medium changes. To evaluate the osmolar effect, the cells were also exposed to iso-osmolarity to 27.8 mmol/l glucose (FCS-free DMEM with 5.5 mmol/l glucose plus 22.3 mmol/l mannitol).

To evaluate the relationship between PKC activation and high glucose-induced alteration of PSF mRNA expression, further studies were conducted as follows. After culture with FCS-free DMEM for 24 h, almost confluent SMCs were exposed to either normal glucose (5.5 mmol/l) or high glucose (27.8 mmol/l) for 3 days. The final 8 h of 3 days incubation was done with or without a PKC activator, phorbol-12-myristate-13-acetate (PMA) (Sigma Chemical Co. St. Louis, Mo., USA) (100 nmol/l), or a specific PKC inhibitor, GF109203X (GFX) (Calbiochem-Novabiochem Corp., La Jolla, Calif., USA) (5 μmol/l). Before adopting 8 h as an incubation time in the present experiment, we performed preliminary studies. We confirmed that the time-dependent effect of PMA and GFX on PSF mRNA expression in the cells was observed and cell damage was not found in the present condition.

We also confirmed no significant increase of lactate dehydrogenase activity in each culture medium and more than 95 % of SMCs showing trypan blue exclusion in each culture dish. Therefore, there was no apparent cell damage in any experimental conditions of the present study.

Northern blot analysis. To prepare RNA, the SMCs were lysed in 1 ml of ISOGEN solution (Wako Chemical, Tokyo, Japan) after washing with phosphate-buffered saline (pH 7.4). Total RNA was extracted according to the method described by Chomczynski and Sacchi [33] and according to the manufacturer's instructions for ISOGEN (Wako). In brief, after extraction with chloroform (Katayama Chemical, Osaka, Japan), precipitation in isopropanol (Katayama), and successive washes with 75 % ethanol (Katayama), the pellet was dissolved in diethylpyrocarbonate (Sigma)-treated water. After quantification by measurement of the absorbance at 260 nm, 20 μg of total RNA was applied to each lane and electrophoresed on 1 % agarose gel (Sigma) containing 2.2 mol/l formaldehyde (Katayama) [34]. The RNA was then transferred to a Hybond N filter (Amersham, Buckinghamshire, UK) and fixed by baking at 80 °C for 2 h. PSF cDNA was obtained according to the method described previously by Yamauchi et al. [27]. The filter was hybridized with α-^{32}P (deoxycytidine 5′-[α-^{32}P] triphosphate; Amersham)-labelled probe (265-bp pair PvuII / SmaI-digested PSF cDNA fragment) using a multiprimer DNA-labelling system (Amersham) at 42 °C for 16 h in a solution

prepared with hybridization buffer tablets (Amersham) containing 50 % formamide (Katayama). Filters were washed with 2 × SSPE (1 × SSPE = 150 mmol/l NaCl, 10 mmol/l NaH_2PO_4, and 1 mmol/l EDTA, pH 7.4) plus 0.1 % SDS at room temperature (three times for 10 min each) and then washed with 1 × SSPE plus 0.1 % SDS at 42 °C for 15 min and exposed to a Fuji imaging plate (Fuji Photo Film Co., Ltd., Tokyo, Japan) at room temperature for 30 min. The radioactivity of PSF mRNA was calculated by a Bio Imaging Analyzer (Fuji Photo Film). The same filter was stripped as described previously [35], and then rehybridized with α-^{32}P-dCTP labelled 28S ribosomal RNA probe (Oncogene, Cambridge, M. A., USA) as the RNA loading control [36]. The radioactivity ratio (PSF mRNA/28S rRNA) was used for comparison among each set of culture conditions.

Statistical analysis. All data are expressed as means ± SEM. Comparisons between two groups were done by the Student's unpaired *t*-test. ANOVA and the Newman-Keuls test were used to assess the statistical significance of multiple comparisons. A p value less than 0.05 was taken to indicate a significant difference.

Results

The effect of high glucose on PSF mRNA expression was evaluated by determining the radioactivity ratio of PSF mRNA to 28S rRNA in bovine aortic SMCs cultured with FCS-free DMEM containing 27.8 mmol/l glucose or 5.5 mmol/l glucose. The PSF mRNA / 28S rRNA ratio was decreased in a time-dependent fashion when vascular SMCs were cultured with 27.8 mmol/l glucose. After 3 days of culture, the ratio was significantly decreased to 66 ± 6 % of control value ($p < 0.01$, $n = 4$) in the vascular SMCs cultured with 27.8 mmol/l glucose as compared to the value on 0 day of culture. However, there was no significant change in the ratio when vascular SMCs were cultured with 5.5 mmol/l glucose (Fig. 1). After 3 days of culture, the PSF mRNA/28S rRNA ratio of vascular SMCs cultured with a high glucose level (27.8 mmol/l) was significantly lower than that of vascular SMCs with a normal glucose level (5.5 mmol/l) (66 ± 6 vs 89 ± 6 % of control value, $n = 4$, $p < 0.01$, Fig. 1). After 4 days of culture, the ratio was also significantly lower in vascular SMCs cultured with high glucose, although the decrease was not significant as compared to that after 3 days of culture (data not shown). Therefore, the PSF mRNA/28S rRNA ratio was determined using vascular SMCs cultured for 3 days in subsequent experiments. As shown in Figure 2 a, the PSF mRNA/28S rRNA ratio in vascular SMCs was decreased by elevated glucose levels in a dose-dependent fashion. When glucose level in the culture medium was elevated to 11.1 mmol/l, the ratio was decreased to 88 ± 6 % of control value. Whereas, there was no significant change in the ratio when the cells were cultured with 5.5 mmol/l glucose. When glucose level was elevated to 27.8 mmol/l, the ratio was significantly decreased to 69 ± 3 % of control value ($n = 4$, $p < 0.01$, Fig. 2 b). Our preliminary study indicated that the decreased ratio was restored to the former level after 3 days of post-incubation with 5.5 mmol/l glucose. Therefore, the effect of glucose on PSF mRNA expression was thought to be reproducible.

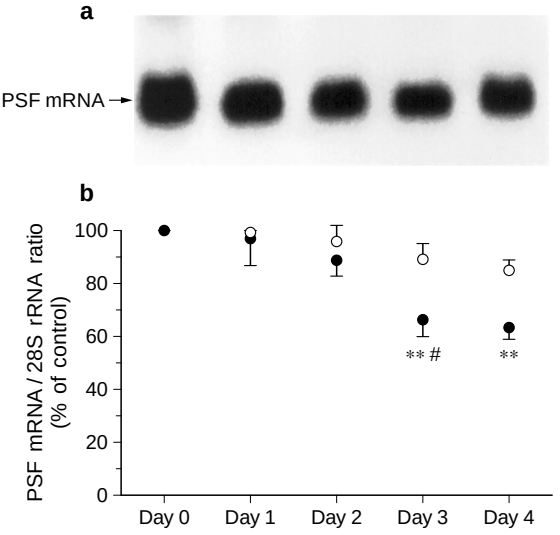

Fig. 1 a, b. Effect of a high glucose concentration on PSF mRNA expression in cultured vascular SMCs. The cells were exposed to FCS-free DMEM containing 5.5 mmol/l (○) or 27.8 mmol/l (●) glucose for 1 to 4 days. **a**) Northern blot analysis was performed using a PSF cDNA probe and the PSF mRNA/28S rRNA ratio was calculated as described in Materials and methods. **b**) Radioactivity ratio was found to decrease significantly in time-dependent fashion. Each experiment was performed in triplicate using 3 culture dishes ($n = 4$). Results are expressed as the mean percentage of the control ± SEM of the radioactivity ratio. ** $p < 0.01$ vs 5.5 mmol/l glucose, # $p < 0.01$ vs day 0

In addition, to elucidate the effect of high osmolarity on PSF mRNA expression, the cells were cultured with iso-osmolar milieu to 27.8 mmol/l glucose (5.5 mmol/l glucose plus 22.3 mmol/l mannitol). The ratio was significantly decreased to 83 ± 5 % of control value ($n = 4$, $p < 0.05$, Fig. 2 b), when the cells were cultured with iso-osmolar milieu to 27.8 mmol/l glucose. However, there was still a significant difference between the ratios cultured with 27.8 mmol/l glucose and cultured with the iso-osmolar milieu ($n = 4$, $p < 0.05$, Fig. 2 b).

The mechanism of the high glucose-induced decrease of PSF mRNA expression in vascular SMCs was examined focusing on glucose-induced PKC activation in the diacylglycerol-PKC pathway. The effect of a specific PKC inhibitor, GFX, on the high glucose-induced decrease of PSF mRNA expression in vascular SMCs was examined (Fig. 3 a). The decreased PSF mRNA/28S rRNA ratio of vascular SMCs cultured with 27.8 mmol/l glucose was increased to 97 ± 5 % of control value ($n = 4$) by addition of GFX to the culture medium (Fig. 3 b). In addi-

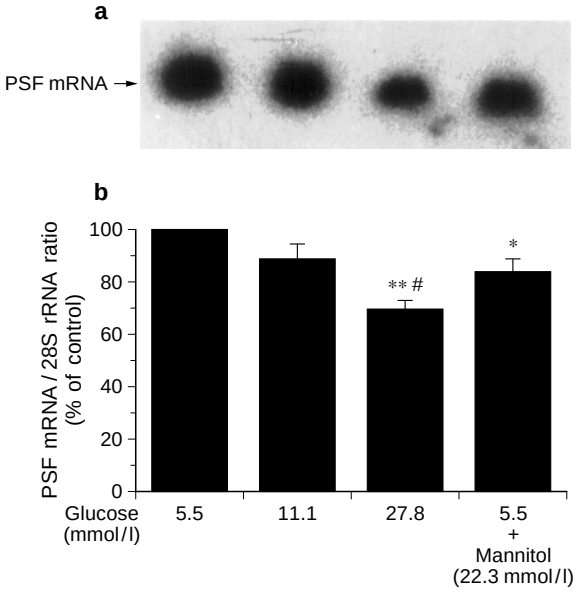

Fig. 2 a, b. Dose-dependent effect of a glucose concentration and osmolar effect on PSF mRNA expression in cultured vascular SMCs. The cells were exposed to FCS-free DMEM containing 5.5 mmol/l glucose, 11.1 mmol/l glucose, 27.8 mmol/l glucose, and 5.5 mmol/l glucose plus 22.3 mmol/l mannitol for 3 days. Northern blot analysis was performed and the PSF mRNA/28S rRNA ratio was calculated as described in Materials and methods. **a**) Representative PSF mRNA message of cultured SMCs in each glucose concentration and glucose plus mannitol medium. **b**) Radioactivity ratios of each culture conditions are shown. Each experiment was performed in triplicate using 3 culture dishes ($n = 4$). Results are expressed as the mean percentage of the control ± SEM of the radioactivity ratio. * $p < 0.05$ vs 5.5 mmol/l glucose, ** $p < 0.01$ vs 5.5 mmol/l glucose, # $p < 0.05$ vs 5.5 mmol/l glucose plus 22.3 mmol/l mannitol

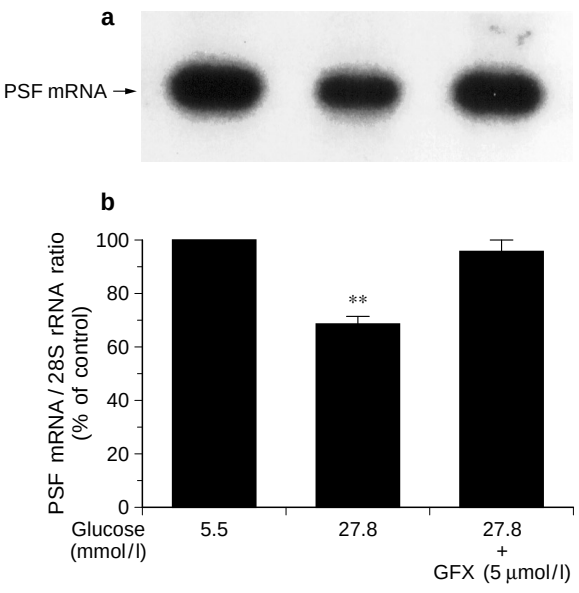

Fig. 3 a, b. Recovery of the high glucose-induced reduction of PSF mRNA expression in cultured vascular SMCs by a specific PKC inhibitor, GFX. The cells were exposed to FCS-free DMEM containing 27.8 mmol/l glucose for 3 days The final 8 h incubation was performed with or without GFX. Northern blot analysis was performed and the PSF mRNA/28S rRNA ratio was calculated as described in Materials and methods. **a**) Representative PSF mRNA message in each culture condition. **b**) Decreased radioactivity ratio of cultured SMCs with high glucose was increased by the addition of GFX. Results are expressed as the mean percentage of the control ± SEM of the PSF mRNA/28S rRNA ratio. ** $p < 0.01$ vs 5.5 mmol/l glucose

tion, the PKC activator, PMA, was found to mimic the effect of high glucose on PSF mRNA expression in vascular SMCs (Fig. 4 a and b). The PSF mRNA/28S rRNA ratio was significantly decreased to 72 ± 5 % of control value ($n = 4$, $p < 0.05$) by addition of PMA to the normal glucose medium (Fig. 4 b). The decreased radioactivity ratio of vascular SMCs in the presence of PMA was restored to 96 ± 7 % of control value ($n = 4$) by addition of GFX (Fig. 4 b).

Discussion

There have been several hypotheses proposed to explain the effect of hyperglycaemia on the development of diabetic vascular complications [1–12]. We have focused on the abnormalities of PG metabolism in diabetic patients and animals [37–43]. In particular, decreased PGI_2 synthesis commonly found in patients and experimental animals with diabetes might influence the progression of vascular damage such as diabetic microangiopathy and macroangiopathy.

The in vivo effect of hyperglycaemia on PG metabolism is complex, because diabetic patients show various kinds of abnormalities connected with circulating metabolic factors. Similarly, the in vitro effects of a high glucose concentration on PGI_2 production by cultured vascular ECs have also been controversial. Aanderud et al. [40] reported that high glucose had no effect on PGI_2 production by cultured human ECs. On the other hand, our previous studies have demonstrated that long-term culture of bovine aortic ECs in high glucose concentration reduces PGI_2 production, suggesting that the high glucose concentration is one of the important factors in the development of vascular dysfunction for PGI_2 synthesis [44].

Recently, we succeeded in cloning the cDNA of a novel bioactive peptide, PGI_2-stimulating factor (PSF) [20]. The major biological in vivo action of PSF, which is mainly produced by vascular SMCs, is thought to stimulate the production of PGI_2 by vascular ECs for the maintenance of vascular homeostasis [28–30]. A decreased PSF expression has been found in the kidneys of diabetic rats and patients with diabetes and atherosclerosis [45, 46]. Therefore, the decreased PSF synthesis in the vessel wall might be a possible pathogenesis of an imbalance of prostanoid

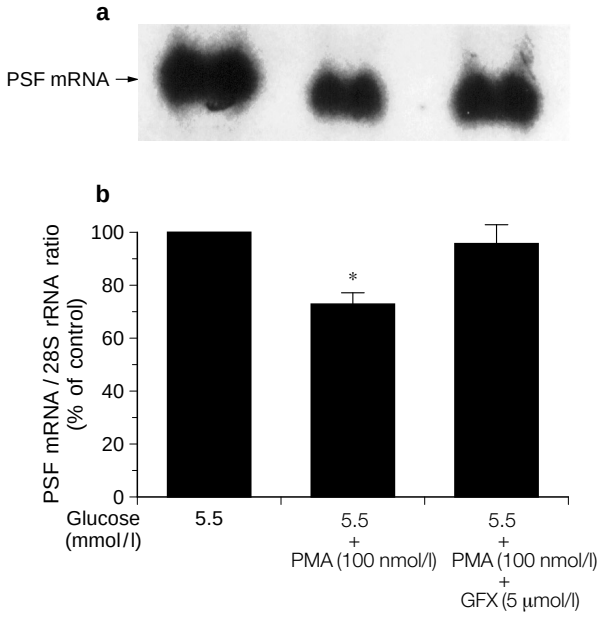

Fig. 4 a, b. Similar effect of a PKC activator, PMA, to high glucose on PSF mRNA expression in cultured vascular SMCs. The cells were exposed to 5.5 mmol/l glucose for 3 days. The final 8 h incubation was performed by addition of PMA, without PMA, with PMA plus GFX. Northern blot analysis was performed and the PSF mRNA/28S rRNA ratio was calculated as described in Materials and methods. **a**) Representative PSF mRNA message in each culture condition. **b**) Decreased radioactivity ratio by addition of PMA was increased by a co-incubation of GFX. Each experiment was performed in triplicate with 3 culture dishes ($n = 4$). Results are expressed as the mean percentages of the control ± SEM of the radioactivity ratio. * $p < 0.05$ vs 5.5 mmol/l glucose

synthesis involving the development of vascular damage such as diabetic angiopathy.

In the present study, we demonstrated that a high glucose concentration decreased the expression of PSF mRNA in cultured bovine aortic SMCs in a time- and dose-dependent fashion. A high osmolarity adjusted by the addition of mannitol also decreased the expression of PSF mRNA as compared with the normal glucose concentration (5.5 mmol/l). However, PSF mRNA level was still significantly lower ($n = 4$, $p < 0.05$) in the cells cultured in high glucose (27.8 mmol/l) than those with iso-osmolarity (5.5 mmol/l glucose plus 22.3 mmol/l mannitol) to high glucose. Although the precise mechanisms of osmolar effect by mannitol on PSF mRNA expression are unknown, it seems that the decreased expression of PSF mRNA in the cells cultured in high glucose may be due partly to an increased osmolarity. The effect of osmolarity was also confirmed using Western blotting and immunohistochemical study under similar experimental conditions [46]. Cell damage due to a high glucose concentration or mannitol was not observed in the present experimental conditions. Therefore, these findings suggest that a high glucose concentration itself reduced the expression of PSF mRNA and glucose had much more effect than the osmolarity control by addition of mannitol. Additionally, our previous Western blot analysis demonstrated that the intensity of specific band corresponding to PSF in cell lysate from high glucose culture was decreased compared to that of normal glucose [46]. Thus, high glucose-induced decrease of PSF mRNA expression might lead to the decreased production of PSF protein in vascular SMCs. We suggest that decreased PSF synthesis by vascular resident cells resulting from a high glucose concentration might be one reason for the decreased PGI_2 synthesis observed in vessels of diabetic patients.

Furthermore, to clarify the regulatory mechanisms of PSF gene expression by a glucose concentration, we focused on the diacylglycerol-PKC pathway. As regards the adverse effect of hyperglycaemia on the vasculature, there were some reports demonstrating a high glucose-induced PKC activation through increased de novo synthesis of diacylglycerol in vascular cells [9–16]. Since PKC modulates vascular permeability, contractility, and cell proliferation, abnormal activation of PKC in the vascular tissues of diabetic patients seems to be one possible cause of the development of diabetic angiopathy. We have previously confirmed the activated PKC levels in vascular SMCs cultured under high glucose conditions or in PMA added culture medium as well as the present experimental condition [11]. We also confirmed the viability of the cells as described in Materials and methods. In the present study, the PKC activator PMA mimicked the effect of high glucose levels on PSF mRNA expression in vascular SMCs. Furthermore, the effects of both high glucose levels and PMA on PSF mRNA expression in vascular SMCs were almost abolished by co-incubation with a PKC-specific inhibitor, GFX. These findings suggest that high glucose-induced PKC activation acts as a possible regulator for PSF gene expression in vascular SMCs. Further studies using other activators and inhibitors in correlation to the diacylglycerol-PKC pathway are needed, because there may be many factors which are altered by a high glucose concentration in this pathway.

Including the osmolar effect, there may be many possible candidates to regulate PSF gene expression. Therefore, to clarify the precise mechanisms of high glucose-induced regulation for PSF gene expression, we should study the relationship between the high glucose level-induced intracellular alterations such as transcription factor levels and abnormalities of PSF expression.

In conclusion, a high glucose concentration itself reduced the PSF gene expression in vascular SMCs and glucose had much more effect than appropriate osmolarity control. Decreased expression of PSF mRNA by high glucose level is suggested to be due

partly to the high glucose-induced PKC activation. The diacylglycerol-PKC pathway was suggested to participate in the regulation of PSF gene expression in part. We hypothesize that decreased expression of PSF mRNA, leading to the reduction of PSF synthesis by vascular resident cells, results in the decreased PGI_2 synthesis in vessel wall. These changes might be an important candidate leading to the development of diabetic angiopathy.

Acknowledgements. This work was supported by a Grant-in-Aid for Scientific Research (No. 01570646) from the Ministry of Education, Science, and Culture of Japan. This work was presented in part by Dr. M. Kunisaki, co-worker, at 56th Annual Meeting and Scientific Sessions of the American Diabetes Association, San Francisco, California, June 1996.

References

1. Mandarino LJ (1992) Current hypotheses for the biochemical basis of diabetic retinopathy. Diabetes Care 15: 1892–1901
2. Shami SK, Chittenden SJ (1991) Microangiopathy in diabetes mellitus: features, complications and investigation. Diabetes Res 17: 157–168
3. The Diabetes Control and Complications Trial Research Group (1993) The effect of intensive treatment of diabetes on development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 320: 977–986
4. Greene DA, Lattimer SA, Sima AF (1987) Sorbitol, phosphoinositides, and sodium-potassium-ATPase in the pathogenesis of diabetic complications. N Engl J Med 316: 599–606
5. Brownlee M, Cerami A, Vlassara H (1989) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 320: 1161–1165
6. Williamson JR, Chang K, Frangos M, et al. (1993) Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 42: 801–813
7. Larkins RG, Dunlop ME (1992) The link between hyperglycaemia and diabetic nephropathy. Diabetologia 35: 499–504
8. Tesfamariam B, Brown ML, Keykin D, Cohen RA (1990) Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta. J Clin Invest 85: 929–932
9. Kunisaki M, Bursell SE, Clermont AC et al. (1995) Vitamin E prevents diabetes-induced abnormal retinal blood flow. Am J Physiol 269:E239–E246
10. Inoguchi T, Battan R, Handler E, Sportsman JR, Haeth W, King GL (1992) Preferential elevation of protein kinase C isoform βII and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. Proc Natl Acad Sci USA 89: 11059–11063
11. Kunisaki M, Bursell SE, Umeda F, Nawata H, King GL (1994) Normalization of diacylglycerol-protein kinase C activation by vitamin E in aorta of diabetic rats and cultured rat smooth muscle cells exposed to elevated glucose levels. Diabetes 43: 1372–1377
12. Craven PA, DeRubertis (1989) Protein kinase C is activated in glomeruli from streptozotocin diabetic rats. J Clin Invest 83: 1667–1675
13. Xia Pu, Kramer RM, King GL (1995) Identification of the mechanism for the inhibition of Na^+, K^+-adenosine triphosphatase by hyperglycemia involving activation of protein kinase C and cytosolic phospholipase A2. J Clin Invest 96: 733–740
14. King GL, Shiba T, Oliver J, Inoguchi T, Bursell SE (1994) Cellular and molecular abnormalities in the vascular endothelium of diabetes mellitus. Ann Rev Med 45: 179–188
15. Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL (1994) Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. Diabetes 43: 1122–1129
16. Williams B, Schrier RW (1993) Glucose-induced protein kinase C activity regulates arachidonic acid release and eicosanoid production by cultured glomerular mesangial cells. J Clin Invest 92: 2889–2896
17. Christensen NJ (1972) Plasma catecholamines in long-term diabetes with and without nephropathy and in hypophysectomised subjects. J Clin Invest 51: 779–787
18. Christlieb AR, Kaldany A, D'Elia JA (1976) Plasma renin activity and hypertension in diabetes mellitus. Diabetes 25: 969–974
19. Moncada S, Gryglewski R, Bunting S, Vane JR (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature 263: 663–665
20. Moncada S, Vane JR (1978) Unstable metabolites of arachidonic acid and their role in haemostasis and thrombosis. Br Med Bull 34: 129–135
21. Gimeno MJ, Sterin-Morda L, Borda ES (1980) Human plasma transforms prostacyclin (PGI_2) into a platelet antiaggregatory substance which contracts isolated bovine coronary arteries. Prostaglandins 9: 907–916
22. Blasko G, Nemesanszky E, Szabo G, Stadler I, Palos LA (1980) The effect of PGI_2 and PGI_2 analogues with increased stability on platelet cAMP content and aggregation. Thromb Res 17: 673–681
23. Watanabe J, Umeda F, Inoguchi T, Ibayashi H (1985) Decreased prostacyclin production and platelet abnormalities in diabetes mellitus. J Japanese Diabetes Soc 28: 1229–1334
24. Inoguchi T, Umeda F, Watanabe J, Ibayashi H (1987) Stimulatory activity on prostacyclin production decreases in sera from streptozotocin-induced diabetic rats. Diabet Res Clin Pract 3: 243–248
25. Inoguchi T, Umeda F, Ono H, Kunisaki M, Watanabe J, Nawata H (1989) Abnormality in prostacyclin-stimulatory activity in sera from diabetics. Metabolism 38: 837–842
26. Yamauchi T, Umeda F, Masakado M, Ono Y, Nawata H (1993) Serum-free conditioned medium of human diploid fibroblast cells contains an activity that stimulates prostacyclin production by cultured bovine aortic endothelial cells. Biochem Mol Biol 31: 65–71
27. Yamauchi T, Umeda F, Masakado M, Isaji M, Mizushima S, Nawata H (1994) Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. Biochem J 303: 591–598
28. Masakado M, Umeda F, Yamauchi T, Ishii H, Ono Y, Nawata H (1994) Human fibroblast cells produce a factor that stimulates prostacyclin synthesis by vascular endothelial cells. Thromb Res 76: 513–524
29. Ono Y, Hashimoto T, Umeda F et al. (1994) Expression of prostacyclin-stimulating factor, a novel factor, in tissues of Wistar rats and in cultured cells. Biochem Biophys Res Commun 202: 1490–1496

30. Masakado M, Umeda F, Takei A, Hashimoto T, Sueishi K, Nawata H (1995) Immunohistochemical localization of a novel peptide, prostacyclin-stimulating factor (PSF), in human tissues. Thromb Haemostas 74(6):1407–1410
31. Ross R (1971) The smooth muscle cells. II. Growth of smooth muscle cells in culture and formation of elastic fibers. J Cell Biol 50: 172–186
32. Sakakibara F, Hotta N, Koh N, Sakamoto N (1993) Effect of high glucose concentration and epalrestat on sorbitol and myo-inositol metabolism in cultured rabbit aortic smooth muscle cells. Diabetes 42: 1594–1600
33. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
34. Maniatis T, Fritsch EF, Sambrook J (1989) Molecular cloning: a Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory, NY
35. Kawakami M, Ishibashi S, Ogawa H, Murase T, Takaku F, Shibata S (1986) Cachectin/TNF as well as interleukin-1 induces prostacyclin synthesis in cultured vascular endothelial cells. Biochem Biophys Res Commun 141: 482–487
36. Bhatia P, Taylor WR, Greenberf H, Wright JA (1994) Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28S-ribosomal RNA gene expression as RNA loading controls for Northern blot analysis of cell lines of varying malignant potential. Anal Biochem 216: 223–226
37. Johnson M, Harrison HE, Raftery AZ (1979) Vascular prostacyclin may be reduced in diabetes in man. Lancet 1: 325–326
38. Silberbauer K, Schernthaner G, Sinzinger H, Piza-Katzer H, Winter M (1979) Decreased vascular prostacyclin in juvenile-onset diabetes. N Eng J Med 300: 366–367
39. Dollery CT, Friedman LA, Hensby CN, et al. (1979) Circulating prostacyclin may be reduced in diabetes. Lancet 2: 1365
40. Aanderud S, Krane K, Nordy A (1985) Influence of glucose, insulin and sera from diabetic patients on prostacyclin synthesis in vitro in cultured human endothelial cells. Diabetologia 28: 641–644
41. Dusting GJ, Moncada S, Vane JR (1977) Disappearance of prostacyclin in the circulation of the dog. Br J Pharmacol 62: 414–415
42. Crall F, Roberts WC (1978) The extramural and intramural coronary arteries in juvenile diabetes mellitus. Am J Med 64: 221–230
43. Sinzinger H, Feigl W, Silberbauer K (1979) Prostacyclin generation in atherosclerotic arteries. Lancet 2: 469
44. Ono H, Umeda F, Inoguchi T, Nawata H (1989) Decrease in insulin binding to aortic endothelial cells cultured with high glucose concentration. Diabet Res Clin Pract 6: 115–119
45. Ono Y, Umeda F, Sekiguchi N, Hashimoto T, Masakado M, Nawata H (1997) Reduced expression of a novel peptide, prostacyclin-stimulating factor (PSF), in the kidneys of streptozotocin-induced diabetic rats. J Diab Comp (in press)
46. Sekiguchi N, Umeda F, Masakado M, Ono Y, Hashimoto T, Nawata H (1997) Immunohistochemical study of prostacyclin-stimulating factor (PSF) in the diabetic and atherosclerotic human coronary artery. Diabetes 46: 1627–1632