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Role of PKC and TGF-β Receptor in Glucose-Induced Proliferation of Smooth Muscle Cells

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The role of protein kinase C (PKC) and transforming growth factor (TGF)-β in the proliferation of vascular smooth muscle cells (SMCs) under a high glucose condition was investigated. [^{3}H]-thymidine incorporation under 20 mM glucose was significantly accelerated compared with that under 5.5 mM glucose, and this increase was inhibited by an anti-TGF-β antibody or a PKC-β specific inhibitor, LY333531. The amount of active and total TGF-β1 in the conditioned media did not differ between 5.5 and 20 mM glucose. However, the expression of TGF-β receptor type II under 20 mM glucose was significantly increased, but that of the TGF-β receptor type I was not. This increased expression of the TGF-β receptor type II was prevented by LY333531. These observations suggest that the increased expression of the TGF-β receptor type II via PKC-β plays an important role in the accelerated proliferation of SMCs under a high glucose condition, leading to the development of diabetic macroangiopathy. © 2001 Academic Press

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Diabetes mellitus accelerates atherosclerosis, resulting in diabetic macroangiopathy, which includes myocardial infarction, angina pectoris, and cerebrovascular disease, and is the major cause of mortality in diabetic patients (1). Various factors that may contribute to the development of diabetic macroangiopathy have been proposed, including hyperglycemia (2), hyperlipidemia (3), hyperinsulinemia (4), increased oxidative stress (5), platelet hyperaggregation (6), and fibrinolytic inbalance. The United Kingdom Prospective Diabetes Study (UKPDS) has demonstrated that strict glycemic control can decrease the incidence of diabetic macroangiopathy, but can not completely prevent it (7). Therefore, it is very important to clarify further the pathogenesis of diabetic macroangiopathy and to establish appropriate therapeutic approaches.

Several theories including polyol pathway hyperactivity (8), enhanced non-enzymatic glycation (9), increased oxidative stress (5), and activation of protein kinase C (PKC) (10) have been proposed to explain the adverse effects of hyperglycemia on the macrovasculature as well as the microvasculature. Among these, glucose-induced PKC activation has recently drawn much attention. Among PKC isoforms, the preferential activation of PKC-β in diabetic tissues such as the aorta, heart, retina, and renal glomeruli (10) or in cells cultured with high glucose such as SMCs, endothelial cells and mesangial cells (11) has been reported, the importance of which has been confirmed by the fact that a PKC-β selective inhibitor, LY333531, improved various deficits in such tissues (12, 13) and cells (14).

The hyperproliferation of vascular SMCs is one of the characteristic features of diabetic macroangiopathy (15). Previous studies have reported that SMCs from diabetic animals or humans have more potential to proliferate than those from normal animals or humans (16). Various growth factors such as PDGF, TGF, and bFGF are considered to be related to the glucose-induced hyperproliferation of SMCs (17). We have previously reported that SMCs cultured with high glucose exhibit accelerated proliferation through the overexpression of the PDGF-β receptor, which is mediated through a polyol pathway sensitive mechanism (18). It has been also reported that the expression of the TGF-β receptor is increased in aortic SMCs of diabetic rats (19), and that the glucose-induced increase in TGF-β1 mRNA is related to the PKC activation in mesangial cells (14). However, the relationship between TGF-β and PKC and the proliferation of SMCs under a high glucose condition has not been investigated well. Therefore, in the present study, the effect of high glucose and PKC inhibition by LY333531 on TGF-β and its receptor was investigated in cultured rat aortic SMCs.

MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources: rat aortic SMCs, A10 cells (ATCC CRL1496) from American Type Culture Collection (Manassas, VA); Dulbecco's modified Eagle me-

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dium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) from Gibco (Grand Island, NY); sodium dodecyl sulfate (SDS) and trichloroacetic acid (TCA) from Sigma (St. Louis, MO); Whatman GF/C filter from Whatman International (Maidstone, UK); polyclonal anti- TGF-β receptor type I antibody, type II antibody, control peptides, and TGF-β receptor type II expressed in E. coli as a 68 kDa polyhistidine tagged fusion protein from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal neutralizing anti-TGF-β antibody from R&D systems (Minneapolis, MN); [γ-^{32}P]ATP and [^{3}H]-thymidine from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, UK); 3 $\times$ 3 cm phosphocellulose papers (P-81) from Whatman International (Maidstone, UK); LY333531 was kindly supplied by Eli Lilly (Indianapolis, IN).

Cell culture. SMCs were grown in DMEM containing 5.5 mM glucose, penicillin (100 U/ml)-streptomycin (100 mg/ml) and 10% FBS, pH 7.40, at 37°C in a humidified 5% CO_2/95% air atmosphere. Third- or fourth-passage cells from the purchase were allowed to grow for three weeks in DMEM containing 5.5 or 20 mM glucose with or without LY333531 (2–200 nM), and were used in the following experiments. Subculture after tripsinization was performed on a weekly basis since the cells became confluent in a week. The medium was replaced with fresh medium every other day.

Determination of DNA synthesis in SMCs. Cells were plated on 6-well plates (9.4 cm^2/well) at a density of 10 $\times$ 10^3 cells/cm^2 and grown in each experimental medium as described above. The proliferation activity of SMCs was assessed by the determination of [^{3}H]-thymidine incorporation into DNA as previously described (18). To analyze the effect of anti-TGF-β antibody on the DNA synthesis, anti-TGF-β antibody (final concentrations: 1 μg/ml, 5 μg/ml, and 10 μg/ml) was added every time the medium was changed in the last week.

Assay of PKC activities. Cells were plated on 12-well plates (3.8 cm^2/well) at a density of 8 $\times$ 10^3 cells/cm^2 and grown in each experimental medium as described above. The cells were washed twice and preincubated for 1 h in serum-free DMEM. PKC activities in SMCs were determined with the method of Heasley and Johnson (20) with minor modifications. Briefly, cells were rinsed twice with 2 ml of DMEM containing 20 mM Hepes (pH 7.4) and then with 2 ml of salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 5.5 mM glucose, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM $CaCl_2$, and 20 mM Hepes, pH 7.4). The cells were preincubated with the salt solution for 10 min at 37°C and incubated for another 15 min in the presence or absence of 100 μM PKC-specific peptide substrate, RKRTLRRL (21), with 50 μg/ml digitonin and 100 μM [γ-^{32}P]ATP (<1500 cpm/pmol). The reaction was terminated with 5% TCA (final concentration). Aliquots of the reaction mixture were spotted on 3 $\times$ 3 cm phosphocellulose papers and washed with three changes of 75 mM phosphoric acid and one change of 75 mM sodium phosphate (pH 7.5). The radioactivity of the phosphorylated substrate was determined by liquid scintillation counting. Protein contents were measured by the BCA method (22) and PKC activities were expressed in pmol/min/mg protein.

TGF-β1 assay. After incubation with each experimental medium for an additional week, the conditioned medium was obtained. Active TGF-β1 was measured using the enzyme-linked immunosorbent assay (ELISA) (TGF-β1 Emax ImmunoAssay System, Promega, Madison, WI). This system is designed for the detection of biologically active TGF-β1 in an antibody sandwich format. To measure total TGF-β1, which is the sum of active TGF-β1 and latent TGF-β1, the samples were acidified by the addition of HCl to give a pH of 2.0 to activate latent TGF-β1, left for 15 min at room temperature, and neutralized with NaOH. The kit allows for the quantification of rat TGF-β1, as indicated in the manufacturer's protocol.

Immunoblot analysis of TGF-β receptor type I and type II protein. After incubation with each experimental medium for an additional week, the cells grown on 150 mm-dishes were washed with ice-cold PBS three times, scraped with a scraper, and sonicated. Cells were then lysed in a buffer containing 50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF at 4°C. After determination of the protein concentrations, samples containing the same amount of protein (20 μg) were electrophoresed on SDS–PAGE (8% acrylamide gel) and transferred to a PVDF membrane. The membrane was blocked overnight with ovalbumin at 4°C and incubated with a polyclonal anti-TGF-β receptor type I or type II antibody overnight at 4°C followed by incubation with an anti-rabbit polyclonal IgG antibody. These antibodies react with rat TGF-β receptor type I or type II (23, 24). Receptor ligand complexes were visualized by using ECL chemiluminesence detection kits (Amersham Pharmacia Biotech UK Limited). Protein expressions were quantified by densitometry. The specificity of immunoreactions was examined by the blocking effect of the control peptide provided by the manufacturer.

RNA preparation and reverse transcription and quantitative real-time polymerase chain reaction (PCR) for analyses of TGF-β receptor type I and type II mRNA. After incubation with each experimental medium for an additional week, the cells grown on 6-well plates were washed three times with ice-cold PBS. Then, total RNA was extracted using an ISOGEN RNA extraction kit (Nippon Gene, Tokyo, Japan). The amount of TGF-β receptor type I and type II mRNA was quantified by the reverse transcription and polymerase chain reaction (RT-PCR) method. Total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (P-E Applied Biosystems, Foster City, CA) and random hexamer. The condition of RT was as follows: 30 min at 42°C and 5 min at 99°C. The synthesized cDNA was quantified by using SYBR Green PCR and RT-PCR Reagents and the Applied Biosystems Prism 7700 (P-E Applied Biosystems) according to the manufacturer's protocol. The primers used here were a forward primer for TGF-β receptor type I (5′-ATTCCCCGAGACAGGCCATTTGTTT-3′), a reverse primer for TGF-β receptor type I (5′-TGCCTCGCCAAACTTCTCCAAACC-3′), a forward primer for TGF-β receptor type II (5′-AGTTCACCTACCAC-GGCTTCACTCTG-3′), a reverse primer for TGF-β receptor type II (5′-CCGTCACTTGGATAATGACCAGCA-3′), a forward primer for β-actin (5′-ACTATCGGCAATGAGCGGTTC-3′), and a reverse primer for β-actin (5′-GCCACCAATCCACACAGAGTA-3′). The expected products are 444, 194, and 287 bp in size, respectively. We analyzed the expression of β-actin mRNA as a housekeeping gene and used it as an inner control. The condition of PCR was as follows: 2 min at 50°C, 10 min at 95°C and 40 cycles of 40 s at 94°C, 60 s at 60°C and 60 s at 72°C.

Statistical analysis. Results are presented as means ± SEM of at least three determinations for a representative experiment. Each experiment was replicated in triplicate. Differences among experimental groups were detected by analysis of variance, and the differences between groups were assessed by Student-Newman-Keuls test. Significance was defined as P value <0.05.

RESULTS

Effects of High Glucose and LY333531 on DNA Synthesis

SMCs cultured with 20 mM glucose demonstrated a significant acceleration of DNA synthesis, which was significantly prevented by LY333531 in a dose dependent fashion (5.5 mM glucose, 12.3 ± 1.50 dpm/mg protein; 20 mM glucose, 22.8 ± 1.10; 20 mM glucose + 20 nM LY333531, 16.9 ± 1.90; 20 mM glucose + 200 nM LY333531, 12.5 ± 0.36) (Fig. 1A).

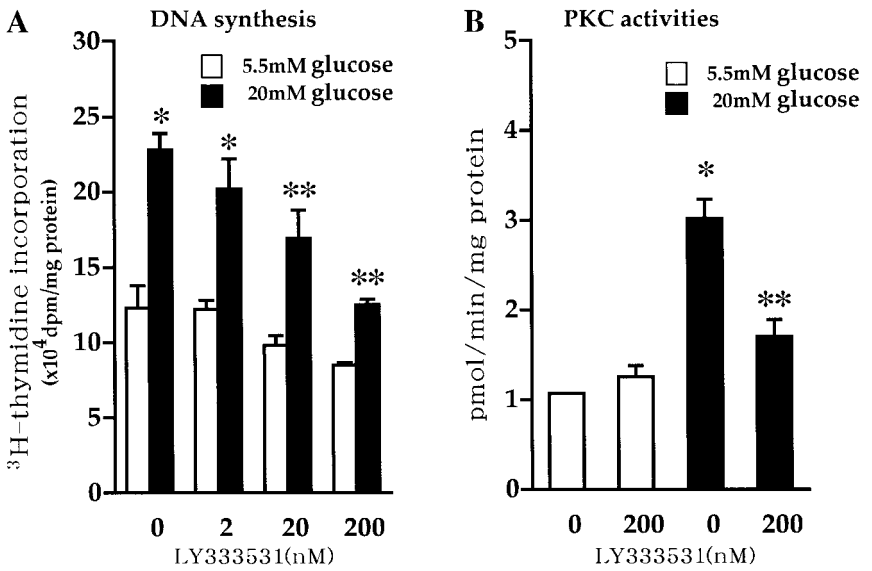

FIG. 1. Effect of LY333531 on DNA synthesis (A) and PKC activities (B) in rat aortic SMCs cultured with 5.5 or 20 mM glucose. Values are means ± SEM. * $P < 0.05$ vs 5.5 mM glucose without LY333531, ** $P < 0.05$ vs 20 mM glucose without LY333531.

Effects of High Glucose and LY333531 on PKC Activities

SMCs cultured with 20 mM glucose demonstrated significantly increased PKC activities compared with those cultured with 5.5 mM glucose, and this increase was significantly prevented by LY333531 (5.5 mM glucose, 1.07 ± 0.13 pmol/min/mg protein; 20 mM glucose, 3.02 ± 0.19, $P < 0.05$; 20 mM glucose + 200 nM LY333531, 1.70 ± 0.12, $P < 0.05$) (Fig. 1B). However, LY333531 had no effects on the PKC activities under the 5.5 mM glucose condition.

Effect of High Glucose and Anti-TGF-β Antibody on DNA Synthesis

DNA synthesis in SMCs measured by [^{3}H]-thymidine incorporation under the 20 mM glucose condition was significantly accelerated compared with that under the 5.5 mM glucose condition (5.5 mM, 28.0 ± 1.41 dpm/mg protein; 20 mM, 44.3 ± 1.61, $P < 0.05$). Anti-TGF-β antibody significantly inhibited DNA synthesis under 20 mM glucose. This inhibitory effect of anti-TGF-β antibody was exerted in a dose dependent fashion (1 μg/ml, 36.5 ± 2.45; 5 μg/ml, 34.3 ± 1.19; 10 μg/ml, 28.2 ± 1.12). There were no significant differences in DNA synthesis between 5.5 mM glucose alone and 5.5 mM glucose with anti-TGF-β antibody (Fig. 2).

Effects of High Glucose and LY333531 on the TGF-β1 Concentrations in the Conditioned Media

There were no significant differences in either the active TGF-β1 or the total TGF-β1 concentrations between 5.5 and 20 mM glucose. LY333531 also had no effects under the 5.5 or 20 mM glucose conditions (Fig. 3).

Effect of High Glucose and LY333531 on TGF-β Receptor Protein Expression

There were no significant differences in the protein expression of TGF-β receptor type I between 5.5 and 20 mM glucose, and LY333531 had no effects under the 20 mM glucose condition (Fig. 4A). As shown in Fig. 5A, on the other hand, the protein expression of TGF-β

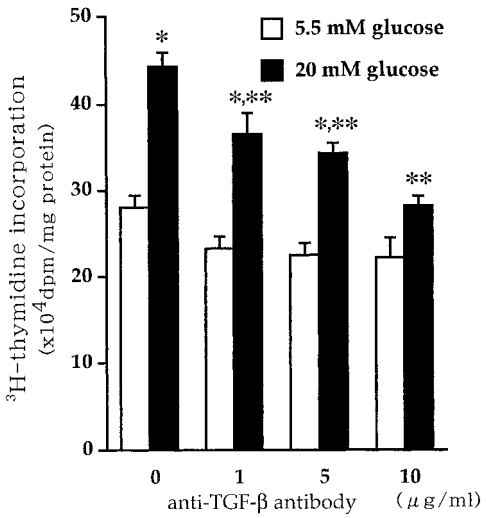

FIG. 2. Effect of anti-TGF-β antibody on DNA synthesis in rat aortic SMCs cultured with 5.5 or 20 mM glucose. Values are means ± SEM. * $P < 0.05$ vs 5.5 mM glucose without anti-TGF-β antibody, ** $P < 0.05$ vs 20 mM glucose without anti-TGF-β antibody.

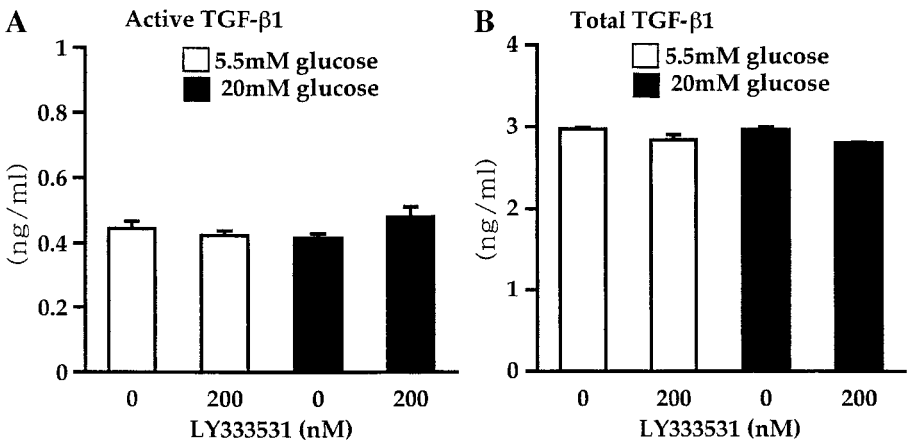

FIG. 3. Effects of LY333531 on the active (A) and total (B) TGF-β1 concentrations in the conditioned media with 5.5 or 20 mM glucose. Values are means ± SEM.

receptor type II was significantly increased in SMCs cultured with 20 mM glucose (153 ± 16%, $P < 0.05$) compared with those cultured with 5.5 mM glucose (100%). LY333531 significantly prevented this overexpression of TGF-β receptor type II protein by 20 mM glucose in a dose dependent fashion (20 nM, 118 ± 10%; 200 nM, 108 ± 3%, $P < 0.05$).

Effect of High Glucose and LY333531 on TGF-β Receptor mRNA Expression

No significant differences were shown in the mRNA expression of TGF-β receptor type I between 5.5 and 20 mM glucose. LY333531 (200 nM) had no effects under the 5.5 or 20 mM glucose condition (Fig. 4B). The mRNA expression of TGF-β receptor type II was significantly increased in SMCs cultured with 20 mM glucose (157 ± 11%, $P < 0.05$) compared with those cultured with 5.5 mM glucose (100%). LY333531 inhibited this increase in the expression of TGF-β receptor type II mRNA by 20 mM glucose in a dose dependent fashion (20 nM, 111 ± 4%; 200 nM, 105 ± 10%, $P < 0.05$). LY333531 (200 nM) had no effects under the 5.5 mM glucose condition (Fig. 5B).

DISCUSSION

In the present study, rat aortic SMCs cultured with 20 mM glucose demonstrated hyperproliferation and increased PKC activities and these deficits were ameliorated by a PKC-β specific inhibitor, LY333531. These observations are consistent with previous studies (11, 12). In addition, in spite of the fact that there

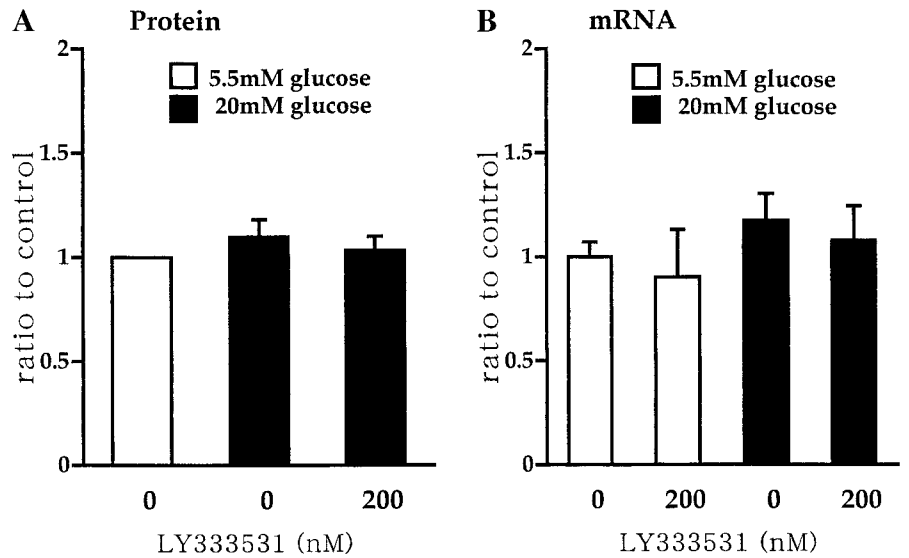

FIG. 4. Effect of 20 mM glucose and LY333531 on the expression of TGF-β receptor type I in rat aortic SMCs. Values are means ± SEM. (A) Immunoblot analysis; (B) real time RT-PCR analysis.

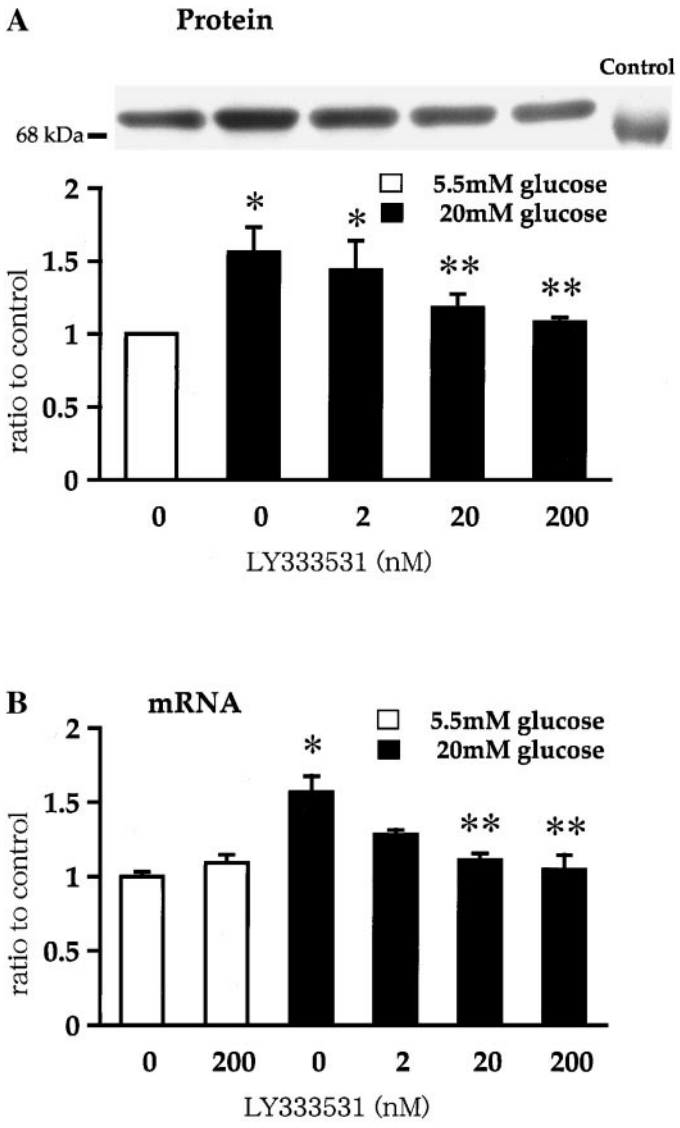

FIG. 5. Effect of 20 mM glucose and LY333531 on the expression of TGF-β receptor type II in rat aortic SMCs. Values are means ± SEM. (A) Immunoblot analysis (control, TGF-β receptor type II expressed in *E. coli* as a 68-kDa polyhistidine tagged fusion protein). (B) Real time RT-PCR analysis. * $P < 0.05$ vs 5.5 mM glucose, ** $P < 0.05$ vs 20 mM glucose without LY333531.

were no differences in the concentrations of active or total TGF-β1 in the conditioned media between 5.5 and 20 mM glucose, the expression of TGF-β receptor type II protein and mRNA was increased by high glucose, and these abnormalities were prevented by LY333531.

Three TGF-β isoforms (TGF-β1, -β2, and -β3) are present in mammals. In A10 cells, TGF-β1 is dominant among the three isoforms in the amount of mRNA (data not shown). TGF-β is a multifunctional cytokine that transmits various cellular responses such as cell proliferation (25) and extracellular matrix production (26). The present study was conducted in the presence of 10% FBS which contains various growth factors such as PDGF, EGF, bFGF, and insulin. Nevertheless, anti-TGF-β antibody normalized the glucose-induced increase in DNA synthesis. The effect of anti-TGF-β antibody was not exerted under the normal glucose condition. These observations suggest that TGF-β plays a crucial role in the hyperproliferation of SMCs in the high glucose condition.

In the vascular tissues, TGF-β1 is contained in endothelial cells, SMCs, macrophages, and platelets, and is secreted from these cells as an inactive latent complex. Before initiating its biological effect, the 25 kDa receptor-binding region must be dissociated from the latency associated peptide (LAP), a process referred to as "biologic activation" (27). The local bioavailability of TGF-β1 may be affected by a variety of factors, including ambient glucose concentration (28), activation of latent TGF-β1 (29), binding of TGF-β1 to matrix proteoglycans (30), and autoinduction of TGF-β1 itself (31). Furthermore, glucose may indirectly increase the active TGF-β1 levels via the release of plasmin or other proteases (29, 32, 33). Therefore, it can be speculated that the active TGF-β1 concentrations in the conditioned media may be elevated by high glucose. However, no increment was observed in this study. The FBS used in the present study contains large amounts of TGF-β1 (34, 35). In addition, TGF-β1 has a short half-life time and the stimulation of TGF-β transcriptional activity by glucose is exerted only for a short period (28). These facts may explain our negative results on the TGF-β concentrations.

The effect of TGF-βs is elicited by the activation of two types of membrane receptors containing serine/threonine kinase activity. In general, all TGF-β isoforms bind and signal primarily through TGF-β receptor type II, which recruits and phosphorylates a dimer of 53 kDa TGF-β receptor type I. Phosphorylation of TGF-β receptor type I activates a kinase, initiating a downstream signal via intracellular proteins. However, TGF-β receptor type I and type II are not always expressed in the same manner. For instance, Ward *et al.* demonstrated that exposure of SMCs to TGF-β1 increased both the expression of TGF-β receptor type I and type II, but that a tyrosine kinase inhibitor, genistein, attenuated the TGF-β1-induced expression of TGF-β receptor type I but not that of TGF-β receptor type II (36). In addition, Kanzaki *et al.* reported that the expression of TGF-β receptor type II was increased in aortic SMCs from streptozotocin diabetic rats but that of TGF-β receptor type I was not (19), which is consistent with our observation in the present study. Therefore, the expression of TGF-β receptor type I and type II may be differently regulated, but the precise differences are not clear.

The glucose-induced overexpression of TGF-β receptor type II in SMCs would be independent of the increased endogenous TGF-β1 bioactivity as reported in mesangial cells (37), and dependent on the transcrip-

tional activation of TGF-β receptor type II by high glucose per se. The TGF-β receptor type II promoter region contains a transcriptional factor AP-1 binding site (38, 39), and it has been reported that high glucose stimulates AP-1 binding and the expression of c-fos and c-jun, components of the AP-1 complex, in cultured mesangial cells (40, 41). In addition, the transcription of these genes, which leads to an increase in AP-1 binding activity, is induced by the PKC activation (42–44). Therefore, it can be hypothesized that high glucose per se induces the overexpression of TGF-β receptor type II through PKC activation, which was confirmed by the present observation that the glucose-induced overexpression of TGF-β receptor type II protein and mRNA was prevented by a PKC inhibitor, LY333531.

In summary, high glucose induced the accelerated proliferation of cultured rat aortic SMCs, which was prevented by anti-TGF-β antibody and was accompanied by the overexpression of TGF-β receptor type II protein and mRNA. These deficits by high glucose were ameliorated by a PKC-β inhibitor, LY333531. These observations suggest that the increased expression of TGF-β receptor type II plays an important role in the accelerated proliferation of SMC by high glucose, leading to the development of diabetic macroangiopathy.

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