Diabetic GK Rat Plasma but Not Normal Wistar Rat Plasma Induces Insulin-Stimulated DNA Synthesis in Primary Cultured Smooth Muscle Cells in GK Rat Aorta

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Received August 26, 1993 Accepted December 22, 1993

ABSTRACT—We investigated the effect of diabetic plasma on insulin-stimulated DNA synthesis in primary cultured aortic smooth muscle cells (SMC) of the GK rat, a model of non-insulin-dependent diabetes mellitus, and compared it with that of Wistar normal rat plasma. We measured the incorporation of 3H-thymidine into cultured SMC. The diabetic plasma (3%) of GK rat, but neither the plasma (3%) of Wistar normal rat nor the plasma (3%) (not containing both insulin-like growth factor-I (IGF-I) and corticosterone) of Wistar hypophysectomized rat induced insulin-stimulated DNA synthesis in GK rat SMC. The responsiveness of SMC to insulin, not to IGF-I, was decreased remarkably by the diabetic state. The diabetic plasma of GK rat remarkably enhanced and the plasma of Wistar hypophysectomized rat weakly enhanced insulin-stimulated DNA synthesis in Wistar normal rat SMC. Corticosterone (20 nM) increased insulin-stimulated DNA synthesis in GK rat SMC but decreased it in Wistar normal rat SMC, using the plasma of Wistar hypophysectomized rat. Corticosterone levels were lower in GK rat plasma than in normal Wistar rat plasma. These results demonstrate that the enhancement of insulin-stimulated DNA synthesis in diabetic SMC by the diabetic plasma of GK rat may be due to neither IGF-I nor corticosterone but due to other factors.

Keywords: Diabetic plasma (GK rat), Insulin, Smooth muscle cell (rat aorta), Corticosterone, DNA synthesis

Diabetes mellitus, predominantly insulin action-deficient diseases, is one of the risk factors for atherosclerosis, which is caused by the abnormal proliferation of intimal smooth muscle cells (SMC) (1). Serum-stimulated proliferation (2) and explant outgrowth (3) of aortic SMC is enhanced in the BB rat, a typical rat model of insulin-dependent diabetes mellitus (IDDM), compared with non-diabetic rats. The effect of insulin on explant outgrowth is less in chronically diabetic SMC of the BB rat than that with acutely diabetic SMC (3). However, the effect of insulin on the proliferation of primary cultured SMC in a model with non-insulin-dependent diabetes mellitus (NIDDM) remains unknown.

The serum isolated from patients with IDDM induces greater proliferation of cultured rabbit SMC than non-diabetic serum (4). Serum contains many factors associated with cell proliferation, including platelet derived growth factor (PDGF), epidermal growth factor, insulin, insulin-like growth factor-I (IGF-I) and glucocorticoid. Insulin is a growth factor (a progression type) different from PDGF (a competence type) (5). Several defined serum-free media contain insulin (6, 7) because insulin is considered to be essential for cell proliferation. The GK (Goto-Kakizaki) rat, established from the Wistar rat, is a non-obese model of spontaneous NIDDM (8, 9). Resting plasma insulin levels in the GK rat are rather higher than those in the normal Wistar rat (10). Low (5.6 mM) and high (20 mM) glucose-stimulated insulin secretions from isolated pancreas are at higher and lower levels in the GK rat than in Wistar normal rat, respectively (11). Streptozocin-induced diabetic swine (12) and rats (13) have lower levels of IGF-I in their sera. The alloxan mouse and streptozocin rat have high levels of plasma corticosterone (14, 15). Plasma corticosterone levels and their effects on cell proliferation of GK rat have not yet been studied.

In this study, we investigated whether corticosterone and IGF-I were involved in the enhancing effects of diabetic plasma on the insulin-stimulated DNA synthesis in
primary cultured aortic SMC of the GK rat.

MATERIALS AND METHODS

Cell culture

Rat aortic SMC in primary culture were prepared by the method described by Chamley et al. (16) with some modifications, as described below. The thoracic aorta of Wistar normal rats or diabetic GK rats was placed into Hanks' solution (pH 7.3; 136.8 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 0.4 mM KH2PO4, 1.3 mM CaCl2, 0.3 mM Na2HPO4, 5.6 mM glucose and 4.2 mM NaHCO3); and blood, fat and connective tissue were removed. The aorta was placed in Hanks' solution containing 1 mg/ml collagenase type I (Sigma, St. Louis, MO, USA) and 0.1-0.8 U/ml elastase type I (Sigma) and incubated for 30 min at 37°C. The adventitia was cleanly stripped off, and the remaining medial tissue was placed in Hanks' solution containing 6 mg/ml collagenase (Wako, Osaka) and 0.25-2.5 U/ml elastase type I for 1.5 hr at 37°C with gentle shaking in order to obtain single cells and small cell clumps. The suspension of cells was centrifuged at 150 x g for 10 min, resuspended with 5% FBS at a density of 3 x 10⁴ cells/0.5 ml/well in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo), which was supplemented with 160 U/ml penicillin G potassium (Banyu Seiyaku, Tokyo), 100 pg/ml streptomycin sulfate (Meiji Seika, Tokyo) and 4.2 mM NaHCO₃ (complete DMEM) with fetal bovine serum (FBS; Whittaker-Bioproducts, Walksville, MA, USA), and aliquots of the suspension were injected into a 24-well plate (16-mm; Corning, New York, NY, USA). The SMC were cultured for 6 days at 37 °C in a humidified atmosphere consisting of 5 % CO₂ in air to obtain nearly confluent growth. The fresh medium was exchanged every other day. After FBS-starvation for 2 days, cultured cells required 12-15 hr to start DNA synthesis following stimulation with 10% FBS (data not shown). The cultured cells were morphologically elongated, grew in a hill and valley pattern at confluence (17), and stained positively with anti-a-smooth muscle actin monoclonal antibody (Progen, Heidelberg, Germany) by immunocytochemistry.

Assay of DNA synthesis in SMC

Serum-deprived SMC were washed with complete DMEM, and then they were incubated with complete DMEM containing 0.037 MBq ³H-thymidine (925 GBq/mmol, continuous labeling; Amersham Japan, Tokyo) plus 5% normal, diabetic or hypophysectomized rat plasma and insulin (porcine; Calbiochem, La Jolla, CA, USA) or IGF-I (human, recombinant; Amersham Japan) for 24 hr at 37°C. Then the ³H-thymidine incorporation was stopped by removing the medium containing ³H-thymidine and by adding 5% trichloroacetic acid. After incubation for 30 min at 4°C, ³H-labeled SMC were washed 3 times with 5% trichloroacetic acid at 4°C and lysed in 0.5 ml of 1 N NaOH. The cell lysate was neutralized, and the radioactivity of the sample was counted with a scintillation counter (LS 3801; Beckman, Fullerton, CA, USA).

Preparation of rat plasma

Normal Wistar and diabetic GK rats were anesthetized with ether and decapitated. The blood was collected at 4°C. Sodium citrate (the final concentration: 0.35%, Wako) was added, and the blood was immediately centrifuged (100 x g, 20 min, 4°C, 10 times). Part of the plasma of Wistar hypophysectomized rats was purchased from the Imamichi Institute for Animal Reproduction (Ibaragi). The plasma was dialyzed at 4°C for 24 hr x 3 times against a phosphate-buffered solution (139 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄; pH 7.5) using molecularporous membranes (cut-off point of M.W. 25,000, Spectra/Por CE; Spectrum Laboratory Products, Los Angeles, CA, USA). As determined by radioimmunoassay, hypophysectomized rat plasma (32 ng/ml) contains much lower IGF-I levels than the plasma of age-matched control rats (Wistar) (500 ng/ml) (5). The supernatant was collected by centrifugation (25,000 x g, 4°C, 30 min) and was immobilized at 56°C for 30 min.

Plasma corticosterone radioimmunoassay

Corticosterone radioimmunoassay was performed using the manufacturer’s protocols (ICN Biomedicals, Costa Mesa, CA, USA) and antibody that is specific for corticosterone (Cosmo Bio, Tokyo). Unlabeled corticosterone (Sigma), and corticosterone [1,2-³H(N)] (1924.0 GBq/mmol; NEN Research Products, Tokyo) were used as a reference standard and a tracer, respectively. Blood (20 μl) was collected from the tail vein of a conscious rat within 2 min. The blood was immediately added to 60 μl of heparinized saline solution (20 U heparin/ml, Sigma) and centrifuged (1880 x g, 10 min, 4°C). The plasma thus obtained was stored −80°C. The sensitivity limits of the radioimmunoassay was 25 pg corticosterone/tube and 10,000 dpm/tube. The interassay variations assessed using standard solutions of 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 ng/ml were 3.3, 2.2, 1.8, 6.3, 7.7, and 6.8%, respectively (n=4).

Animals

Wistar normal (9- to 12-week-old, 290.6±3.1 g, n = 37) and hypophysectomized (10 days) (10-week-old, 244.7±3.0 g, n=4) male rats were purchased from Sankyo Labo Service (Tokyo). GK (Goto-Kakizaki) rats (SPF) were donated by Dr. Ken-ichi Suzuki (Faculty of
Medicine, Tohoku University, Sendai), inbred in our laboratory and used at 9–14 weeks of age (272.2±5.0 g, n=16). Streptozocin-Wistar rats (9-week-old, 168.0±8.0 g, n=3) were prepared by injecting 6-week-old rats with streptozocin (60 mg/kg, i.v., Sigma). Rats were singly housed in a temperature-controlled room with a 12-hr light and 12-hr dark cycle (lights on from 7:00–19:00 hr) and allowed free access to Laboratory chow (CE-2; Clea Japan, Inc., Tokyo) and water. The blood glucose levels of Wistar normal, Wistar hypophysectomized, GK and Wistar streptozocin rats were 119±6 (n=37), 101±9 (n=4), 239±8 (n=16) and 375±22 (n=3) mg/dl, respectively.

Statistical analysis
The significant differences were judged by one-way analysis of variance (ANOVA, Scheffe and Tukey) at P=0.05 or P=0.01.

RESULTS

Diabetic state enhances FBS-stimulated DNA synthesis in primary cultured SMC of GK rat aorta
We investigated the effect of FBS on 3H-thymidine incorporation into the primary cultured SMC of diabetic GK rat (Fig. 1). Both 5% and 10% FBS-stimulated 3H-thymidine incorporations into the SMC of diabetic rat were 1.3-fold greater than those into the SMC of normal Wistar rat at day 10 of culture. Significant enhancement was observed on days 7 to 10 and on days 9 and 10 following 5% and 10% FBS-stimulation, respectively. The proliferation following FBS-stimulation was enhanced in the SMC of GK (diabetic) rat compared with those of Wistar normal rat.

Diabetic plasma enhances insulin-stimulated DNA synthesis in primary cultured SMC of GK rat aorta
To elucidate the effect of diabetic plasma on insulin-stimulated DNA synthesis, we used serum-deprived SMC because the insulin action is greater than that in serum-not-deprived SMC (data not shown). Insulin (0.167–1.67 μM) stimulated 3H-thymidine incorporation into diabetic SMC, with 3% diabetic plasma of GK rat but not with 3% plasma of normal Wistar rat (Fig. 2a). Insulin (16.7 nM–1.67 μM)-stimulated 3H-thymidine incorporation into normal Wistar SMC was enhanced by 3% diabetic plasma of GK rat (Fig. 2b). The extent of stimulation with diabetic plasma was greater in normal SMC than in diabetic SMC. We found that the diabetic plasma of GK rat enhanced insulin-stimulated SMC proliferation, suggesting that the diabetic plasma may lack inhibitory factors against or contain growth factors for the action of insulin.

To exclude the influence of IGF-I and corticosterone, pituitary-dependent factors, on the action of insulin, we used the plasma derived from hypophysectomized Wistar rat. The plasma lacks IGF-I and corticosterone. Insulin plus 3% hypophysectomized plasma stimulated 3H-thymidine incorporation into normal SMC in a concentration-dependent manner (2.02±0.36×10^5 dpm/well/24 hr at 1.67 μM insulin, n=7) (Fig. 2b). However, insulin (1.67 μM) plus 3% hypophysectomized plasma produced only a slight incorporation of 3H-thymidine into diabetic SMC (0.27±0.09×10^5 dpm/well/24 hr, n=3) (Fig. 2a). This suggests that IGF-I, richer in normal rat plasma than in hypophysectomized rat plasma, may compete with insulin, explaining in part the fact that the insulin action of normal SMC with Wistar hypophysectomized plasma was greater than that with Wistar normal plasma (Fig. 2b). However, the low levels of IGF-I in diabetic plasma (12, 13) are not involved in the enhanced insulin action in diabetic SMC.

The responsiveness of diabetic SMC to IGF-I in DNA synthesis were not changed by the diabetic state
As the responsiveness of SMC to insulin with the plasma of hypophysectomized Wistar rat was remarkably suppressed by the diabetic state (Fig. 2, a and b), we investigated the possibility that the change in the sensitivity of
Diabetic SMC to IGF-I was involved in the low sensitivity of diabetic SMC to insulin. IGF-I concentration-dependent (up to 12.9 nM) stimulated 3H-thymidine incorporation into the serum-deprived SMC of normal Wistar rats and GK rats with the plasma of Wistar hypophysectomized rat (Fig. 3). The extent of IGF-I-stimulated 3H-thymidine incorporation was lessened by the diabetic state only at 12.9 pM IGF-I, but was not significantly different at the other concentrations of IGF-I. As the sensitivity of SMC to IGF-I was not remarkably changed by the diabetic state, the responses to IGF-I is not involved in the low sensitivity of diabetic SMC to insulin.

Corticosterone increases insulin-stimulated DNA synthesis in primary cultured SMC from GK rat aorta but decreases it from normal Wistar rat aorta, with hypophysectomized Wistar rat plasma.

We investigated the effect of corticosterone, one of the pituitary-dependent factors, on insulin-stimulated DNA synthesis, using 3% plasma of hypophysectomized Wistar rat. Corticosterone 21-acetate generated in a concentration-dependent manner the action of insulin on diabetic SMC (2.17 x 10^5 dpm/well/24 hr at 1.67 pM insulin plus 20 nM corticosterone 21-acetate, n=3) (Fig. 4a), but decreased the action of insulin on normal Wistar SMC (Fig. 4b).

Corticosterone levels are lower in GK rat plasma than in normal Wistar rat plasma.

Plasma corticosterone levels in tail veins (a circadian rhythm in a day) were compared among normal Wistar, Wistar-streptozocin, GK and hypophysectomized Wistar rats. Diabetic GK rats had lower, but streptozocin-diabetic rats had higher corticosterone levels than normal Wistar rats did (Fig. 5). Hypophysectomized Wistar rats had a zero level of plasma corticosterone (data not shown). When we observed the circadian rhythm of plasma corticosterone levels in the tail veins, GK rats showed a small and flat peak value from 7 p.m. to 3 a.m., whereas Wistar normal rats showed a steep peak value at 7 p.m. In streptozocin-rats, plasma corticosterone levels became higher than those in normal rats, and the circa-
than rhythm became undetectable (Fig. 5).

DISCUSSION

Diabetic rat plasma markedly produced insulin-induced DNA synthesis in diabetic SMC, although the sensitivity of diabetic SMC to insulin was rather suppressed in normal rat plasma and hypophysectomized rat plasma. Diabetic rat plasma was dissimilar to the hypophysectomized rat plasma that is deficient in IGF-I and corticosterone. We have investigated whether the enhancing effect of diabetic plasma on insulin-induced DNA synthesis is associated with the sensitivity of diabetic SMC to or the plasma levels of IGF-I or corticosterone.

Plasma levels of IGF-I are dependent on growth hormone released from the pituitary. However growth hormone does not have any growth promoting activity in Balb/c 3T3 cells (5). Normal rat plasma contains higher levels of IGF-I than hypophysectomized non-diabetic rat plasma (5). IGF-I in normal rat plasma may compete with insulin in the DNA synthesis of normal SMC. Insulin action in normal SMC was enhanced by hypophysectomized rat plasma (low levels of IGF-I) rather than by normal plasma. However, insulin action in both normal and diabetic SMC with hypophysectomized rat plasma was weaker than that with diabetic plasma, although hypophysectomized rat plasma contains low levels of IGF-I. Therefore, diabetic plasma-induced enhancement of the insulin action in diabetic SMC seems not to be caused by the deficiency of IGF-I.

The insulin action on the proliferation of SMC may be mediated by the IGF-I receptor (18). In the present study, with hypophysectomized rat plasma, the action of insulin was markedly suppressed in diabetic SMC, although the sensitivity of IGF-I in diabetic SMC was the same as that in normal SMC. This suggests that insulin action on SMC proliferation does not depend on the IGF-I receptor but depends on the insulin receptor.

Hydrocortisone increases insulin receptor mRNA by increasing the rate of transcription in IM9 human lympho-
cytes (19), suggesting that the number of insulin receptors may be regulated by the level of not only insulin (20, 21) but also that of glucocorticoid. In the present study, corticosterone increased insulin-stimulated DNA synthesis in diabetic SMC, but decreased it in normal SMC. Mean plasma corticosterone concentrations are elevated in streptozotocin (22) and alloxan (23)-induced diabetic rats, but not in GK rats where the plasma corticosterone levels were lower than those of normal Wistar rats. These results suggest that the enhancing factors of growth in diabetic rat plasma are not glucocorticoids.

In conclusion, the action of insulin on diabetic SMC is enhanced by the in vivo environment, diabetic rat plasma, despite the low sensitivity of diabetic SMC to insulin. The enhancement of insulin-stimulated proliferation in diabetic SMC may not be due to corticosterone levels, but may be due to other factors in diabetic plasma, which cause macroangiopathy or atherosclerosis in diabetic plasma in vivo.

Acknowledgments

We thank Dr. Ken-ichi Suzuki and Dr. Yoshio Goto for kindly supplying the GK rats. This work was supported in part by a Scientific Research Grant (No. 63113004) from the Ministry of Education, Science and Culture, Japan.

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