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Dysfunction of aorta involves different patterns of intracellular signaling pathways in diabetic rats

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Abstract

Rat models of insulin-dependent (streptozotocin-induced) and independent (Otsuka Long-Evans Tokushima Fatty (OLETF)) diabetes had sustained and transient increases in blood glucose levels. Over-contraction due to norepinephrine was seen exclusively in streptozotocin rat aorta. Contraction was enhanced under high-glucose conditions in OLETF rats. In order to understand the association between these patterns of changes, total diacylglycerol was measured as a key element of phosphatidylinositol-turnover due to the conversion of some incorporated glucose into diacylglycerol. Streptozotocin rats had enhanced basal diacylglycerol. Both diacylglycerol kinase (metabolic enzyme of diacylglycerol) and total phosphatidylinositol turnover activities also increased on norepinephrine stimulation, independent of extracellular glucose level. On the other hand, diacylglycerol, diacylglycerol kinase and phosphatidylinositol turnover in OLETF rats increased under high glucose conditions in the absence of norepinephrine treatment. These results indicated that diacylglycerol and diacylglycerol kinase-mediated phosphatidylinositol turnover acceleration was influenced by an increase in glucose levels in OLETF rats or by receptor-mediated signals in streptozotocin rats including glucose desensitization based on submaximal incorporation. We suggest that the alteration of vascular dysfunction is induced by different factors in each type of diabetes.

Keywords: Diabetes mellitus; Vascular contraction; Diacylglycerol; Otsuka Long-Evans Tokushima Fatty (OLETF), rat; Streptozotocin

1. Introduction

Dysfunction of vascular smooth muscle contractility in diabetes mellitus has been documented (Ozturk et al., 1996); moreover, it has been suggested that the mechanism(s) of the dysfunction is associated with neuronal factors as well as with intracellular signaling pathways (Abebe et al., 1990). However, the detailed mechanisms are poorly understood.

We previously reported that aortic smooth muscle contractility increased in an insulin-dependent diabetic mellitus model involving streptozotocin-induced diabetic rats (Nobe et al., 1998, 2002). Furthermore, we suggested that this dysfunction, which involves acceleration of phosphatidylinositol-turnover, was mediated by hyper-reactivity of diacylglycerol kinase. On the other hand, little is known regarding the alteration of phosphatidylinositol turnover in non-insulin dependent diabetes mellitus as no useful model had been developed. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat was produced in 1992 as a non-insulin dependent diabetes mellitus model (Kawano et al., 1992). Following several investigations, the OLETF strain of rats was accepted as a suitable model of human type non-insulin dependent diabetes mellitus (Yagi et al., 1997).

The current study used OLETF rats to detect alterations of aortic vascular contractility in non-insulin dependent diabetes mellitus models. In addition, we attempted to assess whether other factors are associated with alteration of phosphatidylinositol turnover. Consequently, it was suggested that alteration of activity indicated different factor-mediated patterns in insulin dependent and non-insulin dependent diabetes mellitus models.

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2. Materials and methods

2.1. Materials

Carrier- and HCl-free radioactive inorganic phosphate ([^{32}P]Pi) was purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). Norepinephrine and streptozotocin were from Sigma (St. Louis, MO, USA). Dioctanoyl-*sn*-glycerol (diC8) was from Avanti Polar Lipids (Alabaster, AL, USA). 1-(2-(5′-carboxyoxazol-2′-yl)-6-amino-benzofuran-5-oxy)-2-(2′-amino-5′-methy l-phenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid penta-acetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR, USA). Biologically synthesized human insulin (insulin) was acquired from Novo Nordisk Pharma (Tokyo, Japan).

2.2. Preparation of experimental diabetic rats

An insulin dependent diabetes mellitus model was induced in Wistar rats (males, 220–260 g body weight) by treatment with streptozotocin (60 mg/kg body weight). Insulin-treated streptozotocin rats were also prepared as described previously (Nobe et al., 1998). Blood glucose levels were determined with a Tidex glucose analyzer (Bayer-Sankyo, Tokyo, Japan). OLETF and Long-Evans Tokushima Otsuka (LETO) rats, the letter as controls for OLETF animals (males, 2 weeks of age) were provided as gifts by Otsuka Pharmaceutical. Four-week-old LETO and OLETF rats were housed at constant room temperature (20 ± 2 °C) with a 12-h light and dark cycle. The rats were fed standard rat chow including 5% fat (Oriental Yeast, Tokyo, Japan). Food and water were available ad libitum and the rats grew satisfactorily. At 28–30 weeks of age, LETO and OLETF rats were used for experiments.

2.3. Oral glucose tolerance test

One week prior to killing, an oral glucose tolerance test was performed. The rats were fasted overnight (12 h) prior to the test. A basal glucose sample was obtained from each animal following administration of a 1 g/kg glucose solution by oral gavage. Blood samples were obtained at 10, 20, 30 and 60 min post gavage (Mathis et al., 2000). The glucose level in each sample was determined with a Tidex glucose analyzer (Bayer-Sankyo).

2.4. Preparation of aortic smooth muscle tissue

The diabetic rats were killed in a CO_2-saturated chamber and aortic tissue was removed. Following resection, fat adhesion and connective tissue were removed from the aorta. Internal surfaces of the aorta were gently rubbed with a wooden rod to eliminate the inhibitory influence of vascular endothelial cells. The tissues were then equilibrated in physiological salt solution (PSS) supplemented with 118 mM NaCl, 5.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.4 mM NaH_2PO_4, 21.4 mM $NaHCO_3$ and 11.1 mM glucose aerated with 95% O_2 and 5% CO_2 at 37 °C.

2.5. Fura-2 loading for measurement of in intracellular calcium concentration ($[ca^{2+}]_i$) and force development

Tissues were initially incubated in tubes filled with 2.0 ml of 3-morpholinopropanesulfonic acid (MOPS)-buffered PSS (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 0.02 mM EDTA, 11.1 mM glucose and 20 mM MOPS, pH7.4) containing 5 μM fura-2/AM dissolved in dimethylsulfoxide (DMSO; final concentration was 1.0%) (Nobe et al., 1993). The non-cytotoxic detergent, pluronic F_{127} (final concentration, 0.5%), was added in order to increase the solubility of fura-2/AM. Loading was done at 18 °C for 4 h. Tissues were subsequently transferred from the loading solution to PSS.

These experiments were performed within 30 min after rinsing. Changes in fluorescence intensities were monitored utilizing a fluorometer specifically designed to measure surface fluorescence of living tissue (CAF-100, Japan Spectroscopic, Tokyo, Japan). The ratio of fluorescence (500 nm), which was attributable to excitation at 340 nm (F_{340}) and at 380 nm (F_{380}) was calculated from successive illumination periods. This value is referred to as $R_{340/380}$. The tissue was positioned horizontally in a temperature-controlled 3-ml organ bath. One end of the tissue was connected to a strain gauge transducer (Type T-7-8-240, Orienteck, Tokyo, Japan) to monitor contractile responses. Aortic contractions were normalized to vascular cross-sectional area with the following equation.

Cross-sectional area (mm^2)=(2 × wet weight (mg))/(1.06 × circumference (mm)), where 1.06 is vascular tissue density (mg/mm^3).

2.6. Assay of diacylglycerol kinase activity

Diacylglycerol kinase activity in tissue was determined via measurement of [^{32}P]dioctanoyl-phosphatidic acid ([^{32}P]diC8-PA) accumulation from diC8 in radioactive inorganic phosphate ([^{32}P]Pi) and diC8-pre-labeled tissues. Simultaneously, the endogenous phosphatidic acid level was determined by measuring [^{32}P]phosphatidic acid ([^{32}P]PA) accumulation in each tissue. For this assay, diC8 was dissolved in chloroform and stored at −40 °C as a stock solution. Prior to use, the stock solution was dried under Ar gas at room temperature and dissolved in a 50% ethanol solution (final concentration of 0.03%). The diC8 ethanol solution was added to PSS supplemented with 2.7 mg/ml bovine serum albmin (diC8 solution). Tissues (25–50 mg wet weight tissue per tube) were initially incubated with 2.22 MBq/ml [^{32}P]Pi in 1 ml of diC8 solution for 90 min at 37 °C, followed by washing (two repetitions) with 10 ml of PSS. The reaction was initiated by the addition of 0.8 ml of PSS containing various compounds at 37 °C. Addition of 3

ml of ice-cold chloroform/methanol/10 M HCl (100:200:1, v/v/v) stopped the reaction. Subsequently, the specimens were homogenized with a glass homogenizer in ice-cold water. One milliliter of chloroform and 1 ml of 0.1 M HCl solution were added to the homogenate; the resulting mixture was shaken vigorously, followed by centrifugation for 20 min at 1000 × *g* to allow phase separation. The lower phase was carefully removed and dried under N_2 gas at room temperature. The residue was then re-dissolved in 40 μl of chloroform for thin layer chromatography (TLC) analysis. The re-dissolved chloroform phase was spotted on high-performance TLC plates (Merck, silica gel 60 with concentrating zone), which were developed with an organic phase consisting of ethyl acetate/isooctane/acetic acid/water (18:10:6:2 by vol.). In this system, diC8-PA (Rf 0.27) and PA (Rf 0.51) were separated from one another and from major phospholipids. The spots corresponding to [^{32}P]diC8-PA and [^{32}P]PA localized by autoradiography (−20 °C, overnight) were scraped; subsequently, radioactivity was counted by liquid scintillation spectrometry (Nobe et al., 1994). Results were expressed as cpm/mg wet weight tissue.

2.7. Measurement of total mass of diacylglycerol

Isolated tissues were incubated in 0.8 ml of PSS solution containing various compounds at 37 °C. The reaction was terminated by the addition of 3 ml of ice-cold chloroform/methanol (1:2 by vol.). The tissues were homogenized in ice-cold water, followed by the addition of 1 ml of chloroform and 1 ml of water to the homogenate. The mixture was shaken vigorously, followed by centrifugation at 1000 × *g*. The lower phase was carefully removed and dried under N_2 gas. Subsequently, the residue was re-dissolved in chloroform (2 μl/mg wet weight tissue) and the chloroform phase was spotted on HPTLC plates. Diacylglycerol was separated, using an organic phase consisting of diethylether/heptane/acetic acid (75:25:1). The plates were dried and stained with 0.03% coomassie brilliant blue solution containing 30% methanol and 100 mM NaCl for 60 min; destaining was done in 30% methanol solution containing 100 mM NaCl (Nakamura and Handa, 1984) for 5 min. The TLC plates were scanned with a Dual-Wavelengh TLC-scanner (Shimadzu CS900, Kyoto, Japan). Total masses of diacylglycerol were determined from each standard curve. Diolein was used as a standard compound for TLC (Nobe et al., 1993).

2.8. Measurement of [^{3}h]myo-inositol incorporation

Isolated tissues were incubated with PSS containing 3.7×10^4 Bq/ml (1 μCi/ml) [^{3}H]*myo*-inositol (Conrad et al., 1991). Unlabeled *myo*-inositol was added to produce a final concentration of 50 μM. The [^{3}H]*myo*-inositol pre-labeled tissue was incubated in the presence or absence of each reagent for 10 min. Norepinephrine (10 μM) was subsequently added for 5 min. The reaction was stopped; tissues were homogenized in 1.0 ml of 0.6 N $HClO_4$. Following centrifugation at 3200 × *g* for 10 min, supernatants were neutralized with 140 μl of 5.0 N KHO. $KClO_4$ was removed via an additional centrifugation step. Radioactivity of the supernatant was determined for [^{3}H].

2.9. Data analysis

Data were expressed as means ± S.E.M. Statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni's *t*-test for multiple comparisons.

3. Results

3.1. Basic factors of experimental diabetic rats

Basic factors were determined in streptozotocin (6–7 weeks after injection) and OLETF (28–30 weeks of ages) rats (Table 1). Significant decreases in body weight and increased resting blood glucose levels were observed in streptozotocin rats. These values were 22.1% and 352.9% relative to Wistar rats, respectively. These changes in streptozotocin rats were partially restored by treatment with insulin (90.3% and 129.6% of Wistar rats, respectively). As a typical phenomenon of diabetes, changes in blood glucose levels following food intake were also investigated. Oral

Table 1
Basic data of experimental diabetic rats

Experimental groups	Animal	Body weight (g)	Blood glucose (mg/ml)	OGTT (60 min after treat.)	Aortic weight (mg wet wt./cm)
Wistar	16	388.6 ± 6.3	122.1 ± 11.2	147.0 ± 26.3	2.78 ± 0.5
STZ rat	10	302.6 ± 10.2[a]	431.0 ± 28.1[a]	468.6 ± 22.6[a]	2.19 ± 0.9
STZ rat + Insulin	5	351.0 ± 9.75[a,b]	158.2 ± 14.6[a,b]	n.d.	2.58 ± 0.7
LETO	12	458.2 ± 18.2	135.3 ± 17.2	148.6 ± 18.6	3.12 ± 1.2
OLETF	14	648.1 ± 21.9[a]	198.3 ± 22.2	418.2 ± 12.6[a,c]	3.22 ± 1.3

Values in Wistar and streptozotocin (STZ) rats were measured at 6–7 weeks following injection. Values in LETO and OLETF rats were measured at 28–30 weeks of age. Oral glucose tolerance test was performed as described in methods. n.d.; not determined.

[a] $P < 0.05$ vs. control rats.

[b] $P < 0.05$ vs. streptozotocin rats.

[c] $P < 0.05$ vs. non-treated blood glucose level.

glucose tolerance test caused no changes in blood glucose levels in Wistar (20.4% of resting level) and streptozotocin (8.7% of resting level) rats. The increased blood glucose levels in streptozotocin rats were maintained during the oral glucose tolerance test. In OLETF rats, body weight increased significantly (141.4% in comparison to that of LETO rats); however, as shown in the table, blood glucose levels increased by 146.5% in OLETF rats in comparison with LETO rats. The oral glucose tolerance test evidenced a significant increase in blood glucose level exclusively in OLETF rats (210.9% of resting level). This increase was time-dependent and the peak value was detected at 60 min after glucose administration. On the other hand, no differences were observed in aortic wet weight in comparisons of the various rat types.

3.2. Norepinephrine-induced changes in $[ca^{2+}]_i$ and force development in diabetic rat aorta

Norepinephrine-induced changes in $[Ca^{2+}]_i$ and force development were measured in fura-2-loaded control and diabetic rat aortae (Fig. 1). Typical records of force development and $R_{340/380}$ are presented as indicators of $[Ca^{2+}]_i$. In both Wistar and streptozotocin rat aortae, the increase in $R_{340/380}$ following 10 μM norepinephrine treatment preceded the increase in force development; moreover, the increases in both parameters were sustained (Fig. 1A). There was a positive correlation between $R_{340/380}$ and force development induced by norepinephrine in Wistar and streptozotocin rats (Fig. 1C left panel). However, for a given value of $R_{340/380}$, force development in streptozotocin

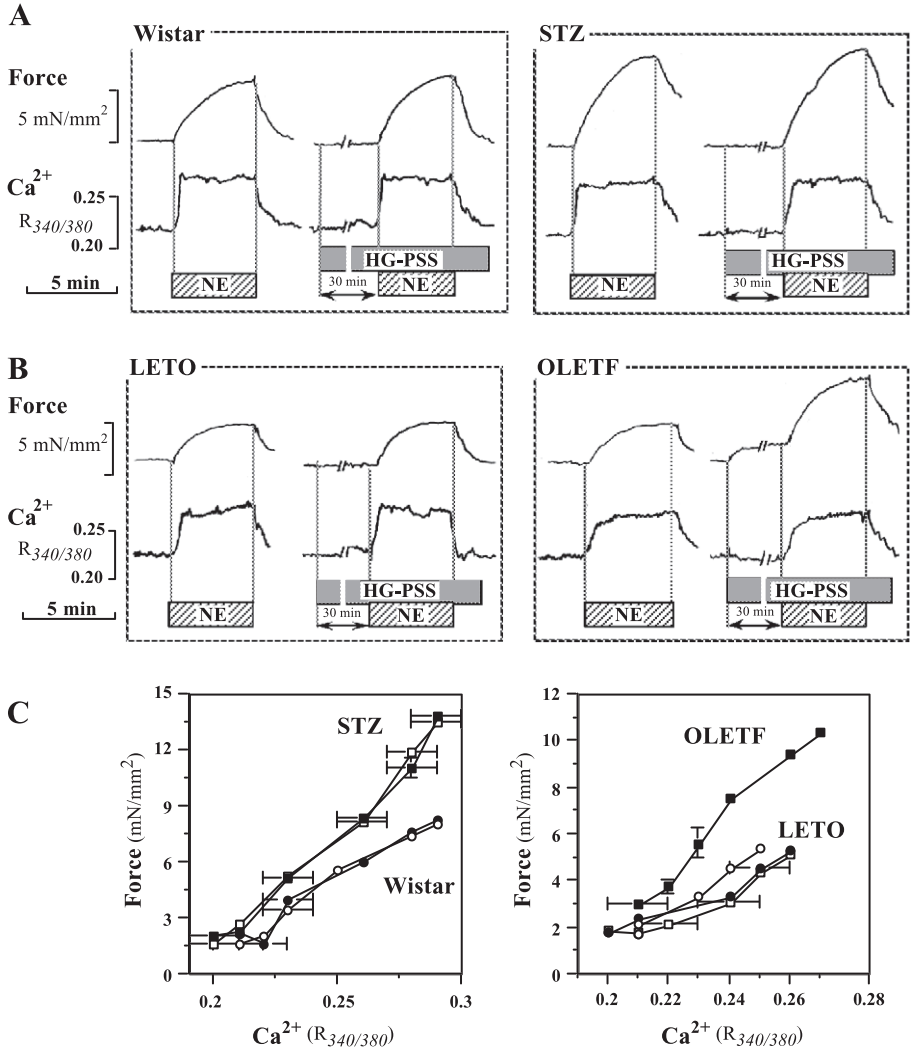

Fig. 1. $[Ca^{2+}]_i$ and force development responses in diabetic rat aorta. Fresh tissues isolated from control and diabetic rats were pre-incubated with 5 μM fura-2/AM at 18 °C for 4 h. Force development in these tissues was recorded. $[Ca^{2+}]_i$, which was determined via measurement of fura-2 fluorescence at 500 nm due to excitation at either 340 or 380 nm, is presented as the ratio of these fluorescence values ($R_{340/380}$). In Wistar and streptozotocin (STZ) rats (A) and LETO and OLETF rats (B), typical values of force development and $R_{340/380}$ stimulated by 10 μM norepinephrine (NE) in normal and high glucose-PSS (30-min pre-incubation; HG-PSS) are shown. The relationships between $R_{340/380}$ and force development in the presence of differing concentrations of norepinephrine (0.3–30 μM) under normal (open) and high glucose-PSS (closed) conditions in each control (circle) and diabetic (square) rat are shown (C). Values were indicated as absolute changes in $R_{340/380}$ and force development (μN/mm^2). Each value represents the mean ± S.E.M. of at least five independent determinations.

rats was consistently greater than that of Wistar rats (176.8% at 10 μM norepinephrine stimulation). Pre-treatment of tissues with 22.2 mM glucose containing PSS (high-glucose-PSS; twofold greater than normal concentration) for 30 min did not affect the norepinephrine-induced increases in $[Ca^{2+}]_i$ and force development in either strain. The enhanced force development in streptozotocin rats remained.

Similar experiments were performed with LETO and OLETF rats (Fig. 1B). Although norepinephrine-induced sustained increases in $[Ca^{2+}]_i$ and force development were detected in LETO and OLETF rats, the maximal responses were similar (4.6 ± 0.1 and 4.3 ± 0.1 mN/mm^2, respectively). In high glucose-PSS, enhancement of force development, in the absence of an effect on $[Ca^{2+}]_i$ response, was observed under basal conditions and upon norepinephrine treatment exclusively in OLETF rats. These increases in high glucose-PSS were 45.5% (basal) and 232.7% (norepinephrine) of the response to norepinephrine in LETO rats. A positive correlation was also detected between $R_{340/380}$ and force development in LETO and OLETF rats (Fig. 1C right panel). This relation in high glucose-PSS-treated OLETF rat aorta was significantly different from the relation in normal PSS.

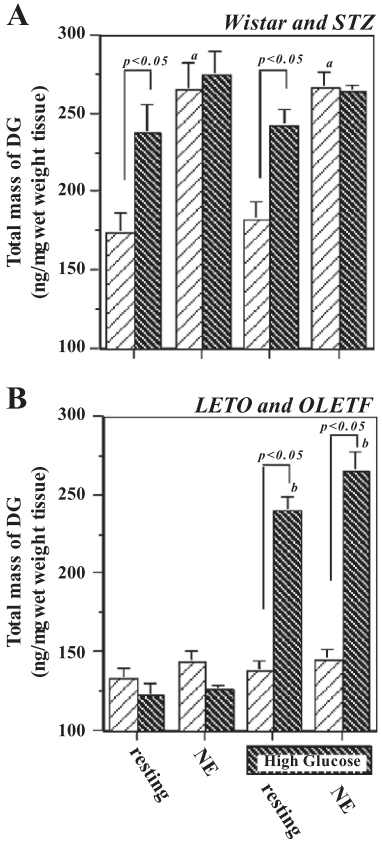

Fig. 2. Alteration of total mass of diacylglycerol in diabetic rat aorta. Fresh aortae isolated from control (Wistar and LETO; ▨) and diabetic (streptozotocin and OLETF; ▩) rats were pre-incubated in normal or high glucose-PSS (High glucose) for 30 min, followed by stimulation in the presence (NE) or absence (resting) of 10 μM norepinephrine for 10 min. These reactions were terminated and total masses of diacylglycerol were quantified as described in Materials and methods. Results are presented as ng/mg wet weight tissue. Each value represents the mean ± S.E.M. of at least five independent determinations. $^aP<0.05$ vs. resting levels and $^bP<0.05$ vs. responses in normal PSS.

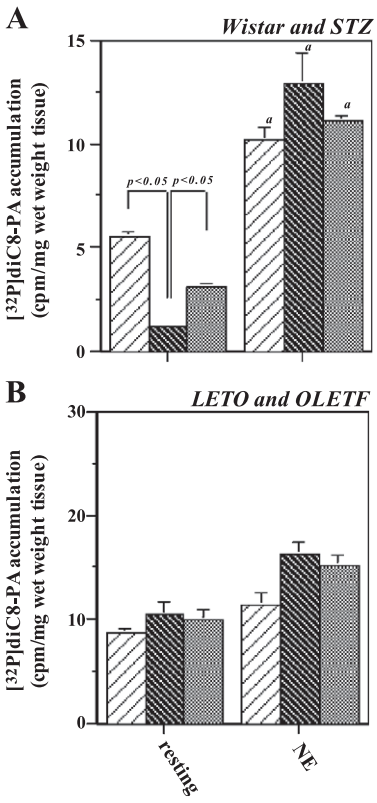

Fig. 3. Effects of norepinephrine treatment on [^{32}P]diC8-PA accumulation in insulin dependent (A) and non-insulin dependent diabetes mellitus (B) rat aortae. Tissues were isolated from control (Wistar and LETO; ▨) and diabetic (streptozotocin and OLETF; ▩) rats; subsequently, [^{32}P]Pi and diC8 were loaded. Some of the diabetic tissue received 30 units/ml insulin for the final 30 min of the incubation (▩). Ten minutes prior to termination of the incubation, 10 μM norepinephrine was added to some of the tissues (NE). [^{32}P]diC8-PA was quantified as described in Materials and methods. Each value represents the mean ± S.E.M. of at least 10 independent determinations. $^aP<0.05$ vs. non-stimulated resting level.

3.3. Norepinephrine-induced changes in total mass of diacylglycerol in diabetic rats

Total mass of diacylglycerol was measured. The resting level of diacylglycerol mass was 173.6 ± 12.7 ng/mg wet weight tissue in Wistar rat aorta (Fig. 2A). Norepinephrine stimulation (10 μM, 10 min) induced a significant increase in diacylglycerol levels (264.7 ± 17.6 ng/mg wet weight tissue). The elevated diacylglycerol returned to its resting level upon PSS rinse of the tissue (data not shown). In streptozotocin rats, the resting level of the total mass of diacylglycerol indicated a significant increase (237.3 ± 18.5 ng/mg wet weight tissue) compared with that of Wistar rats. This value was close to the norepinephrine-induced maximal response in Wistar rats. No significant increases in total mass of diacylglycerol were detected on norepinephrine

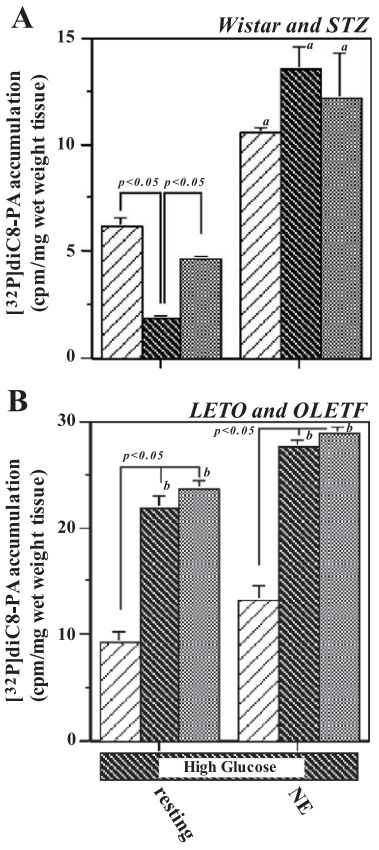

Fig. 4. Effects of high glucose-PSS treatment on norepinephrine-induced [^{32}P]diC8-PA accumulation in insulin dependent (A) and non-insulin dependent diabetes mellitus (B) rat aortae. Tissues were isolated from control (Wistar and LETO; ▨) and diabetic (streptozotocin and OLETF; ▩) rats; subsequently, [^{32}P]Pi and diC8 were loaded. After washing, tissues were incubated in high glucose-PSS at 37 °C for 30 min. Some of the diabetic tissue received 30 units/ml insulin for the final 30 min of the incubation (▩). 10 min prior to termination of incubation, 10 μM norepinephrine was added to some of the tissues (NE). [^{32}P]diC8-PA was quantified as described in Materials and methods. Each value represents the mean ± S.E.M. of at least 10 independent determinations. $^{a}P<0.05$ vs. non-stimulated resting level and $^{b}P<0.05$ vs. values in control rats.

treatment in streptozotocin rats (275.0 ± 14.8 ng/mg wet weight tissue). Treatment of tissues with high glucose-PSS for 30 min did not affect the resting or norepinephrine-stimulated total masses of diacylglycerol in streptozotocin rats.

In LETO and OLETF rats, resting levels of diacylglycerol mass were 133.0 ± 6.7 and 123.0 ± 7.4 ng/mg wet weight tissue, respectively (Fig. 2B); however, no significant norepinephrine-induced increase in the total mass of diacylglycerol was detected in these rats. In high glucose-PSS (at 37 °C for 30 min), however, there was a significant enhancement of the total mass of diacylglycerol in the presence and absence of norepinephrine exclusively in OLETF rats (265.7 ± 11.2 and 240.0 ± 8.0 ng/mg wet weight tissue, respectively). With high glucose-PSS, treatment with norepinephrine did not induce an additional increase in OLETF rats.

3.4. Norepinephrine-induced [^{32}p]dic8-pa accumulation

In Wistar rats, treatment with 10 μM norepinephrine induced an increase in [^{32}P]diC8-PA accumulation (relative to the resting value 5.56 ± 0.23 to 10.28 ± 0.53 cpm/mg wet weight tissue) (Fig. 3A). Although the resting level remained low, norepinephrine induced significant increases in streptozotocin rats (relative to the resting value 1.22 ± 0.07 to 12.89 ± 1.45 cpm/mg wet weight tissue). Preincubation of streptozotocin rat aorta with 30 units/ml insulin led to a significant increase in the resting level (3.11 ± 0.11 cpm/mg wet weight tissue); moreover, the 10 μM norepinephrine-induced maximal response, which was similar to the response in Wistar rats (11.1 ± 0.23 cpm/mg wet weight tissue), was reached.

In LETO rat aorta, the resting level of [^{32}P]diC8-PA accumulation (8.76 ± 0.41cpm/mg wet weight tissue) was not significantly influenced by treatment with 10 μM norepinephrine (11.52 ± 1.01 cpm/mg wet weight tissue) (Fig. 3B). OLETF rat aorta exhibited increases upon norepinephrine treatment relative to the resting values (10.64 ± 0.98 to 16.33 ± 1.11 cpm/mg wet weight tissue). Pre-treatment of OLETF rat aorta with 30 units/ml insulin did not affect the resting and norepinephrine-treated accumulation.

3.5. Effect of high glucose condition on norepinephrine-induced [^{32}p]dic8-pa accumulation

Tissues isolated from normal and diabetic rats were preincubated with high glucose-PSS at 37 °C for 30 min with each reagent added subsequently. The resting and the norepinephrine-treated levels of [^{32}P]diC8-PA accumulation in Wistar, streptozotocin and insulin-pretreated streptozotocin rats were similar to these with normal PSS (Fig. 4A).

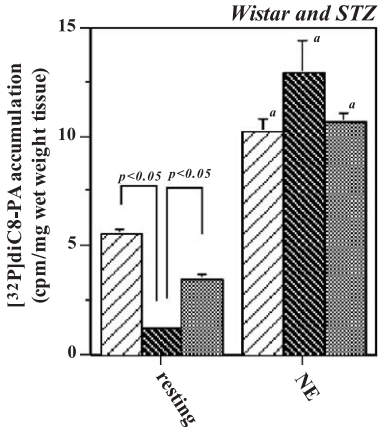

Fig. 5. Effects of in vivo insulin treatment on norepinephrine-induced [^{32}P]diC8-PA accumulation in streptozotocin rat aorta. Aortic smooth muscle was isolated from Wistar (▨), streptozotocin (▩) and insulin-treated streptozotocin (▩) rats. Insulin treatment was described in Materials and methods. Norepinephrine stimulation of the tissues and [^{32}P]diC8-PA accumulation were conducted as described in Fig. 3. Each value represents the mean ± S.E.M. of at least 10 independent determinations. $^{a}P<0.05$ vs. control rats.

However, high glucose-PSS treatment caused a significant increase in the resting level of [^{32}P]diC8-PA in OLETF rats (21.99 ± 0.95 cpm/mg wet weight tissue) (Fig. 4B). Treatment with 10 μM norepinephrine under high glucose conditions led to an increase in [^{32}P]diC8-PA accumulation in OLETF rats (27.73 ± 0.39 cpm/mg wet weight tissue). These norepinephrine-induced changes in [^{32}P]diC8-PA accumulation in OLETF rats under high glucose conditions were not affected by insulin preincubation (28.88 ± 0.65 cpm/mg wet weight tissue).

3.6. Effects of in vivo insulin treatment on [^{32}p]dic8-pa accumulation in streptozotocin rat aorta

Alteration of [^{32}P]diC8-PA accumulation in insulin-treated streptozotocin rat aorta was investigated (Fig. 5). The resting level of [^{32}P]diC8-PA accumulation in insulin-treated streptozotocin rat aorta was 3.54 ± 0.11 cpm/mg wet weight tissue. This value was significantly different from the resting level in streptozotocin rats. Treatment with 10 μM norepinephrine induced significant increases in accumulation (10.72 ± 0.33 cpm/mg wet weight tissue). These values reached levels observed in Wistar rats.

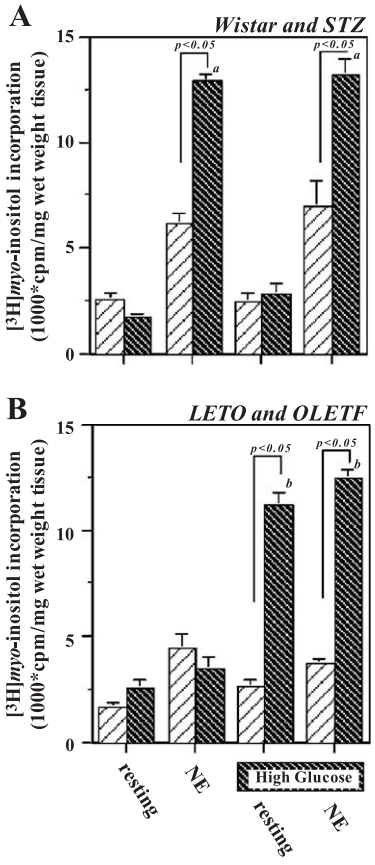

Fig. 6. Effect of high glucose-PSS treatment on norepinephrine-induced [^{3}H]*myo*-inositol incorporation in insulin dependent (A) and non-insulin dependent diabetes mellitus (B) rat aortae. Tissues were isolated from control (Wistar and LETO; ▨) and diabetic (streptozotocin and OLETF; ▩) rats, followed by subsequent incubation of [^{3}H]*myo*-inositol prelabeled tissues in the presence (NE) or absence (resting) of 10 μM norepinephrine for 5 min. Upon termination of treatment, radioactivity of [^{3}H]phosphoinositides was measured as described in Materials and methods. Each value represents the mean ± S.E.M. of at least five independent determinations. [a]$P < 0.05$; values significantly different from resting level. [b]$P < 0.05$; values significantly different from values in normal PSS.

3.7. Norepinephrine-induced increase in [^{3}h]myo-inositol incorporation in diabetic rats

Incorporation of [^{3}H]*myo*-inositol into phosphoinositides was measured in control and diabetic rat aorta to evaluate phosphatidylinositol turnover. In Wistar rat aorta, the resting (non-stimulated) incorporation was 2566.7 ± 260.3 cpm/mg wet weight tissue (Fig. 6A) and 10 μM norepinephrine induced a significant increase in incorporation (6200.0 ± 416.3 cpm/mg wet weight tissue). In streptozotocin rats, the resting level of incorporation was slightly reduced (1766.7 ± 145.3 cpm/mg wet weight tissue) and norepinephrine induced a large increase in incorporation. The value in streptozotocin rats (1290.0 ± 321.5 cpm/mg wet weight tissue) was significantly elevated relative to that in Wistar rats. In order to confirm the effect of high glucose conditions on [^{3}H]*myo*-inositol incorporation, the tissues were pretreated with high glucose-PSS for 30 min. However, no significant effects were detected in resting or in norepinephrine-treated tissues.

Using an identical plan, [^{3}H]*myo*-inositol incorporation was measured in LETO and OLETF rats (Fig. 6B). Resting levels of incorporation were 1733.3 ± 176.4 and 2600.0 ± 378.6 cpm/mg wet weight tissue, respectively. No norepinephrine-induced increases in incorporation were detected; however, high glucose-PSS pretreatment enhanced incorporation significantly only in OLETF rats. Resting and norepinephrine-stimulated values in high glucose-PSS were 11266.7 ± 536.5 and 12466.7 ± 405.5 cpm/mg wet weight tissue, respectively.

4. Discussion

The present study demonstrated that the dysfunction of smooth muscle contractility mediated by phosphatidylinositol turnover activity in aorta isolated from diabetic rats displayed different patterns in insulin dependent and in non-insulin dependent diabetes mellitus models. Our findings suggested that different factors (pattern of blood glucose level and/or receptor stimulation) influenced the alterations mediated by diacylglycerol and diacylglycerol kinase activity.

Dysfunction of tissues in diabetes has been investigated in experimental diabetic models (Bolli, 2001; Russell, 2001). The rat with streptozotocin-induced diabetes is widely used as a typical insulin dependent diabetes mellitus model (Malhotra and Sanghi, 1997; Ozturk et al., 1992; Mahler, 1981). Alterations of contractile and/or relaxant responses of vascular tissue were also reported for this

model (Hashim et al., 2002; Mori et al., 2002; Ozyazgan et al., 2000). We have also documented an enhancement of aorta smooth muscle contractility in streptozotocin rats (Nobe et al., 2002). However, the majority of diabetic patients present with non-insulin dependent diabetes mellitus; unfortunately, proper care for this type of diabetes has not been established. Regarding dysfunction of vascular contractility as a complication of diabetes, the differences between insulin dependent and non-insulin dependent diabetes mellitus are poorly understood. Therefore, we investigated the alteration of aortic contractility in a spontaneous non-insulin dependent diabetes mellitus model; OLETF rats were compared to streptozotocin rats.

The model involving OLETF rats has a typical fatty type of non-insulin dependent diabetes mellitus (Kawano et al., 1992). Moreover, this model is accompanied by human type complications (Yagi et al., 1997), while LETO rats, which are identical in terms of strain to OLETF rats, do not have the fat and the symptoms of non-insulin dependent diabetes mellitus. In this study, the basic aspects of diabetes (body weight, water consumption, volume of urine and food intake) were initially considered in both streptozotocin and OLETF rats. Both models showed typical changes in these basic aspects (Table 1 and further data not shown). These results were consistent with those in previous reports (Watanabe et al., 2001; Kai et al., 2000). Therefore, these prepared experimental diabetic rats were employed as useful models with respect to the understanding of tissue dysfunction.

Blood glucose level is the most typical variable in diabetes; consequently, blood glucose levels were also measured. The values in streptozotocin rats remained at submaximal levels, independent of food intake; in contrast, levels in OLETF rats increased only after food intake (Table 1). Kai et al. (2000) also described a large, transient increase in blood glucose levels in an oral glucose tolerance test (2 g/kg body weight) seen exclusively in OLETF rats. Therefore, blood glucose levels showed different revealed distinct patterns of change between streptozotocin and OLETF rats. We hypothesized that these differences were involved in the dysfunction of vascular tissues in diabetes.

Dysfunction of aortic smooth muscle contractility as a complication was investigated in both diabetic models (Fig. 1). In order to examine the dysfunction, aortic endothelial cells were removed in the present study as alterations of endothelial cell function involving calcium metabolism have been reported by several groups (Kamata et al., 1995; Kamata and Nakajima, 1998; Bishara et al., 2002; Zou et al., 2002). Interaction of aortic smooth muscle from endothelial cell could not be excluded; however, mytogenic dysfunction in non-insulin dependent diabetes mellitus is poorly understood. Therefore, changes in smooth muscle cells were studied in under simple preparation, and characterized by the absence of an effect on endothelial cell regulation. Although enhancement of smooth muscle contractility during norepinephrine stimulation was detected in endothelial cell-replaced aortic smooth muscle tissue, both diabetic models showed different patterns. Enhancement of norepinephrine-induced contractions, including sensitization of intracellular calcium concentration, in streptozotocin rats was independent of extracellular glucose levels (Fig. 1A); moreover, an enhancement was detected only under high glucose conditions in OLETF rats. These patterns of enhancement of aortic smooth muscle contractility overlapped strongly the changes in blood glucose levels. Aortic smooth muscle tissue was isolated from streptozotocin rats that had been treated under high glucose conditions for eight weeks or more. Therefore, it was thought that short-term (30 min) high glucose treatment in vitro had not affected contractility as the tissue was desensitized to an increase in extracellular glucose level. On the other hand, aorta of OLETF rats was treated under high glucose conditions in vivo solely after feeding. Therefore, short-term high glucose treatment in vitro also altered contractility exclusively in OLETF rats. These results indicated that the pattern of blood glucose level might be associated with aortic smooth muscle dysfunction in both diabetic models which findings supported our hypothesis.

The present findings raised questions regarding the mechanism governing the relation between changes in extracellular glucose level and alteration of vascular contraction. We believed that overlaps might be associated with tissue dysfunction mediated by the intracellular signaling pathway; however, details pertaining to a relationship are unknown. phosphatidylinositol turnover is an essential signal transduction system in many types of tissue; consequently, the current investigation focused on this aspect (Itoh et al., 1990). In some key elements of phosphatidylinositol turnover, significant alterations were detected under normal glucose conditions in streptozotocin rat aorta; total mass of diacylglycerol in the resting state was significantly enhanced in comparison with that in Wistar rats (Fig. 2A). This enhancement was submaximal; furthermore, it was close to the peak value in the norepinephrine stimulation. Diacylglycerol kinase activity also indicated hyper-reactivity, which included both inactivation in the resting state and over-activation upon norepinephrine stimulation (Fig. 3A) as previously reported (Nobe et al., 2002). These results suggested that a continuous increase in blood glucose levels in streptozotocin rats induced these alterations in aorta.

Long-term treatment of tissue under high glucose conditions stimulated the increased glucose incorporation in the cells; additionally, a portion of incorporated glucose was converted into diacylglycerol (Inoguchi et al., 2000). This conversion was confirmed in rat aorta in our preliminary trials (data not shown). Moreover, we previously reported that accumulation of diacylglycerol in streptozotocin rat aorta did not affect the consequences of treatment with phospholipase C inhibitor (Nobe et al., 2002). Similar results were also detected in OLETF rats (data not shown). These findings suggested that accumulation of diacylglycerol under high glucose conditions did not involve activation of phospholipase C. Therefore, endogenous diacylglycerol levels had been enhanced at the time the tissue was isolated;

moreover, these levels had not been affected by short-term treatment with normal PSS in vitro.

It was reported that in cultured bovine endothelial cell, long-term high glucose treatment desensitized the responses (Yorek and Dunlap, 1989). Moreover, decreased glucose incorporation was documented in diabetic vascular tissue (Atkins et al., 2001). Therefore, we believed that the enhancement of diacylglycerol levels detected in diabetic aorta might be attributable to activation of a step in the conversion of incorporated glucose to diacylglycerol. Diacylglycerol functions as an endogenous protein kinase C activator; furthermore, protein kinase C serves to activate diacylglycerol kinase (Nobe et al., 1995; Kanoh et al., 1989). It was thought that the resting state of streptozotocin rat aorta in normal PSS functioned in the activation of the protein kinase C-diacylglycerol kinase pathway. Only receptor stimulation might be lacking. Therefore, diacylglycerol kinase over-activation occurred immediately following norepinephrine stimulation. During stimulation under normal glucose conditions, *myo*-inositol incorporation, as a parameter of total phosphatidylinositol turnover activity, was enhanced as well (Fig. 6A). The possibility existed that aorta in streptozotocin rats had been treated under high glucose conditions in vivo and that the dysfunction of contractility involving diacylglycerol, diacylglycerol kinase and phosphatidylinositol turnover activation was maintained in the normal glucose state in vitro. These alterations of diacylglycerol and diacylglycerol kinase activities in streptozotocin rat aorta were not influenced under high glucose-PSS conditions (Figs. 2A and 4A). These findings may have been due to the activation of some factors of phosphatidylinositol turnover prior to each measurement. Alterations of diacylglycerol kinase activity in streptozotocin rats were recovered by insulin treatment in vitro (Figs. 3A and 4A) and in vivo (Fig. 5). These results indicated that the alterations were characteristic responses in the insulin dependent diabetes mellitus models. Our findings suggested that the alteration of aortic smooth muscle contractility in streptozotocin rats was mediated by acceleration of phosphatidylinositol turnover involving both diacylglycerol accumulation and diacylglycerol kinase activation. It was induced by long-term treatment under high glucose conditions in vivo.

On the other hand, blood glucose levels in OLETF rats increased spontaneously following food intake (Table 1); additionally, diacylglycerol levels increased under high glucose conditions (Fig. 2). These results indicated that pathways of glucose incorporation and/or conversion into diacylglycerol were also dependent on the extracellular glucose concentration. In LETO and OLETF rats, differences were not evident in diacylglycerol kinase (Fig. 3B) and total phosphatidylinositol turnover activities (Fig. 6B) between resting levels and levels of norepinephrine-stimulation under normal glucose conditions. This observation indicated that alteration of diacylglycerol kinase and total phosphatidylinositol turnover activities in OLETF rats did not depend strongly on a receptor-mediated signaling pathway. High glucose-PSS treatment led to increases in basal diacylglycerol kinase (Fig. 4B) and total phosphatidylinositol turnover (Fig. 6B) activities exclusively in OLETF rats. These findings suggested that changes in intracellular diacylglycerol level directly influence diacylglycerol kinase and total phosphatidylinositol turnover activities in the absence of receptor stimulation. Data indicating that high glucose-induced over-activation was not affected by insulin treatment (Figs. 3B and 4B) suggested that the glucose-dependent enhancement of diacylglycerol levels did not involve an insulin-sensitive mechanism. Moreover, we believed that over activation of diacylglycerol kinase and total phosphatidylinositol turnover activities in OLETF rats might respond to large, rapid elevations in diacylglycerol level independent of the total amount of diacylglycerol. This transient increase in intracellular diacylglycerol level might induce dysfunction of the aortic smooth muscle contraction mediated by activation of diacylglycerol kinase and total phosphatidylinositol turnover. Increases in basal vascular tonus under high glucose-PSS in OLETF rat aorta were also in favor of this possibility (Fig. 1B).

The present investigation found that the alteration of intracellular signaling systems was influenced by different factors in insulin dependent and non-insulin dependent diabetes mellitus rat aorta. In insulin dependent diabetes mellitus, receptor-mediated signals influenced diacylglycerol kinase and phosphatidylinositol turnover activities based on stable enhancement of diacylglycerol level dependent on maintenance of a high blood glucose level. In non-insulin dependent diabetes mellitus model, total phosphatidylinositol turnover involving diacylglycerol kinase activity was directly influenced by the transient increase in blood glucose level mediated by increased diacylglycerol levels, but not by receptor stimulation. Over activation of phosphatidylinositol turnover in both insulin dependent and non-insulin dependent diabetes mellitus induced an enhancement of aortic smooth muscle contractility mediated by intracellular calcium sensitization. This report is the first to indicate differences in the intracellular signaling system with respect to dysfunction of insulin dependent and non-insulin dependent diabetes mellitus rat aorta.

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