Glucose-dependent enhancement of diabetic bladder contraction is associated with a rho kinase-regulated protein kinase C pathway

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   Atr, atropine sulfate; CCh, carbachol; DG, diacylglycerol; EGTA, glycoletherdiaminetetracetic acid; HG, high-glucose; [$Ca^{2+}$]i, intracellular calcium concentration; PI, phosphatidylinositol; PIP2, phosphatidylinositol-bisphosphate; PKC, protein kinase C; PSS, physiological salt solution; SHR, spontaneously hypertensive rat

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ABSTRACT

Urinary bladder dysfunction, which is one of the most common diabetic complications, is associated with alteration of bladder smooth muscle contraction. However, details regarding the responses under high-glucose (HG) conditions in diabetes are poorly understood. The objective of this study was to identify a relationship between extracellular glucose level and bladder smooth muscle contraction in diabetes.

Bladder smooth muscle tissues were isolated from spontaneously type-II diabetic (ob/ob mouse; 16-20 weeks of age, male) and age-matched control (C57Bl mouse) mice. Carbachol (CCh) induced time- and dose-dependent contractions in ob/ob and C57Bl mice; however, maximal responses differed significantly (14.34±0.32 and 12.69±0.22 mN/mm² following 30 µM CCh treatment, respectively; n=5-8). Pretreatment of bladders under HG conditions (22.2 mM glucose; concentration is twice that of normal glucose for 30 min) led to enhancement of CCh-induced contraction solely in diabetic mice (15.9±0.26 mN/mm²; n=5). Basal extracellular glucose-dependent enhancement of bladder contraction in diabetes was documented initially in this study. The correlation between intracellular calcium concentration and contraction was enhanced only in ob/ob mouse. This enhancement of contraction and total protein kinase C (PKC) activity were inhibited by pretreatment with not only a PKC inhibitor (rottlerin) but also with a rho kinase inhibitor (fasudil). These reagents also suppressed the differences between ob/ob and C57Bl mouse bladder contractions under HG conditions. The data indicated that glucose-dependent enhancement of contraction in diabetic bladder is involved in the activation of the rho kinase and calcium-independent PKC.
pathways. This dysfunction may contribute to bladder complications such as detrusor over-activity and reduced bladder capacity in diabetes.
INTRODUCTION

Currently, it is estimated that diabetes mellitus affects more than 150 million people worldwide (Engelgau et al., 2003); moreover, this number is expected to increase to approximately 300 million by 2025 (Zimmet et al., 2001; Permutt et al., 2005). Diabetic patients display numerous diabetic complications (e.g., retinopathy, nephropathy and neurosis) dependent on the extent of the damage caused by disease progression; furthermore, these individuals are threatened by complications leading to cardiovascular damage (Gillies and Su, 1993; Stockand and Sansom, 1997). Diabetic bladder dysfunction is among the most common and costly consequences of diabetes; dysfunctions include decreased bladder sensation, increased bladder capacity and impaired bladder emptying with resultant increased post-void residual urine. As a result, urinary incontinence in the diabetic patient has been most commonly attributed to over-flow incontinence as a sign of voiding problems. Estimates of the prevalence of dysfunction range from 25-85% (Starer and Libow, 1990; Ueda et al., 1997; Brown et al., 2005). While this condition is not life-threatening, it is associated with several debilitating symptoms, which disrupt patient quality of life. However, therapy for diabetic bladder dysfunctions has not been established.

Assessment of bladder smooth muscle contractility is important in terms of understanding the mechanisms governing diabetic dysfunctions due to the involvement of neuronal dysfunction in diabetes (Poladia and Bauer, 2005). Alterations of contractile responses have been documented and correlations between receptor activity and/or intracellular signaling pathways have been suggested in various types of smooth
muscle tissues isolated from diabetic models (Arun et al., 2005; Schulingkamp et al., 2005; Ma et al., 2008). We have also reported increased vascular smooth muscle contraction in a type-II diabetic mouse model (Nobe et al., 2002; Nobe et al., 2003a). This increased contraction was enhanced under high-glucose (HG) conditions (Nobe et al., 2004). Moreover, spontaneous contraction in diabetic portal vein lacked extracellular glucose-dependency. Therefore, we suggested that both diabetic dysfunction and extracellular glucose-dependency might be responsible for various patterns of changes in each smooth muscle tissue. Increased carbachol (CCh)-induced contraction was demonstrated in bladder smooth muscle in streptozotocin-induced diabetic rat (Yang et al., 2007); in contrast, reduced contraction was noted in combined hypertensive and hyperlipidemic rat (Nobe et al., 2008). Extracellular glucose-dependencies have not been evaluated in these bladder dysfunctions. Based on these findings, we hypothesized that detection of bladder smooth muscle dysfunctions and elucidation of the underlying mechanisms under normal and HG conditions are essential in order to establish novel therapeutic regimes in diabetic patients.

The objective of this study was to identify alterations of contractile responses and intracellular mechanisms in bladder smooth muscle tissue isolated from type-II diabetic mouse models under normal and HG conditions.
Methods

Reagents. Carbachol (CCh), atropine sulfate (Atr) and 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo-(2,3-α)pyrrolo(3,4-+c)-carbazole (Gö6976) were obtained from Sigma-Aldrich (St. Louis, MO). 1-(5-isoquinolinesulfonyl)homopiperazine HCl (fasudil) and (R)-(+)trans-N-(4-pyridyl)-4- (1-aminoethyl)-cyclohexanecarboxamide (Y27632) were procured from Wako Pure Chemical Co. (Osaka, Japan). Calphostin C and mallotoxin (rottlerin) were acquired from Calbiochem-Novabiochem (San Diego, CA). Fura-PE3 acetoxymethyl ester (fura-PE3/AM) and pluronic F127 were purchased from TEF Lab, Inc. (Austin, TX). Ionomycin was procured from Seikagakukogyo Corp. (Tokyo, Japan). 3-Morpholinopropanesulfonic acid (MOPS) was obtained from Dojindo (Kumamoto, Japan). All other reagents, which were of the highest purity, were purchased from Sigma-Aldrich except as noted. Calphostin C and Gö6976 were dissolved in dimethyl sulfoxide (DMSO); no effects of vehicle were noted when total vehicle concentration was 0.03% or less.

Animals. Male C57Bl/6J obese mice (ob/ob) and their lean littermates (+/?; C57Bl/6J) were purchased from Nippon Clea Corp. (Tokyo, Japan) at 16-20 weeks of age. Mice were housed at constant room temperature (20 ± 2 °C) with 12 hr light and dark cycles. Mice were fed standard mouse chow, which included 5% fat (Oriental Yeast Corp., Tokyo, Japan). Food and water were available ad libitum and mice grew satisfactorily. At 18-22 weeks of age, animals were utilized for experiments. Prior to
experiments, water intake and urine volume of each mouse were measured employing metabolic cages (Osawa, Co., Tokyo, Japan). All procedures were performed according to the quilting principles for the care and use of laboratory animals of the Japanese Pharmacological Society.

**Blood Collection and Plasma Biochemical Assays.** Blood samples were obtained from the inferior vena cava under ether anesthesia. The plasma supernatant was utilized for the detection of plasma glucose (P-glucose), phospholipids, free fatty acids, triacylglycerol (TG) and total cholesterols (Total-Cho) in clinical laboratory tests conducted by SRL Inc. (Tokyo, Japan).

**Bladder Smooth Muscle Preparation.** The urinary bladder was isolated from ether-anesthetized mice. Bladders were rinsed in physiological salt solution (PSS); subsequently, fat and connective tissue were removed from both sides. PSS, which was supplemented with 118 mM NaCl, 5.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.4 mM NaH₂PO₄, 21.4 mM NaHCO₃ and 11.1 mM glucose, was aerated with 95% O₂ and 5% CO₂ at 37°C. Prior to measurements, the wet weight of each tissue was determined. To assess tissue responses under HG conditions, pretreatment of the tissue with 22.2 mM glucose (twice the glucose concentration of normal-PSS) contained within PSS (HG-PSS) at 37°C for 30 min was introduced in this study.

**Isometric Force Measurement.** Each tissue was positioned vertically in a temperature-controlled 5 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. Bladder contractions were normalized to cross-sectional area.
with the following equation:

\[
\text{Cross-sectional area (mm}^2) = \frac{\text{wet weight (mg)}}{1.06 \times \text{circumference (mm)}},
\]

where 1.06 represents smooth muscle tissue density (mg/mm\(^3\)).

Resting tension was set at 10 mN, where the tissue length was established in the optimal range for force generation.

**Simultaneous Measurement of Force Development and Intracellular Calcium Concentration ([Ca}^2+\text{])\text{].}** The urinary bladder strips were pre-incubated in test tubes filled with the intracellular calcium indicator loading solution containing 0.3 mL of MOPS-PSS supplemented with 13.3 µM fura-PE3/AM dissolved in DMSO (final concentration was 0.03%). MOPS-PSS, which contained 140 mM NaCl, 4.70 mM KCl, 1.2 mM NaH\(_2\)PO\(_4\), 20.0 mM MOPS, 0.02 mM EDTA, 2.5 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 21.4 mM NaHCO\(_3\) and 11.1 mM glucose, was adjusted with NaOH to pH 7.4 at 37°C. The non-cytotoxic detergent pluronic F\(_{127}\) (final concentration was 0.1%) was added to increase the solubility of fura-PE3/AM. Tissues were incubated at room temperature for 5-6 hr, after which they were rinsed in 37°C PSS for 15 min to remove free dye. Subsequently, bladder strips were mounted in a measurement chamber. One end of the tissue was connected to the force transducer and the other end was fixed to the front-surface fluorimeter (CAM-230, Japan Spectroscopic Co., Tokyo). Resting tension was set at 10 mN, where the tissue length was established to be in the optimal range for force generation. The chamber was placed in the holder (water-jacketed, 37°C) of the CAM-230 dual wavelength spectrofluorimeter. Fluorescence was excited at 340 and 380 nm, and emission was measured at 510 nm. As previously described, the
fluorescence ratio was determined by dividing the emission intensity generated at the 340 nm excitation wavelength by that at 380 nm ($R_{340/380}$). $R_{340/380}$ was calculated and calibrated to absolute values of [Ca$^{2+}$]$_i$ (nM) as reported by Grynkiewicz et al. (Grynkiewicz et al., 1985). Ionomycin (10 µM) and Ca$^{2+}$-EGTA solutions were used to establish $R_{\min}$ and $R_{\max}$; furthermore, Mn$^{2+}$ served to quench the fura-PE3 fluorescence for the background fluorescence of the bladder; the K$_d$ value was set at 224 nM.

**Measurement of PKC Activity.** Fresh tissues were treated under various conditions and then homogenized with a polytrone homogenizer in 0.5 mL of ice-cold solution consisting of 20 mM 3-(N-morpholino)propane sulfonic acid (pH 7.2), 250 mM sucrose, 1 mM dithiothreitol, 1 mM EGTA, 1 µg/mL pepstatin, 1 µg/mL leupeptin and 50 µg/mL trypsin inhibitor (Buffer A). The homogenates were centrifuged (1,000xg for 5 min) to remove the nuclei. Supernatants were decanted and pellets were washed once with Buffer B (sucrose-free Buffer A). The combined supernatants were centrifuged a second time (2,000xg for 30 min). Finally, the membrane and cytosol fractions were collected by centrifugation (100,000xg for 60 min). PKC activity was measured using the PepTag Protein Kinase Assay (Promega, Madison, WI). The assay is based on the highly specific phosphorylation of a fluorescent PepTag C1 peptide substrate by PKC. Collected fractions (2.5 µg protein/mL) were incubated with 2 µg of PepTag, a PKC substrate, and a PKC activating solution (phosphatidyl serine, 5 µg) for 30 min at 30°C. The reaction was terminated by boiling the assay medium in a water bath for 10 min. The samples were further electrophoresed on an
0.8% agarose horizontal gel at 100 V for 15 min, which induced migration of the phosphorylated peptide toward the anode, while non-phosphorylated peptide migrated toward the cathode. The ratio of phosphorylated to non-phosphorylated peptide was quantified with a densitometer (BioRad Gel Doc 1000 Darkroom, Hercules, CA). PKC activity was expressed as phosphorylation rate (ng/min/mg protein).

**Measurement of Rho A Activity.** In this study, active rho A was determined employing a Rho G-LISA™ assay as recommended by the manufacturer (Cytoskeleton Inc., Denver, CO). The membrane fraction was prepared as described above. Prepared membrane fraction was added to the wells of the Rho G-LISA plate coated with rho-GTP-binding protein. The plate was placed on a cold microplate shaker set at 400 rpm at 48 °C for 30 min. The plate was washed three times with Wash Buffer at room temperature; subsequently, anti-rhoA primary antibody (diluted 1:250) was introduced to each well and left on the shaker for 45 min. After three washes, diluted horse radish peroxidase (HRP)-labeled secondary antibody (1:250) was added to the wells and placed on the shaker at room temperature for 45 min. After 3 washes, HRP detection reagent (provided in the enhanced chemiluminescence kit) was added to the wells, after which the luminescence signal was detected with a microplate luminescence reader. Results were detected as relative luminescence units (RLU) over background signal (background incubation with assay reagents alone instead of cellular fractions).

**Data Analysis.** Values are presented as the mean ± SEM obtained from at least 4-5 animals. Statistical differences for multiple comparisons were assessed with one-way analysis of variance (ANOVA) for repeated measurements followed by the
Student-Newman-Keuls (SNK) test (Y-Stat Program; Igaku Tosyo Shuppan, Co. Ltd, Tokyo, Japan).
Results

Basic Characteristics of Experimental Diabetic Models. A significant difference in body weight was observed in ob/ob mice at 16-20 weeks of age relative to C57Bl mice (Table 1). As is typical with type-II diabetes, change in blood glucose level was examined. Following a 12 hr fast, blood glucose levels in ob/ob mice were higher than those in controls (185% of the C57Bl mouse). Levels of phospholipids and total cholesterol were also significantly higher (164 and 192% of the C57Bl mouse, respectively). However, meaningful differences in free fatty acids and triglyceride between these animals were not detected. Alterations in water intake and urine volume were also measured in the ob/ob mouse at 16 weeks of age as typical diabetic parameters (Table 2). Marked increases in these parameters were evident in the ob/ob mouse (267 and 287% of the C57Bl mouse, respectively).

Alteration of CCh-induced Isometric Force Responses in C57Bl and ob/ob Mouse Bladder under Normal and HG Conditions. Resting levels of isometric force in C57Bl and ob/ob mouse bladders averaged 2.07 ± 0.09 and 2.21 ± 0.01 mN/mm² (n = 5-8), respectively. Cumulative addition of CCh induced significant increases in isometric force. Typical responses of individual C57Bl and ob/ob mouse bladders are shown in Figure 1A and 1B; the averaged concentration-response relationships from these experiments for both bladder types are summarized in Figure 1C. The submaximal increase in force response in C57Bl mouse bladder in the presence of 30 µM CCh was 12.69 ± 0.22 mN/mm² (n = 8). At higher CCh levels, force development declined. In the ob/ob mouse, the maximal increase in isometric
force was enhanced considerably \( (14.34 \pm 0.32 \text{ mN/mm}^2; n = 5) \) in comparison to the C57Bl mouse. Significant increases were also evident in the range of 1.0-300 \( \mu \text{M} \) CCh. EC\(_{50}\) values for C57Bl and \( \text{ob/ob} \) mouse bladders were 3.0 and 1.8 \( \mu \text{M} \), respectively. The dose-response curve for the \( \text{ob/ob} \) mouse displayed substantial enhancement in comparison to that of the C57Bl mouse. The responses to CCh were responsible for this shift following a 15 min rise.

In order to demonstrate alteration of bladder contractility in hyperglycemia, CCh-induced changes in isometric force development were measured under HG conditions. Diabetes, which is characterized by elevated extracellular glucose levels, was created by pretreatment of the bladder with HG-PSS at 37 °C for 30 min. This condition was introduced in our previous studies (Nobe et al., 2003b; Nobe et al., 2004). Prior to measurements under HG conditions, several basic factors were examined in the current investigation. We confirmed that the effects of 2-fold HG-PSS were submaximal and that these effects did not differ from those in 3- and 4-fold HG-PSS. Moreover, the HG-PSS effects were time- and dose-dependent and a 30 min pretreatment period was submaximal. Over-night incubation of tissues under HG conditions led to similar responses in comparison to 30 min pretreatment (data not shown).

In order to evaluate the effect of osmotic changes under HG conditions, 11.1 mM sucrose was added to normal-PSS. However, this sucrose-supplemented (total, 22.2 mM; 11.1 mM glucose + 11.1 mM sucrose) PSS did not affect agonist-induced responses. Pretreatment of C57Bl mouse bladder with HG-PSS for 30 min did not
alter the non-stimulated resting level (2.04 ± 0.07 mN/mm²; n = 8). The CCh-induced increase in force development in C57Bl mouse was not affected by extracellular glucose accumulation. Force developments induced by 30 µM CCh in normal- and HG-PSS were 12.69 ± 0.22 and 12.39 ± 0.27 mN/mm² (n = 8), respectively. However, the CCh-induced increase in ob/ob mouse bladder in HG-PSS displayed significant enhancement without affecting the level of the resting state (2.12 ± 0.03 mN/mm²; n = 5). Apparent enhancement in comparison to the responses in normal-PSS was detected following treatment with 0.3-300 µM CCh. The submaximal response induced by 30 µM CCh was 15.9 ± 0.29 mN/mm² (n = 5). The EC₅₀ value in HG-PSS was 0.75 µM.

The muscarinic receptor antagonist atropine (Atr; 3, 10, 30, 100 and 300 nM, 10 min pre-incubation) inhibited the CCh-induced increase in isometric force in C57Bl and ob/ob mice. In C57Bl mouse bladder, dose-response curves were shifted to higher concentrations of CCh (data not shown). Similar responses were detected under HG conditions. pA² values of Atr in normal- and HG-PSS were 9.27 and 9.26, respectively. Under normal and HG conditions, CCh-induced dose-response curves in the ob/ob mouse were also shifted to higher concentrations of CCh. Differences between normal and HG conditions remained in the presence of Atr. pA² values of Atr in normal- and HG-PSS were 9.14 and 9.30, respectively.

**Effects of Calcium Channel Antagonist on CCh-induced Increase in Isometric Force in C57Bl and ob/ob Mouse Bladders.**

In order to identify an association between calcium channel activity and the enhancement of force development in ob/ob mouse, the voltage-dependent calcium
channel antagonist, nicardipine (Nic; 100 nM), was utilized; summarized data are presented in Fig. 2. Pretreatment with Nic for 5 min did not alter isometric force resting level in C57Bl mouse bladder. In contrast, the response was severely blunted by cumulative CCh stimulation; isometric force upon exposure to 30 µM CCh was 77.4 ± 3.28% (n = 5) of that of the control (response in normal-PSS). A similar inhibitory effect was also detected in HG-PSS. The dose-response curve at each glucose level overlapped (Fig. 2A). In ob/ob mouse bladder, pretreatment with Nic also markedly reduced the isometric force response to CCh without affecting the resting level. CCh (30 µM)-induced force developments in the presence of Nic under normal and HG conditions were 83.2 ± 4.61 and 80.6 ± 1.9% (n = 5), respectively. Differences between these extracellular glucose conditions remained following Nic treatment.

**Relationship between [Ca²⁺]ᵢ and isometric force development in C57Bl and ob/ob mouse bladder.** CCh-induced dose-dependent increases in force developments were detected in fura-PE3-loaded bladder as in Figure 1. During the measurements, [Ca²⁺]ᵢ also increased in a dose-dependent manner. When calcium sensitivity of the contraction was analyzed by plotting [Ca²⁺]ᵢ against force development per corresponding concentration of CCh, a correlation between [Ca²⁺]ᵢ and force development emerged under normal and HG conditions (Fig. 3). In C57Bl mouse, resting and 100 µM CCh-induced [Ca²⁺]ᵢ levels were 68.63 ± 6.3 and 746.21 ± 30.0 nM (n = 8), respectively. Pretreatment of tissue with HG-PSS for 30 min did not alter [Ca²⁺]ᵢ levels. The relationship between [Ca²⁺]ᵢ and isometric force development in normal- and HG-PSS exhibited similar correlations: r = 0.995 and 0.991, respectively.
The slopes of the relationship for CCh-induced responses in normal- and HG-PSS were 1.048 and 0.955, respectively. In ob/ob mouse bladder, CCh-induced force development was apparently enhanced relative to the response in C57Bl mouse (Fig. 1). However, $[\text{Ca}^{2+}]_i$ levels could not be distinguished. Subsequently, the slope of the $[\text{Ca}^{2+}]_i$ and isometric force relationship in normal-PSS was 1.236. This result indicated that the $[\text{Ca}^{2+}]_i$–force association is enhanced in ob/ob mouse bladder. Under HG conditions, the correlation between $[\text{Ca}^{2+}]_i$ and force development in ob/ob mouse was also enhanced (Slope = 1.491); however, $[\text{Ca}^{2+}]_i$ level at each CCh concentration could not be distinguished from the corresponding level in normal-PSS.

**Effects of PKC Inhibitors on CCh-induced Bladder Contractility under HG Conditions in C57Bl and ob/ob Mice.** In order to identify a relationship between PKC and bladder contraction in C57Bl and ob/ob mice, three types of PKC inhibitors were introduced. Calphostin C is a general-type (non-isoform-specific type) PKC inhibitor. Gö6976 and rottlerin, a calcium-dependent and -independent PKC inhibitor, respectively, were selected (Fig. 4). To assess the specific inhibitory effects of the aforementioned PKC inhibitors, time- and dose-dependencies of these agents were measured (data not shown). Based on these preliminary trials, each condition was adopted as the minimum concentration in the critical inhibitory range that did not affect non-stimulated resting level. Treatment with 10 µM CCh in normal-PSS increased C57Bl mouse bladder contraction in a manner similar to that of Figure 1 (10.8 ± 0.26 mN/mm²; n = 5). Both Calphostin C and Gö6976 significantly reduced CCh-induced force development (14.0 ± 4.93 and 50.0 ± 6.52% of the control response, respectively;
n = 5). In contrast, rottlerin did not influence the response (89.6 ± 3.54% of the control response; n = 5). Similar trials were performed under HG conditions; however, inhibitory effects were similar to those observed in normal-PSS.

In *ob/ob* mouse bladder, enhancement of force development induced by 10 µM CCh under normal glucose conditions was confirmed (Fig. 4B). Pretreatment of tissues with Calphostin C reduced CCh-induced responses (38.5 ± 3.31% of the control response; n = 5); however, meaningful attenuation of force development was not evident in the presence of Gö6976 (over 80% of control response remained). Pretreatment of bladders with rottlerin significantly reduced force development (39.94 ± 2.64% of the control response; n = 5). This inhibitory effect resembled that of Calphostin C. Effects of CCh and PKC inhibitors were measured under HG conditions; patterns similar to those of the responses in normal-PSS emerged. Extracellular glucose-dependent enhancement of force development was not detected in the presence of either Calphostin C or rottlerin.

**Association of Rho-Rho Kinase Pathway with CCh-induced Increase in Isometric Force in C57Bl and *ob/ob* Mouse Bladders.** In order to identify rho-rho kinase-mediated pathway involvement with respect to the differences between C57Bl and *ob/ob* mice, rho A activity in the membrane fraction and the inhibitory effect of a specific rho kinase inhibitor, fasudil, were measured. The resting level of rho A activity in C57Bl mouse was 159.3 ± 23.4 RLU (n = 5); moreover, this level increased dramatically following the introduction of 10 µM CCh (753.3 ± 79.2 RLU; n = 5) (Fig. 5). Pretreatment of the bladder with HG-PSS at 37 °C for 30 min did not alter rho A
activities in the presence or absence of CCh. In ob/ob mouse, a CCh-induced increase in rho A activity was detected (1139.3 ± 125.1 RLU; n = 5); furthermore, the activity was higher than that in C57Bl mouse. Under HG conditions, significant enhancement of rho A activities relative to those values in normal-PSS were evident not only in bladders following treatment with CCh (1633.5 ± 100.4 RLU; n = 5) but also during the non-stimulated resting state (436.7 ± 51.6 RLU; n = 5). Significant differences between C57Bl and ob/ob mice were also observed under HG conditions. The rho A activities were apparently reduced by pretreatment of bladder tissues with the rho inhibitor, C3-exoenzyme (10 µg/mL, 5 hr; data not shown).

In the C57Bl mouse, pretreatment with 1 µM fasudil significantly reduced the isometric force attributable to CCh; moreover, the resting level was unaffected (Fig. 6). Isometric force induced by 30 µM CCh in the presence of fasudil was 3.55 ± 0.16 mN/mm² (14.3% of the control response; n = 5). A similar inhibitory effect of fasudil was evident in ob/ob mouse bladder. Dose-response curves for CCh in the presence of fasudil in C57Bl and ob/ob mice overlapped (Fig. 6C). Differences between C57Bl and ob/ob mice were suppressed by fasudil pretreatment. Enhanced ob/ob mouse bladder contraction under HG conditions was inhibited and differences between normal- and HG-PSS were also suppressed by fasudil treatment. Inhibitory effects on the ob/ob mouse bladder similar to those of fasudil were detected following application of another type of rho kinase inhibitor, namely, Y27632 (data not shown).

**Alteration of Total PKC Activity in Diabetic Mouse Bladder.** In C57Bl mouse, the PKC activity in the total membrane fraction of the non-stimulated resting
state was $2.16 \pm 0.07 \text{ ng/min/mg protein (n = 5)}$ (Fig. 7A). This activity increased significantly upon treatment of tissue with $10 \mu M$ CCh ($10.82 \pm 0.26 \text{ ng/min/mg protein; n = 5}$). Pretreatment with $1 \mu M$ rottlerin and $1 \mu M$ fasudil did not affect CCh-induced PKC activation (in excess of $88\%$ of control activity remained). Similar trials were performed under HG conditions. In the non-stimulated resting state in HG-PSS, PKC activity in the membrane fraction could not be distinguished ($2.39 \pm 0.17 \text{ ng/min/mg protein; n = 5}$). CCh-induced responses were also unaffected. In ob/ob mouse bladder, the resting activity of PKC in normal PSS was similar to the value in C57Bl mouse ($2.24 \pm 0.14 \text{ ng/min/mg protein; n = 5; Fig. 7B}$). Treatment with CCh enhanced PKC activity in ob/ob mice ($11.10 \pm 0.64 \text{ ng/min/mg protein; n = 5}$); fasudil did not affect the response.

Under HG conditions, CCh-induced PKC activity displayed a meaningful increase in comparison to normal-PSS ($15.25 \pm 0.80 \text{ ng/min/mg protein; n = 5}$) without affecting the resting activity. In HG-PSS, this activation diminished significantly in the presence of rottlerin and fasudil ($8.36 \pm 0.86$ and $9.97 \pm 0.65 \text{ ng/min/mg protein; n = 5}$, respectively). The extracellular glucose-dependent increase in PKC activity was abolished by these inhibitors solely in ob/ob mouse bladder.
Discussion

This study revealed the amplification of enhanced contraction in diabetic mouse bladder under HG conditions. Moreover, involvement of the rho A-rho kinase-regulated nPKC pathway in the dysfunction was suggested.

Experimental diabetic models were employed in a variety of studies (Yono et al., 2005; Matsumoto et al., 2006). It is widely accepted that clinical symptoms and organic dysfunctions in these models are dependent on model stage or conditions. The \textit{ob/ob} mouse (16-20 weeks of age) introduced in this investigation exhibited chronic hyperglycemia as well as increases in body weight, plasma phospholipids and cholesterol (Table 1). These parameters indicated that this stage of the \textit{ob/ob} mouse is a suitable model of type-II diabetes with obesity. Typical symptoms of diabetes, particularly polydipsia and polyuria (Nichols, 2001), were also detected at this stage (Table 2). Frequent urination and dysuria, which are typical diabetic dysfunctions, might be associated with the symptoms including alteration of bladder smooth muscle contraction. Therefore, we surmised that analysis of contractile dysfunctions in \textit{ob/ob} mouse bladder could elucidate mechanisms underlying urinary complications in diabetic patients.

CCh-induced bladder smooth muscle contraction displayed significant enhancement in \textit{ob/ob} mice (Fig. 1). Increases in bladder contraction have also been reported in streptozotocin-induced type-I diabetic rat (Yang et al., 2007). According to these reports, CCh-induced maximal responses were 120-140\% of normal responses. Our results were consistent with those obtained in the aforementioned animals. These
enhanced bladder smooth muscle contractions might account for reduction of bladder urine capacity mediated by decreased bladder volume. The reduction in urine capacity in *ob/ob* mice leads to serious urinary dysfunction due to enhancement of total urine volume (Table 2). The *ob/ob* mouse indicated sustained hyperglycemia (Table 1); consequently, the effect of extracellular glucose level on CCh-induced bladder contraction was assessed in this investigation. Based on the fasting plasma glucose level in the *ob/ob* mouse (Table 1), glucose at twice the normal extracellular glucose concentration (22.2 mM) served as the HG condition. The current HG condition consisted of only 30 min HG-PSS treatment; however, we believed that this condition was an excellent approximation of diabetic hyperglycemia. The veracity of the aforementioned HG condition was supported by our preliminary trials in terms of glucose concentration (11.1-44.4 mM) and pretreatment period (10 min-12 hr) dependencies. Under HG conditions, significant enhancement of bladder contraction was detected solely in the *ob/ob* mouse (Fig. 1). Procurement of glucose dependency in diabetic bladder contraction was described initially in this study. Our findings indicated that bladder contraction increases in diabetes and that increased contraction is enhanced in hyperglycemia. Accordingly, aggravation of bladder dysfunction in hyperglycemia was suggested.

Alterations of bladder smooth muscle contraction have been reported in some types of diseases, e.g., “lifestyle-related diseases” (Andersson and Arner, 2004; Drake et al., 2006; Nobe et al., 2007). Enhancement of contraction in bladder smooth muscle is well known in spontaneous hypertensive rat (SHR) (Rajasekaran et al., 2005; Drake et
al., 2006). We also confirmed similar results in stroke-prone SHR (unpublished data). Contractile responses in \textit{ob/ob} mice were similar to those responses in the aforementioned models. Mechanisms governing the enhancement of contractions have been suggested in the SHR. An increase in the total number of muscarinic receptors and/or the level of sensitization of the receptor induced the dysfunction (Sherer et al., 2000; Stevens et al., 2006). In addition, bladder dysfunction in the absence of alterations of muscarinic receptor was also documented (Schneider et al., 2005). Therefore, an association between the muscarinic receptor and enhanced contraction of SHR bladder could not be concluded. Increased activation of another regulatory factor, rho kinase, was also suggested (Rajasekaran et al., 2005). On the basis of these findings in SHR bladder, this study assessed alteration of the muscarinic receptor in \textit{ob/ob} mouse bladder. However, enhancement of CCh-induced contraction in the \textit{ob/ob} mouse remained in the presence of muscarinic receptor antagonist (Atr); furthermore, pA$_2$ values in C57Bl and \textit{ob/ob} mouse bladders were similar. An increase in muscarinic receptor expression was documented in type-I diabetic rat bladder (Cheng et al., 2007); thus, the association of receptor expression level with enhanced contraction in \textit{ob/ob} mouse bladder could not be refuted. However, we believed that an increase in the number of muscarinic receptors and/or level of sensitization of the receptor is not a major contributor to enhanced contraction of \textit{ob/ob} mouse bladder.

Subsequently, in terms of important intracellular contractile factors, the influence of calcium influx from extracellular medium on enhancement of contraction was examined. Treatment with Nic reduced CCh-induced bladder contraction in C57Bl
and *ob/ob* mice; although inhibitory rates were similar, differences between these types of mice were observed (Fig. 2). These data suggested that the meaningful increase in bladder contraction in *ob/ob* mice was not derived from increased activation of calcium channels. Based on these results, we surmised that alteration of both muscarinic receptors and calcium channels do not play a major role in enhanced contraction in *ob/ob* mouse bladder. The relationship between \([Ca^{2+}]_i\) and force development, as intracellular calcium sensitivity of bladder contraction, was examined in order to develop an understanding of the mechanism (Fig. 3). Developed force levels in *ob/ob* mouse bladder were significantly enhanced relative to those of the C57Bl mouse at similar \([Ca^{2+}]_i\) levels. This finding was indicative of enhancement of calcium sensitivity in diabetic bladder contraction. Enhanced calcium sensitivity was also reported in other diabetic models (Waring and Wendt, 2000). Details regarding sensitization are poorly understood; moreover, an increase in myosin light chain phosphorylation associated with calcium sensitization was suggested.

This study focused on the enhancement of calcium sensitization in *ob/ob* mouse bladder under HG conditions (Fig. 3). During sensitization, CCh-induced alteration of \([Ca^{2+}]_i\) could not be distinguished from the corresponding level in C57Bl mouse. These results were indicative of extracellular glucose-dependent enhancement of contraction caused by activation downstream of the intracellular calcium signaling pathway and/or calcium-independent signaling pathway(s). Therefore, activation of PKC, a key factor of the intracellular signaling system located downstream of calcium responses, was evaluated. The PKC family primarily involves calcium-dependent...
(cPKC; PKC-α, β-II and γ) and -independent (nPKC; PKC-δ, ε, η and θ) isoforms (Das Evcimen and King, 2007).

An inhibitor of cPKC, Gö6976, altered CCh-induced contraction only in C57Bl mouse (Fig. 4A). In contrast, extracellular glucose-dependent enhancement of contraction in ob/ob mouse was unaffected (Fig. 4B). These results indicated that the glucose-dependent enhancement of bladder contraction is not involved in calcium-dependent PKC activation; therefore, activation of the calcium-dependent pathway might not contribute to the enhancement of ob/ob mouse bladder contraction. Rather, rottlerin, a nPKC inhibitor, significantly reduced CCh-induced bladder contraction (Fig. 4) and total PKC activity (Fig. 7) in ob/ob mouse. These findings indicated that nPKC plays a role in diabetic mouse bladder dysfunction. Interestingly, extracellular glucose-dependent enhancement of contraction and PKC activation in the ob/ob mouse were also suppressed by rottlerin. These results suggested that diabetic mouse bladder dysfunction is regulated by nPKC and that glucose-dependent enhanced contraction is derived from increased activation of nPKC without affecting the intracellular calcium level.

A typical pathway, namely, rho and the rho kinase pathway, which is involved in vascular and intestinal smooth muscle contractions, was examined to confirm the participation of the calcium-independent signaling pathway. The membrane associated-rho A plays physiological roles (Somlyo and Somlyo, 2003); therefore, rho A activity in the membrane fraction was measured in this study. The CCh-induced increase in rho A activity in ob/ob mouse was enhanced in comparison to the level in
C57Bl mouse (Fig. 5). Extracellular glucose-dependent enhancement of rho A activities was detected exclusively in ob/ob mouse bladder. These patterns of changes were well correlated with the alteration of ob/ob mouse bladder contraction under HG conditions (Fig. 1C). Moreover, the rho kinase inhibitor, fasudil, significantly inhibited CCh-induced bladder contraction in both types of mice (Fig. 6). Enhancement of contraction in the ob/ob mouse under normal and HG conditions was also suppressed in the presence of fasudil. These data indicated that the increased rho A activity in ob/ob mouse led to enhancement of rho kinase activity. Therefore, differences in contraction between C57Bl and ob/ob mouse bladders might be associated with over-activation of the rho A-rho kinase pathway. Results of PKC- and rho kinase-inhibitor treatments (Fig. 4, 6) suggested that the rho A-rho kinase-mediated calcium-independent pathway might play an essential role in diabetic bladder contraction.

How is diabetic bladder dysfunction regulated by both the rho A-rho kinase and nPKC pathways? A relationship between PKCδ (a member of the nPKC sub-family) and rho kinase was suggested by some researchers (Kandabashi et al., 2003; Li et al., 2005). In cerebral artery, rho and the rho kinase pathway activate the PKCδ isoform (Obara et al., 2005), which induces enhancement of vascular contraction and is caused by cerebral vasospasm. In a manner similar to the calcium-independent signaling pathway, we hypothesized that glucose-dependent enhancement of contraction is also involved in the regulation of PKCδ via the rho A-rho kinase pathway. Fasudil-induced rho kinase inhibition attenuated PKC activity (Fig. 7B), which supports this hypothesis.
How does enhancement of extracellular glucose level influence the rho A-rho kinase and nPKC pathways? Recently, it was reported that HG treatment enhances rho kinase activity via increases in rho A level in diabetic aorta (Akiyama et al., 2008). Previously, we suggested that HG-induced vascular endothelial cell dysfunctions participate in glucose-dependent rho kinase activation (Nobe et al., 2006). A similar relationship between glucose and rho kinase was predicted with respect to the diabetic bladder smooth muscle dysfunction under HG conditions; however, a critical target of the enhanced extracellular glucose in relation to rho kinase activity is unknown. Consequently, a target of enhanced extracellular glucose has not been identified; however, identification of the role of the glucose-dependent rho A-rho kinase and nPKC pathways in tissue dysfunctions might be critical in terms of an understanding of diabetic complications.

This study established the involvement of not only over-contraction but also of enhancement of extracellular glucose-dependency in type-II diabetic bladder dysfunction. The current findings suggested the important mechanistic contribution of the rho A-rho kinase-regulated calcium-independent PKC pathway. A thorough understanding of these mechanisms may contribute to the development of novel therapeutic targets for diabetic bladder complications.
References


Nobe K, Yamazaki T, Kumai T, Okazaki M, Iwai S, Hashimoto T, Kobayashi S, Oguchi


Waring JV and Wendt IR (2000) Effects of streptozotocin-induced diabetes mellitus on intracellular calcium and contraction of longitudinal smooth muscle from rat


Footnotes

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Legends for Figures

Fig. 1. Effects of carbachol (CCh) on isometric force under normal and HG conditions in bladder from C57Bl and ob/ob mice. CCh-induced changes in isometric force (mN/mm²) were measured as described in Methods. Typical changes observed in bladder preparations isolated from C57Bl (A) and ob/ob (B) mice. Bladder tissues were pre-incubated in normal-PSS (left panel, open) and HG-PSS (right panel, closed) for 30 min; subsequently, indicated contractions of CCh were introduced. Concentration-response relationships for CCh-induced isometric force responses in bladder of C57Bl (circles) and ob/ob (squares) mice were indicated (C). Each value represents the mean ± SEM of at least five independent determinations. * and # denote values significantly different from C57Bl mouse and responses in normal-PSS, respectively, at p<0.01.

Fig. 2. Effects of nicardipine (Nic) on CCh-induced isometric force under normal and HG conditions in bladder of C57Bl (A) and ob/ob (B) mice. CCh-induced changes in isometric force (mN/mm²) were measured under normal (open) and HG (closed) conditions as described in Fig. 1. Bladder tissues were pre-incubated in the presence (squares) or absence (circles) of the calcium channel antagonist (100 nM Nic) for 10 min; subsequently, indicated contractions of CCh were introduced. Each value represents the mean ± SEM of at least five independent determinations. * and # denote values significantly different from values in normal-PSS and response in the
absence of Nic, respectively, at p<0.01.

**Fig. 3. Effects of extracellular glucose enhancement on [Ca\(^{2+}\)]-force relationship of contractions induced by CCh in C57Bl and ob/ob mice.** CCh-induced changes in [Ca\(^{2+}\)]\(_i\) and isometric force were measured simultaneously as described in Methods. Indicated concentrations of CCh (µM)-induced [Ca\(^{2+}\)]\(_i\) and isometric force responses in bladder of C57Bl (circles) and ob/ob (squares) mice were measured under normal (open) and HG (closed) conditions. During the stimulation, the relationship between [Ca\(^{2+}\)]\(_i\) and isometric force was plotted as the percentage of maximal response in C57Bl mouse under normal-glucose conditions. Calculated absolute value of [Ca\(^{2+}\)]\(_i\) was also indicated. Each value represents the mean ± SEM of at least five independent determinations.

**Fig. 4. Effects of protein kinase C (PKC) inhibitors on CCh-induced isometric force under normal and HG conditions in bladder of C57Bl (A) and ob/ob (B) mice.** CCh (10 µM)-induced changes in isometric force (mN/mm\(^2\)) were measured under normal (open bars) and HG (closed bars) conditions as described in Methods. Bladder tissues were pre-incubated in the presence or absence of 1 µM calphostin C (Cal-C), 1 µM Gö6976 or 1 µM rottlerin for 10 min; subsequently, 10 µM CCh-induced contractions were introduced. Each value represents the mean ± SEM of at least five independent determinations. * and # denote values significantly different from values of resting and CCh-induced control responses, respectively, at p<0.01.
Fig. 5. Alteration of rho A activity in membrane fraction under normal and HG conditions in bladder of C57Bl and ob/ob mice. Bladder tissues were isolated from C57Bl (open bars) and ob/ob (closed bars) mice; subsequently, tissues were pre-incubated in normal- and HG-PSS at 37°C for 30 min. CCh (10 µM) was added for 5 min. These treatments were terminated and membrane fractions were collected as described in Methods. Rho A activity in each sample was assayed with the G-LISA™ assay system. Results are expressed as relative luminescence units (RLU). Each value represents the mean ± SEM of at least five independent determinations. * and # denote values significantly different from resting level and responses in normal-PSS, respectively, at p<0.01.

Fig. 6. Effects of rho kinase inhibitor (fasudil) on isometric force in bladder of C57Bl and ob/ob mice. CCh-induced changes in isometric force (mN/mm²) were measured as described in Fig. 1. Typical changes observed in bladder preparations isolated from C57Bl (A) and ob/ob (B) mice. Bladder tissues were pre-incubated in normal- (open) and HG-PSS (closed) for 30 min. Next, tissues were incubated in the presence (left panels) or absence (right panels) of 1 µM fasudil for 10 min. Finally, indicated contractions of CCh were introduced. Concentration-response relationships for CCh-induced isometric force responses in bladder of C57Bl (circles) and ob/ob (squares) mice were indicated (C). Each value represents the mean ± SEM of at least five independent determinations. * and # denote values significantly different from
C57Bl mouse and responses in the absence of fasudil, respectively, at p<0.01.

**Fig. 7.** Responses of PKC activity in CCh-induced C57Bl (A) and ob/ob (B) mouse bladders. Isolated bladder tissues were pre-incubated in normal- (open bars) and HG-PSS (closed bars) at 37°C for 30 min. These samples were exposed to 1 µM rottlerin (Rottlerin) or 1 µM fasudil (Fasudil) for 5 min; subsequently, 10 µM CCh (CCh) was added for 5 min. These treatments were terminated and membrane fractions were collected as described in Methods. PKC activity in each sample was assayed with the TepTag assay system. Results are expressed as ng/min/mg protein. Each value represents the mean ± SEM of at least five independent determinations. * and # denote values significantly different from resting level and responses in the presence of CCh, respectively, at p<0.01.
Table 1  Body weights and blood parameters of C57Bl and ob/ob mice

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body weight (g)</th>
<th>P-glucose (mg/dL)</th>
<th>Phospholipids (mg/dL)</th>
<th>Free fatty acid (µEQ/L)</th>
<th>TG (mg/dL)</th>
<th>Total-Cho (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl</td>
<td>8</td>
<td>24.8 ± 0.45</td>
<td>88.6 ± 9.63</td>
<td>199.1 ± 22.8</td>
<td>1138.5 ± 90.2</td>
<td>42.1 ± 8.24</td>
<td>97.3 ± 15.5</td>
</tr>
<tr>
<td>ob/ob</td>
<td>8</td>
<td>56.4 ± 0.53*</td>
<td>163.8 ± 5.54*</td>
<td>327.3 ± 25.8*</td>
<td>885.5 ± 54.2</td>
<td>67.1 ± 10.1</td>
<td>186.8 ± 17.2*</td>
</tr>
</tbody>
</table>

Body weight and 12 hr-fasted plasma glucose level (P-glucose) were measured in 16-20-week-old C57Bl and ob/ob mice. Levels of plasma phospholipids, free fatty acids, triacylglycerol (TG) and total cholesterol (Total-Cho) were measured as described in “Methods”. * p<0.01 vs C57Bl mouse (t-test).
Table 2  Alteration of water intake and urine volume in C57Bl and ob/ob mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>n</th>
<th>Water intake (mL/day)</th>
<th>Urine volume (mL/day)</th>
</tr>
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<tbody>
<tr>
<td>C57Bl</td>
<td>4</td>
<td>6.05 ± 0.37</td>
<td>4.13 ± 0.53</td>
</tr>
<tr>
<td>ob/ob</td>
<td>4</td>
<td>16.18 ± 1.25*</td>
<td>11.85 ± 1.65*</td>
</tr>
</tbody>
</table>

Water intake and urine volume were measured in 16-week-old C57Bl and ob/ob mice using the individual cage system as described in “Methods”.  * p<0.01 vs C57Bl mouse (t-test).
**Fig. 1**

**A** *C57Bl* mouse

**B** *ob/ob* mouse

**C**

<table>
<thead>
<tr>
<th>Isometric Force (mN/mm²)</th>
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<tbody>
<tr>
<td>18</td>
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</tbody>
</table>

**CCh (µM)**

- 0.1
- 0.3
- 1
- 3
- 10
- 30
- 100
- 300

High glucose-PSS
A  

*C57Bl mouse*

B  

*ob/ob mouse*

**Fig. 2**
Fig. 3
Fig. 4

A  

C57Bl mouse

B  

ob/ob mouse

Isometric Force (mN/mm²)
Fig. 5
**Fig. 6**

A  **C57Bl mouse**

B  **ob/ob mouse**

C  [Graph showing isometric force vs. CCh (µM) with control and fasudil treatments]
Fig. 7

A  *C57Bl mouse*

![Graph showing PKC activity in C57Bl mice.](image)

B  *ob/ob mouse*

![Graph showing PKC activity in ob/ob mice.](image)

*Fig. 7*