Two types of over-contraction are involved in intrarenal artery dysfunction in type II diabetic mouse

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Running title page

1) Running Title
Intrarenal small artery dysfunction in diabetes

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3) Counts
Text page: 31 pages
Number of tables: 2
Number of figures: 8
Number of references: 30
Number of words in abstract: 234 words
Number of words in introduction: 668 words
Number of words in discussion: 1443 words

4) List of non-standard abbreviations
BUN, blood urea nitrogen; C3-toxin, exoenzyme C3; [Ca\(^{2+}\)], intracellular free Ca\(^{2+}\)
concentration; Ca\(^{2+}\)-free PSS, CaCl\(_2\)-replaced PSS; Cal-C, calphostin C; EC, endothelial cell; FFA, free fatty acid; GFR, glomerular filtration rate; HG, high-glucose; ILA, interlobar artery; NE, norepinephrine; OGTt, oral glucose tolerance test; PE, phenylephrine; PKC, protein kinase C; PL, phospholipids; PSS, physiological salt solution; RA, renal artery; RLU, relative luminescence units; t-Chol, total cholesterol; TG, triacylglycerol.

5) Recommended section

Endocrine and Diabetes
ABSTRACT  Contractile responses in small intrarenal arteries are associated with diabetic nephropathy. However, the mechanisms that induce and maintain altered small vessel contraction are not clearly understood. To further understand intrarenal artery dysfunction in diabetes, phenylephrine (PE)-induced force development was assessed in the intrarenal artery (interlobar artery; ILA) in control (lean) and type II diabetic (ob/ob) mice. PE-induced dose-dependent force development in the ILA was significantly greater in ob/ob mice than in lean mice (592.8 ± 5.2 and 770.1 ± 12.1 µN/mm tissue, respectively, following administration of 30 µM PE, n = 5). Under high-glucose conditions (twice the normal concentration of glucose), PE-induced force development in the ILA was only enhanced in ob/ob mice (946.0 ± 18.2 µN/mm tissue; n = 5). ILA dysfunction reduces blood flow to the glomerulus and may induce diabetic nephropathy. Basal over-contraction of the ILA in ob/ob mice under normal glucose conditions was reduced by pretreatment with rottlerin, a calcium-independent protein kinase C (PKCδ) inhibitor. Total PKC activity was also reduced by rottlerin. Under high-glucose conditions, the enhanced ILA contraction in diabetic mice was suppressed by rho A and rho kinase inhibitors. Our results indicate two types of ILA dysfunction in diabetes: (1) a basal increase in PE-induced contraction under normal-glucose conditions and (2) extracellular glucose-dependent enhancement of PE-induced contraction. We believe that these dysfunctions are mediated by the activation of the PKCδ and rho A–rho kinase pathways, respectively.
Introduction

Treatment of diabetic complications, including controlling blood glucose levels, is a very important component of the management of non-insulin-dependent diabetes mellitus (NIDDM). Many complications in NIDDM patients, including the three major complications of neuropathy, retinopathy and nephropathy, are associated with vascular dysfunction in specific tissues (Bogdanov and Osterud, 2009; Sena et al., 2013). For example, angiogenesis is a key element of diabetic retinopathy. Reduced blood flow is also thought to be the cause of diabetic neuropathy. Thus, it is widely recognized that vascular dysfunction might be the basis for many diabetic complications (Brown, 2008; Porter and Riches, 2013). In diabetic vascular dysfunction, the alterations in vascular endothelial cell (EC) function have been extensively studied because ECs regulate the vascular tone via the release of nitric oxide and endothelial cell-dependent hyperpolarizing factor (EDHF) (Gokina et al., 2013). Some groups have suggested that changes in blood glucose levels, insulin secretion, a reduction in vascular relaxation and/or oxidative stressors induce EC dysfunction (Beer et al., 2008; Brouwers et al., 2010). EC dysfunction might induce diabetic complications in many types of tissues by enhancing vascular tone. Although diabetes-related changes in EC function are well recognized, the association between vascular smooth muscle cell contraction and diabetic complications has not been clearly defined. Researchers have reported over-contraction of agonist-induced vascular responses in a NIDDM mouse model (Kanie and Kamata, 2000). Conversely, a reduction of the contractile response was reported by other groups (Fulton et al., 1991). To understand these contrasting results, we speculated that multiple patterns of change are derived from the variety of vascular
smooth muscle tissues rather than EC dysfunction. Therefore, assessment of microvascular function in specific tissues might be essential to understand these diabetic vascular complications. To reveal local microvascular dysfunction in a NIDDM mouse model, it is necessary to use a system for assessing vascular contraction. However, the forces developed by microvascular contractions are only 10–100 µN, which is difficult to measure. We previously established a microvascular force measurement system that was able to assess vascular dysfunction in the small arteries of diabetic mice (Nobe et al., 2006). In 2012, we used an EC-denuded mouse mesenteric artery second branch (MA2) as a typical small vascular tissue to assess the vascular function in a NIDDM mouse model (ob/ob mice) (Nobe et al., 2012). We found that phenylephrine (PE)-induced MA2 contraction was reduced in diabetic mice, but the contractions induced by prostaglandin F$_{2\alpha}$ and thromboxane A$_2$ (TXA$_2$) were unaffected. These diabetic vascular dysfunctions in MA2 could not be detected in the first branch of the mesenteric artery (MA1). Using MA2 derived from hyperglycemic ob/ob mice, MA2 contractions were also measured under normal- and high-glucose conditions and a reduction of extracellular glucose-dependency in diabetes was found. Based on these findings, we concluded that there are three major factors influencing microvascular dysfunction in NIDDM: 1) the type and location of vascular tissue; 2) the type of stimulant (agonist); and 3) the extracellular glucose level. On the basis of this hypothesis, we tried to measure diabetic vascular dysfunction in small renal arteries because the kidney is composed of many microvascular tissues, which might influence renal function. We compared the contractile responses between the intra- and outer-renal small arteries of non-diabetic (ddY) mice using our microvascular force
measurement system. U46619-induced contraction in the intrarenal artery (interlobar artery; ILA) was dependent on the extracellular glucose concentration, but U46619-induced contraction could not be detected in the outer-renal artery (renal artery; RA) (Nobe et al., 2008). These differences were not detected in PE-induced contraction. Thus, our previous findings suggested the need to assess changes in vascular smooth muscle contraction in each part of the vessel to understand the association between vascular dysfunction and diabetic complications. It is also necessary to evaluate the dependency of contraction on extracellular glucose.

The aim of this study was to assess ILA-specific dysfunction in a NIDDM mouse model and to compare our findings with those of the ILA in normal mice. We also investigated the molecular mechanisms underlying vascular dysfunction in these mice.

**Materials and Methods**

**Animals and Tissue Preparation.** Male B6.V Lep\(^{ob/ob}\) (ob/ob) mice and their lean littermates (B6.V Lep\(^{ob/+/}\); lean) were purchased from CLEA Japan (Tokyo, Japan) at 12 weeks of age. Mice were housed at a constant room temperature (20 ± 2°C), with 12-h light and dark cycles. Mice were fed standard chow, which included 5% fat (Oriental Yeast Corp., Tokyo, Japan). Food and water were available *ad libitum*. Mice were used in experiments at 19–22 weeks of age. All experiments were performed in compliance with the *Guiding Principles for the Care and Use of Laboratory Animals* approved by the Japanese Pharmacological Society and the guidelines approved by the Ethics Committee on Animal Care and Animal Experimentation at Josai University. The number of animals used was kept to the minimum necessary for meaningful interpretation of the
data. Animal discomfort was also minimized.

**Oral Glucose Tolerance Test (OGTT) and Plasma Biochemical Assays.** Fasting blood glucose levels were measured and OGTTs (1 g/kg glucose) were performed as described previously (Nobe et al., 2012). The plasma supernatant was used to measure plasma phospholipids (PL), free fatty acid (FFA), triacylglycerol (TG) and total cholesterols (t-Cho) levels in clinical laboratory tests conducted by SRL Inc. (Tokyo, Japan).

**Vascular Tissue Preparation.** Mice were sacrificed in a pre-charged CO2 chamber. Vascular tissue components, which included the RA and ILA, were isolated. The RA, which was located between the aorta and kidney, was used to assess extrarenal vascular responses. A small-branched vessel was used as the ILA. The ILA, buried in the renal parenchyma; was isolated from connective tissue and renal parenchyma using a dissecting microscope. The lengths of the isolated RA and ILA were 1.5–1.7 mm and 1.0–1.2 mm, respectively, and the internal diameters were 0.2–0.3 mm and 0.1–0.2 mm, respectively. The length of each tissue (≥1 mm) was confirmed with a micrometer. Tissues were rinsed in ice-cold bicarbonate-buffered physiological salt solution (PSS). PSS consisted of: 137 mM NaCl, 4.73 mM KCl, 1.2 mM MgSO4, 0.025 mM ethylenediamine tetra-acetic acid (EDTA), 1.2 mM KH2PO4, 2.5 mM CaCl2 and 11.1 mM glucose (buffering was achieved with 25.0 mM NaHCO3; the pH was 7.4 when the solution was bubbled with 95% O2 and 5% CO2 at 37°C). Connective tissue was removed under a stereoscopic microscope. Because the endothelium is capable of vasoactive mediator release under basal conditions, endothelium-denuded blood vessels were used in all experiments. Denuding was accomplished by rotating the
vascular rings around stainless wires.

**Isometric Force Measurement.** Isometric force measurements were conducted as previously described (Nobe et al., 2008). Briefly, vascular rings were mounted horizontally onto the microvascular force measurement system. In this study, the normal physiological glucose concentration (11.1 mM) was defined as the normal-glucose (NG) condition. To understand the direct effects of extracellular glucose accumulation, a high-glucose (HG) condition was established by pretreating the vascular tissues with HG-PSS (22.2 mM glucose in PSS) at 37°C for 30 min, as previously reported by our laboratory (Nobe et al., 2004).

**Measurement of the Intracellular Free Ca^{2+} Concentration ([Ca^{2+}]_i).** Vascular tissue was prepared as described above. A fluorescent calcium indicator, acetoxyethyl2-[5-[bis[(acetoxymethoxy-oxo-methyl) methyl]amino]-4-[2-[2-[bis[(acetoxymethoxy-oxo-methyl)methyl]amino]-5-methyl-phenoxy]ethoxy]benzofuran-2-yl]oxazole-5-carboxylate (fura-2/AM; 5 µM), was suspended in PSS containing 0.01% pluronic F127, and the ILA was incubated in the solution for 60 min at room temperature. After rinsing with PSS, the tissue was mounted on a fluorescent microscope. Fluorescent images of cells were obtained with excitation at 340 and 380 nm and emission at 510 nm (F340 and F380, respectively) using an Argus-HiSCA system (Hamamatsu Photonics, Hamamatsu, Japan). The F_{340} nm/F_{380} nm ratio (R_{340/380}) was measured and converted to the absolute concentration (nM) using a calcium buffer kit (Molecular Probes, Life Technologies, Carlsbad, CA, USA).

**Measurement of Protein Kinase C (PKC) and rho A Activities.** PKC activity in whole ILA lysates was measured using a colorimetric PKC activity assay kit according to
the manufacturer’s instructions (Stressgen Bioreagents, Victoria, BC, Canada). The results were detected as the absorbance at 450 nm. Rho A activity was determined using a Rho G-LISA™ assay kit as recommended by the manufacturer (Cytoskeleton Inc., Denver, CO, USA). Results were detected as relative luminescence units (RLU) above background signal (incubation with assay reagent alone), as previously described (Nobe et al., 2009).

**Gene Expression Analysis.** Gene expression assays were performed similar to previous reports (Li et al., 2010; Nobe et al., 2013). Total RNA was extracted from ILA using Trizol reagent (Life Technologies) and the concentration was determined by ultraviolet spectrophotometry by measuring absorbance at 260 nm/280 nm. RNA was reverse transcribed using SuperScript First-standard cDNA synthesis system (Invitrogen, Life Technologies, Carlsbad, CA, USA). Sequences of PKCδ and rho kinase (ROCK1) cDNA were obtained from the GenBank database. The following primers were then designed: PKCδ: sense primer, 5′GAGGCACTCACCACAGAC3′ and anti-sense primer, 5′AGGTCCAGCCAGAACTCA3′; rho kinase: sense primer, 5′TAAAGTCACCAAAGCACGCCTAA3′ and anti-sense primer, 5′CATGGAACATTGTTTCTCAGTCTC3′; β-actin control sense primer, 5′ACTATCGGCAATGAGCG3′, and anti-sense primer, 5′GAGCCAGGGGCAGTAATCT3′. The PCR products were amplified in a DNA thermal cycler, followed by electrophoresis through a 1% agarose gel. The amplified cDNA bands were then visualized with GelStar staining (Lonza Ltd., Basel, Switzerland). To quantify the PCR products, an invariant mRNA of β-actin was used as an internal control. The OD values of the kinases, as measured by the Kodak electrophoresis documentation system (Eastman Kodak
Company, Rochester, NY, USA), were normalized to the OD values of β-actin. The ratios are expressed as arbitrary units for quantitative comparison.

**Statistical Analysis.** Data are normalized for identical lengths (1 mm) of vascular rings. Results are presented graphically and as the means ± SEM of 4–5 independent determinations. Statistical analyses for multiple comparisons were conducted with analysis of variance (ANOVA) for repeated measurements followed by the Student-Newman-Keuls (SNK) test using Y-stat software (Igaku Tosho Co. Ltd., Tokyo, Japan). A $P$ value of $p < 0.05$ was considered statistically significant.

**Drugs.** Phenylephrine hydrochloride (PE), fura-2/AM, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole (Gö6976) and (R)-(+)−trans-4-(1-aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate (Y27632) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Calphostin C (*Cladosporium cladosporioides*; Cal-C), exoenzyme C3 (*Clostridium botulinum*; C3-toxin) and rottlerin were acquired from Calbiochem-Novabiochem (San Diego, CA, USA). All other reagents, were of the highest purity, and were purchased from Sigma Chemical Co. except as noted.

**Results**

**Characterization of ob/ob mice.** In this study, vascular tissue was isolated from diabetic (ob/ob) and control (lean) mice as reported in our previous publication (Nobe et al., 2012). The basic characteristics of these genotypes, including increases in body weight, blood glucose levels and lipid parameters, were confirmed (Table 1), and are similar to our previous report. The mice also displayed typical diabetic changes,
including increases in water intake and urine volume (Table 2). Notably, one variable associated with nephropathy, blood urea nitrogen (BUN), was unchanged in ob/ob mice.

**Changes in Isometric Force Development in the RA and ILA Isolated from Lean and ob/ob Mice.** To detect changes in contractions of the RA and ILA in diabetic mice, we first tested 50 mM KCl and PE. The isometric force at rest was 400.1 ± 7.6 and 398.1 ± 7.9 μN/mm tissue (n = 5) in the RA of lean and ob/ob mice, respectively (Fig. 1A). Administration of 50 mM KCl induced similar sustained force developments (data not shown). The cumulative addition of PE significantly increased isometric force. The submaximal increase in the force response in the presence of 30 μM PE was 2117.6 ± 47.2 and 1983.1 ± 30.1 μN/mm (n = 5) in the lean and ob/ob mouse RA, respectively. The EC\textsubscript{50} values for PE in lean and ob/ob mice were 2.05 and 2.02 μM, respectively. To assess the effects of hyperglycemia on RA contractility, PE-induced changes in isometric force development were measured under HG conditions. This condition was established by pretreating the lean and ob/ob mouse RA with HG-PSS at 37°C for 30 min. However, HG did not affect the non-stimulated resting level (398.5 ± 11.2 and 393.0 ± 5.2 mN/mm in lean and ob/ob mice, respectively; n = 5), but it did enhance PE-induced force development in both lean and ob/ob mice. Force development in the RA of C57BL/6 mice was slightly increased by 30 μM PE in HG-PSS to 126.2 ± 7.7% (n = 5) of the response under NG conditions. Extracellular glucose did not enhance force development in the ob/ob mouse RA.

Similar trials were performed using the ILA isolated from lean and ob/ob mice (Fig. 1B). The mean resting force level was 385.9 ± 6.0 and 394.1 ± 7.1 μN/mm tissue in lean and ob/ob mice, respectively (n = 5). Although 50 mM KCl induced similar responses
(data not shown), the PE-induced time- and dose-dependent increases in ILA force development were greater in ob/ob mice than in lean mice. Significant increases were detected after treatment with 0.3–100 µM PE. The mean PE (30 µM)-induced force development was 592.8 ± 5.2 and 770.1 ± 12.1 µN/mm tissue (n = 5) in lean and ob/ob mouse ILA, respectively. The EC50 values in lean and ob/ob mouse ILA were 2.25 and 2.01 µM, respectively. Pretreatment with HG-PSS did not affect PE-induced force development in the ILA from lean mice. The dose–response curves overlapped for the NG and HG conditions and 30 µM PE-induced force development was similar (614.7 ± 14.4 µN/mm tissue; n = 5). However, the PE-induced increase in force development was significantly enhanced in ob/ob mice at 0.3–100 µM PE, without changes in resting force. The mean PE (30 µM)-induced force development was 946.0 ± 18.2 µN/mm tissue (n = 5). Extracellular glucose only enhanced ILA force development in ob/ob mice.

Association between [Ca2+]i and PE-induced Isometric Force in Lean and ob/ob Mouse ILA. To identify an association between [Ca2+]i and enhanced force development in ob/ob mouse ILA, we measured PE-induced responses in CaCl2-replaced PSS (Ca2+-free PSS). To confirm the effect of Ca2+-free PSS pretreatment on force development, the PE-induced changes in the fluorescence ratio of fura-2 (R340/380) were measured as a surrogate for [Ca2+]i (Fig. 2A-inset). In lean mouse ILA, 30 µM PE significantly increased the R340/380 from 0.213 ± 0.002 to 0.285 ± 0.003 (n = 5). This response was suppressed by pretreating the ILA with Ca2+-free PSS at 37°C for 10 min (0.199 ± 0.005; n = 5). Under HG conditions, a similar change in R340/380 was detected in lean mouse ILA. Pretreatment with Ca2+-free PSS did not affect the resting force level in the lean mouse ILA (Fig. 2A). However, the response was partially reduced by
cumulative PE stimulation because the isometric force after exposure to 30 µM PE was 49.4 ± 4.4% (n = 5) of the response in the control (normal-PSS) condition. A similar inhibitory effect was also detected in HG-PSS. In Ca²⁺-free PSS, 30 µM PE-induced force development was 43.8 ± 2.2% (n = 5) of that of the response in HG-PSS. The dose-response curves overlapped between the normal and HG-PSS conditions.

In ob/ob mouse ILA, the R₃₄₀/₃₈₀ was significantly increased by 30 µM PE, from 0.207 ± 0.006 to 0.294 ± 0.008 (n = 5) (Fig. 2B-inset). As in lean mouse ILA, this increase was suppressed in Ca²⁺-free PSS (0.208 ± 0.004; n = 5). HG did not affect the PE-induced R₃₄₀/₃₈₀ response and extracellular glucose did not enhance R₃₄₀/₃₈₀, consistent with isometric force development. Pretreatment of ob/ob mouse ILA with Ca²⁺-free PSS reduced the isometric force response to PE without affecting the resting force level (Fig. 2B). 30 µM PE-induced force development in Ca²⁺-free PSS was reduced under normal and HG conditions to 51.3 ± 2.0% and 61.8 ± 5.7% (n = 5) of the control response, respectively. The differences in PE-induced responses between normal- and HG-PSS were of similar magnitudes to those in Ca²⁺-free conditions.

Effects of PKC Inhibitors on PE-induced ILA Contractility under HG Conditions in Lean and ob/ob Mice. To identify a relationship between PKC and ILA contraction in lean and ob/ob mice, we tested three types of PKC inhibitors (Fig. 3). Calphostin C is a general (nonisoform-specific) PKC inhibitor (Wesselman et al., 2001), Gö6976 is a calcium-dependent PKC (PKCα and β) inhibitor (Kamata et al., 2006), and rottlerin is a calcium-independent PKC (PKCδ) inhibitor (Hsieh et al., 2008). Each concentration of the inhibitor was introduced and had no effect on the baseline response (Suppl. Fig. 1). Treatment with 30 µM PE in normal-PSS increased lean mouse ILA contraction similar
to that shown in Fig. 1 (613.6 ± 14.0 µN/mm tissue; n = 5). Calphostin C partly attenuated PE-induced force development (502.9 ± 9.0 µN/mm; n = 5) (Fig. 3A-inset). By contrast, Gő6976 did not affect the contractile response (617.7 ± 11.6 µN/mm; n = 5).

Under HG conditions, the inhibitory effects of these inhibitors were similar to those observed in normal-PSS. PE-induced force development in lean mouse ILA was slightly inhibited by rottlerin and calphostin C (Fig. 3A). Similar changes was observed under HG conditions (Suppl. Fig. 2).

In ob/ob mouse ILA, 30 µM PE enhanced force development under NG conditions (780.2 ± 34.1 µN/mm tissue; n = 5). Pretreatment with calphostin C significantly reduced the PE-induced response (572.2 ± 27.0 µN/mm tissue; n = 5), but Gő6976 did not (Fig. 3B-inset). The enhanced force development under HG conditions was not affected by pretreatment with calphostin C or Gő6976. However, pretreatment of ob/ob mouse ILA with rottlerin significantly inhibited PE-induced force development in NG- and HG-PSS conditions (Fig. 3B). In the presence of rottlerin, the response to PE in NG and HG conditions was reduced to 44.8 ± 1.9% and 63.3 ± 2.2% of the control response, respectively. Nevertheless, force development remained higher in HG conditions than in NG conditions in the presence of rottlerin.

To confirm the effect of rottlerin on ILA force development, we measured total PKC activities in the ILA. In lean mice, 30 µM PE significantly increased PKC activity (421.4% of resting state) and this activation was suppressed by pretreatment with rottlerin (Fig. 4A). Total PKC activity in the non-stimulated resting state was significantly greater in ob/ob mice compared with lean mice (Fig. 4B; 344.8% of the resting state of lean mouse ILA). Moreover, 30 µM PE induced the activation of PKC. Pretreatment with 1
μM rottlerin reduced the total PKC activity. Although extracellular glucose did not enhance PKC activity in either lean or ob/ob mice, an inhibitory effect of rottlerin was detected and was similar to that in normal-PSS.

Involvement of the Rho A–Rho Kinase Pathway in the PE-induced Increase in Isometric Force in Lean and ob/ob Mouse ILA. To identify whether the rho A–rho kinase pathway contributes to the differences between lean and ob/ob mice, we measured the effect of the rho kinase inhibitor, Y27632 (Freitas et al., 2009), on PE-induced responses. In lean mice, pretreatment with 1 µM Y27632 significantly reduced the isometric force attributable to PE without affecting the resting level (Fig. 5A). The isometric force induced by 30 µM PE in the presence of Y27632 was 464.6 ± 12.0 and 470.1 ± 7.4 µN/mm tissue (n = 5) under normal and HG conditions, respectively. In ob/ob mouse ILA, the PE-induced dose-dependent increase in isometric force was significantly reduced by pretreatment with 1 µM Y27632 (Fig. 5B). The submaximal force development in the presence or absence of Y27632 decreased from 826.4 ± 14.0 to 656.8 ± 10.8 µN/mm tissue (n = 5). Under HG conditions, Y27632 significantly reduced the PE response from 933.3 ± 41.3 to 675.1 ± 6.1 µN/mm tissue (n = 5). Therefore, the differences between normal- and HG-PSS were suppressed by pretreatment with Y27632. The dose-response curves also overlapped.

To determine the contribution of rho A in ILA contraction in lean and ob/ob mice, we examined the effect of the rho A inhibitor, C3-toxin, on PE-induced responses (Fig. 5-inset). Tissues were pretreated with C3-toxin (100 µg/mL) at 37°C for 12 h (PSS containing C3-toxin was replaced every hour). Pretreatment with C3-toxin significantly reduced the 30 µM PE-induced ILA contraction in lean and ob/ob mice (39.1 ± 16.9%
and 51.2 ± 12.8% of the control responses, respectively; n = 5), without affecting the baseline responses (Suppl. Fig. 3). C3-toxin also had inhibitory effects under HG conditions and suppressed the glucose-dependent increase in force development in ob/ob mouse ILA.

To confirm the role of rho A activity in ILA force development, we also measured the effect of C3-toxin on rho A activity in lean and ob/ob mouse ILA (Aktories et al., 1989). Rho A activity in the lean mouse was 63.7 ± 3.8 RLU (n = 5) at rest and was increased by 30 µM PE (125.8 ± 14.6 RLU; n = 5) (Fig. 6A). A PE-induced increase in rho A activity was also detected after pretreatment with HG-PSS (108.6 ± 11.2 RLU; n = 5) similar to the normal-PSS control. Pretreatment of lean and ob/ob mouse ILA with 100 µg/mL C3-toxin at 37°C for 12 h inhibited PE-induced rho A activation under both glucose conditions. After pretreatment with C3-toxin, rho A activity under normal and HG-conditions was reduced to 40.0% and 64.0% of the respective control responses (n = 5). In ob/ob mice, the resting and PE-treated rho A activities did not differ appreciably from those in lean mice (65.3 ± 6.5 and 127.5 ± 10.9 RLUs, respectively; n = 5). Under HG conditions, extracellular glucose caused an increase in rho A activity following PE treatment (178.6 ± 8.9 RLU; n = 5). However, rho A activities under normal- and HG-conditions were significantly reduced by pretreatment with C3-toxin (45.1 ± 3.2 and 59.2 ± 5.7 RLUs; n = 5). Thus, activation of rho A under HG conditions was suppressed by C3-toxin. However, C3-toxin did not affect the resting force level (Suppl. Fig. 4).

**Changes in PKCδ and Rho Kinase mRNA Levels in Diabetic Mouse ILA.** To investigate whether PKCδ and rho kinase contribute to the changes in diabetic mouse ILA contraction, the total mRNA levels of PKCδ and rho kinase were assessed under
normal and HG conditions. The relative expression level of PKC\(\delta\) was significantly enhanced in \(ob/ob\) mouse ILA compared with lean mouse ILA (192.5 \(\pm\) 18.7% of lean mice; \(n = 5\)) (Fig. 7A). The mRNA levels of PKC\(\delta\) in lean and \(ob/ob\) mice were not affected by pretreating the tissues with HG-PSS. The relative expression of rho kinase was also measured (Fig. 7B). Under NG conditions, the relative expression levels of rho kinase were similar in lean and \(ob/ob\) mice. In lean mouse ILA, pretreatment with HG-PSS did not affect rho kinase expression. However, rho kinase expression in \(ob/ob\) mouse ILA was significantly increased by HG-PSS by 213.9% compared with NG conditions (\(n = 5\)).

**Discussion**

This study revealed functional changes in small intrarenal arteries in a type II diabetic mouse model. Diabetes in \(ob/ob\) mice is induced by changes in blood leptin levels and subsequent obesity (Wolf, 2001). The \(ob/ob\) mouse model is a widely used model of type II diabetes (Allen et al., 2004). Therefore, this model was chosen to better understand the mechanisms of diabetic vascular dysfunction because of the increase in the number of diabetic patients with a similar phenotype in recent years (Kiyohara, 2014). As in previous reports (Nobe et al., 2012), increases in blood glucose levels and other diabetic parameters were observed in this study, but a marker of nephropathy, BUN, was not affected (Tables 1 and 2). Based on these findings, our diabetic mouse model seems to represent an intermediate stage of diabetes characterized by diabetic hyperglycemia without accompanying nephropathy. To better understand the detailed mechanisms of the development of nephropathy, we choose this stage of the model.
because it is essential to assess the intrarenal vascular function before overt diabetic nephropathy. The results obtained using this animal model may have important implications on understanding the association of small arteries with nephropathy.

We measured force developments in the mouse RA and ILA as in our previous report (Nobe et al., 2008). PE-induced force developments in lean mice overlapped with the contractions observed in ddY mice, which are often used in this type of study. The removal of ECs from vascular tissue was confirmed by the addition of ACh to PE-induced sustained force development (data not shown). Changes in EC function in the diabetic state have been reported in many experimental models (Brouwers et al., 2010; Sena et al., 2013). We also recognize the importance of ECs in diabetes. However, in this study, we focused on the force responses independently of the regulation of vascular smooth muscles by ECs, one of their main functions. We also surmised that diabetic vascular dysfunction cannot be solely explained by EC dysfunction.

The RA is generally used as a kidney-related vascular tissue (Sanz et al., 2003; Yousif, 2006). However, the RA is not an intrarenal artery and the PE-induced contractile responses in ob/ob mice were not different to those in lean mice (Fig. 1A). Extracellular glucose only slightly enhanced contraction. However, PE-enhanced ILA contraction was increased by NG in ob/ob mice (Fig. 1B, Suppl. Fig. 5). Because ob/ob mouse ILA is exposed to HG conditions in vivo, this over-contraction in the NG condition (ex vivo) is indicative of basal over-contraction without changes in extracellular glucose concentrations. This might be related to dysfunction induced by hyperglycemic conditions in vivo. This over-contraction may cause a reduction in blood flow to the glomerulus. We next investigated the direct effects of the extracellular glucose
concentration on contraction. However, the extracellular glucose-dependency was only observed in ob/ob mouse ILA. This glucose sensitization resulted in an increase in PE-enhanced ILA contraction. Because ob/ob mice showed chronic hyperglycemia, it was important to compare the contractile responses between lean mouse ILA in normal-PSS and ob/ob mouse ILA in HG-PSS, because these glucose conditions are similar to those in vivo. In this comparison, the diabetic ILA contraction was 273.3% of that of the normal ILA contraction. Therefore, we suggest that the combination of diabetes and hyperglycemia impairs intrarenal blood flow and may lead to diabetic nephropathy. In this study, we found two types of intrarenal small artery dysfunction in diabetes: (1) a basal increase in PE-induced contraction under NG conditions and (2) an acquisition of extracellular glucose-dependency in HG-PSS (Illustration). Importantly, these dysfunctions were limited in PE-treated ILA contraction. The contractions induced by other agonists (U46619, PGF2α and 50 mM KCl) were not affected (data not shown). Agonist-specific changes in the vascular responses under NG and HG conditions were previously reported in the ob/ob mouse mesenteric artery (Nobe et al., 2012). These two types of alterations may be typical features of diabetic small artery dysfunction.

To understand the mechanisms involved in over-contraction of the ILA in diabetes, we investigated the cellular signaling systems. Inhibitory effects of the α1-receptor antagonist, prazosin, on PE-induced contraction could not be differentiated between lean and ob/ob mice (data not shown). The responses to pA2 were also similar between the two mouse models. Moreover, similar increases in [Ca^{2+}]] were detected in lean and ob/ob mice (Fig. 2-inset). These results suggest that the signaling system from receptor stimulation to intracellular calcium accumulation was intact in ob/ob mouse ILA.
similar reductions in ILA contraction in lean and ob/ob mice under Ca\textsuperscript{2+}-free conditions also support this suggestion (Fig. 2).

We next investigated the downstream signal transduction pathway involved in intracellular calcium accumulation. Because we previously reported that U46619-induced ILA contraction involves calcium-independent PKC activation (Nobe et al., 2008), we examined the effects of rottlerin, a selective PKC\textdelta inhibitor, on ob/ob mouse ILA contraction. Rottlerin reduced PE-induced contractions in lean and ob/ob mice, and suppressed the differences between these two genotypes (Fig. 3). Total PKC activity was also increased in ob/ob mouse ILA, but this increase was suppressed by rottlerin treatment (Fig. 4). Similar results were detected with calphostin C, but not with Gõ6976 (Fig. 3-inset). These results indicate that basal over-contraction of the diabetic ILA under NG conditions is caused by over-activation of calcium-independent PKC (Illustration), which might be caused by diabetic hyperglycemia \textit{in vivo}. Over-activation of PKC in diabetes was also reported in the rat renal artery (Noh and King, 2007; Kizub et al., 2014). This report showed that the expression of calcium-dependent PKC activity is enhanced in diabetes, and is associated with attenuated coronary artery vasodilation, without affecting voltage-gated calcium channel activity. Although different PKC isoforms were involved in our study, enhanced PKC activity may be a key element in diabetic vascular dysfunction. Moreover, total rho A activity in normal-PSS could not be differentiated between lean and ob/ob mice. These results suggest that rho A activity is not involved in diabetic over-contraction, which supports the importance of calcium-independent PKC activation. Taken together, basal over-contraction in diabetes involves calcium-independent PKC activation. To better understand diabetic ILA dysfunction, we
also assessed the mRNA expression levels of specific PKC isoforms (Fig. 7A). The total mRNA level of PKCδ was significantly enhanced in ob/ob mouse ILA but was not affected by extracellular glucose levels, supporting our hypothesis. Conversely, the extracellular glucose-dependent enhancement of ILA contraction in diabetic mice was not reduced by rottlerin or other PKC inhibitors (Fig. 3). Furthermore, HG-PSS did not affect total PKC activity or the PE-induced intracellular calcium responses (Fig. 4). These results indicate that the glucose-dependent ILA responses are not associated with calcium-independent PKC-mediated ILA over-contraction under NG conditions, as discussed above. To better understand glucose-dependent dysfunction of diabetic ILA, we focused on the activity of the rho A–rho kinase pathway as a candidate calcium-independent pathway (Nobe et al., 2012). Other researchers have described a relationship between the extracellular glucose concentration and the activity of vascular smooth muscle cells (Cicek et al., 2013). This report suggest that an increase in the extracellular glucose concentration affects numerous intracellular signaling factors, including PKC, rho A and CPI-17. Similar effects were also suggested for other cell types (Zhang et al., 2013). We previously reported that the rho A–rho kinase pathway is activated by HG-PSS in mouse mesenteric artery (Nobe et al., 2012). In mouse ILA, pretreatment with C3-toxin and Y27632 partly reduced PE-induced contractions in lean and ob/ob mice under NG conditions. These results indicated that the rho A–rho kinase pathway is involved in normal ILA contraction. Importantly, extracellular glucose-dependent enhancement of ILA contraction was suppressed by C3-toxin and Y27632, but only in ob/ob mice (Figs. 5 and 6). Similar responses were observed for total rho A activity, and the mRNA level of rho kinase was only increased under HG conditions (Fig.
These findings suggested that the rho A–rho kinase pathway is involved in extracellular glucose-dependent ILA over-contraction in diabetes. In diabetic hyperglycemia, chronic extracellular glucose accumulation induces sustained rho A and rho kinase activation. Activation of the rho A–rho kinase pathway leads to a reduction of myosin-light chain phosphatase activity and/or activation of CPI-17. Both of these effects contribute to ILA contraction (Illustration). Because total PKC activity was unaffected by HG-PSS treatment in our study, we suggest that changes in the rho A–rho kinase pathway have a greater effect on extracellular glucose-dependent ILA over-contraction than do changes in the PKC pathway. The mechanisms by which extracellular glucose activates the rho A–rho kinase pathway and how accumulated glucose influences rho A activity are not yet known. However, some possible mechanisms include protein glycation on plasma membranes and/or direct effects of incorporated glucose. However, the precise mechanisms are still forthcoming. Understanding this mechanism may be the next step in understanding glucose-dependent vascular dysfunction in diabetes.

In this study, we found changes in intrarenal small artery contraction in the early stage of diabetes. These changes were caused by adrenalin receptor-mediated over-contraction of the artery under NG conditions and glucose-dependent enhancement of contraction under HG conditions. In diabetic hyperglycemia, both types of ILA alterations may lead to a reduction in glomerular filtration through a reduction in blood flow at the renal glomerulus. These dysfunctions were only characterized in intrarenal small arteries (i.e. ILA), not in outer-renal arteries (i.e. RA). Our results suggest that calcium-independent PKC is involved in basal over-contraction of the ILA under NG
conditions and rho A–rho kinase pathway is involved in the extracellular-glucose dependence of contraction in the diabetic state. Targeting the mechanisms underlying these two types of renal dysfunction may unveil a new approach to the treatment of diabetic nephropathy.

Acknowledgments
We thank Mr. Tsumita and Mr. T. Yamazaki for technical support.

Conflict of interest
The authors declared no conflicts of interest.

Authorship Contributions
Participated in research design: Nobe and Honda.
Conducted experiments: Takenouchi and Hashimoto.
Contributed new reagents or analytical tools: none.
Performed data analysis: Takenouchi and Kasono.
Wrote or contributed to the writing of the manuscript: Nobe, Kasono and Honda.

References


Footnotes
This study was supported by a Grant-in-Aid for the Encouragement of Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology (MEXT; 21590290, 26460346) in Japan (to KN) and a Private University High Technology Research Center Project matching fund subsidy from MEXT (to KH).
Figure Legends

Fig. 1. Phenylephrine (PE)-induced force development in endothelial-denuded renal (RA) and interlobar (ILA) arterial rings under NG and HG conditions. Isometric force developments in the RA (A) and ILA (B) isolated from kean (circles) and ob/ob (squares) mice were measured as described in the “Materials and Methods”. Vascular tissues were pre-incubated under NG (open) or HG (closed) conditions at 37°C for 30 min. Thereafter, the indicated concentrations of PE were cumulatively added. The dose–response relationships for PE-induced responses in RA and ILA were calculated as µN/mm tissue length. Each value represents the mean ± SEM of five independent determinations. * p < 0.05, ** p < 0.01 vs. the response in normal-PSS. # p < 0.05, ## p < 0.01 vs. lean mice.

Fig. 2. Role of the intracellular calcium concentration to the increases in PE-induced force development in ILA rings isolated from Lean (A) and ob/ob (B) mice. Isolated ILA rings were pre-incubated under NG (open) or HG (closed) conditions and were stimulated with the indicated concentrations of PE. Extracellular calcium was replaced by changing PSS to CaCl\(_2\)-replaced PSS (Ca\(^{2+}\)-free-PSS) 10 min before stimulation (normal calcium, circles; calcium-free, squares). Isometric force was measured as described in the “Materials and Methods”. Under NG and HG conditions, the increases in intracellular calcium following administration of 30 µM PE were measured as the changes in R\(_{340/380}\) using fura-2-loaded ILA (inset). Each value represents the mean ± SEM of five independent determinations. ** p < 0.01 vs. the response in NG-PSS. # p < 0.05, ## p < 0.01 vs. the response under normal-calcium conditions. †† p < 0.01 vs. the...
non-stimulated resting level (*inset*).

**Fig. 3.** Effects of protein kinase C (PKC) inhibitors on PE-induced force development in ILA rings isolated from lean (A) and *ob/ob* (B) mice. Isolated ILA rings were pre-incubated under NG (open) or HG (closed) conditions at 37°C for 30 min and were stimulated with the indicated concentrations of PE. 1 µM rottlerin (squares) or vehicle (PSS; circles) were added 10 min before stimulation. Similarly, 1 µM calphostin C (CC) or 1 µM Gö6976 (Gö) were introduced 10 min before stimulation (*inset*). Isometric force was measured as described in the “Materials and Methods”. Each value represents the mean ± SEM of four independent determinations. ** *p* < 0.01 vs the response in NG-PSS. # *p* < 0.05, ## *p* < 0.01 vs. the response in the absence of the PKC inhibitor. †† *p* < 0.01 vs. the non-stimulated resting level (*inset*).

**Fig. 4.** Effects of rottlerin on PE-induced PKC activity in the lysates of ILA isolated from lean (A) and *ob/ob* (B) mice. Isolated ILA rings were pre-incubated under NG (open) or HG (closed) conditions at 37°C for 30 min and were stimulated with 30 µM PE. Rottlerin (1 µM) was added 10 min before stimulation. PKC activity was measured in the ILA lysates as described in the “Materials and Methods”. Each value represents the mean ± SEM of five independent determinations. ** *p* < 0.01 vs the non-stimulated resting level. ## *p* < 0.01 vs. the PE-induced response. †† *p* < 0.01 vs. lean mice.

**Fig. 5.** Effects of rho kinase inhibitors on PE-induced force development in ILA rings isolated from lean (A) and *ob/ob* (B) mice. Isolated ILA rings were pre-incubated under
NG (open) or HG (closed) conditions at 37°C for 30 min and were stimulated with the indicated PE concentrations. 1 µM Y27632 (squares) or vehicle (PSS; circles) was added 10 min before stimulation. ILA rings were pretreated with C3-exoenzyme (C3-toxin; 100 µg/mL) in the force measurement chamber at 37°C for 12 h and then stimulated with 30 µM PE (inset). Isometric force was measured as described in the “Materials and Methods”. Each value represents the mean ± SEM of five independent determinations. ** p < 0.01 vs. the response in NG-PSS. # p < 0.05, ## p < 0.01 vs. the response in the absence of Y27632. †† p < 0.01 vs the non-stimulated resting level (inset).

**Fig. 6.** Effects of C3-exoenzyme (C3-toxin) on PE-induced rho A activity in ILA isolated from lean (A) and ob/ob (B) mice. Isolated ILA rings were pre-incubated in the presence or absence of 100 µg/mL C3-toxin at 37°C for 12 h. In the final 30 min of the incubation, NG (open) or HG (closed) conditions were established and 30 µM PE was added. Total rho A activity in each sample was measured as described in the “Materials and Methods”. Each value represents the mean ± SEM of five independent determinations. ** p < 0.01 vs. the non-stimulated resting level. ## p < 0.01 vs. the PE-induced response.

**Fig. 7.** Changes in PKCδ (A) and rho kinase (B) mRNA expression levels in ILA isolated from lean and ob/ob mice. Isolated ILA rings were pre-incubated under NG (open) or HG (closed) conditions at 37°C for 30 min. Total mRNA levels in each sample were determined as described in the “Materials and Methods”. Each value represents the
mean ± SEM of five independent determinations. ** p < 0.01 vs. lean mice. ## p < 0.01 vs. NG-PSS.

**Illustration.** Illustration showing the two type of mouse ILA over-contraction in diabetes. The typical charts of PE-induced ILA contraction under NG and HG condition and the suggested intracellular mechanisms are shown. The corresponding figure numbers are also indicated. Abbreviations: α1-AR, α1-adrenaline receptor; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PE, phenylephrine; p-MLC, phosphorylated-myosin light chain.
### TABLE 1

**Body weights and blood parameters in Lean and ob/ob mice**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Lean</th>
<th>ob/ob</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Body Weight</strong></td>
<td>(g)</td>
<td>28.6 ± 1.0</td>
<td>54.0 ± 3.8 *</td>
</tr>
<tr>
<td><strong>P-glucose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting (mg/dL)</td>
<td></td>
<td>134.3 ± 6.9</td>
<td>404.6 ± 5.9 *</td>
</tr>
<tr>
<td>OGTT (mg/dL)</td>
<td></td>
<td>204.0 ± 9.0</td>
<td>408.0 ± 10.4 *</td>
</tr>
<tr>
<td><strong>PL</strong></td>
<td>(µg/dL)</td>
<td>202.8 ± 10.3</td>
<td>288.5 ± 13.1*</td>
</tr>
<tr>
<td><strong>t-Cho</strong></td>
<td>(mg/dL)</td>
<td>104.5 ± 9.0</td>
<td>176.4 ± 7.3 *</td>
</tr>
<tr>
<td><strong>FFA</strong></td>
<td>(µEQ/L)</td>
<td>1178.2 ± 66.7</td>
<td>898.1 ± 52.5</td>
</tr>
<tr>
<td><strong>TG</strong></td>
<td>(µEQ/L)</td>
<td>37.2 ± 2.6</td>
<td>42.7 ± 3.0</td>
</tr>
<tr>
<td><strong>BUN</strong></td>
<td>(mg/dL)</td>
<td>22.8 ± 1.9</td>
<td>23.8 ± 1.3</td>
</tr>
</tbody>
</table>

* p < 0.01 vs. lean mice. # p < 0.01 vs. fasting plasma glucose.
**TABLE 2**

Changes in water intake and urine volume in lean and *ob/ob* mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Lean</th>
<th><em>ob/ob</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Water intake (mL/day)</td>
<td>5.98 ± 0.13</td>
<td>15.28 ± 0.95 *</td>
</tr>
<tr>
<td>Urine volume (mL/day)</td>
<td>4.23 ± 0.49</td>
<td>10.66 ± 0.86 *</td>
</tr>
</tbody>
</table>

* *p* < 0.01 vs. lean mice.
Figure 1
A Lean mouse

B ob/ob mouse

Figure 2
**Figure 3**

A Lean mouse

B ob/ob mouse

Legend:
- **Rest**: Resting condition
- **PE**: Prostaglandin E2
- **CC**: Carbachol
- **G6**: Glucose

Graphs depict isometric force (μN/mm) plotted against PE concentration (μM) for both lean and ob/ob mice. Statistical comparisons are indicated with symbols:
- ****: Significant difference compared to Rest
- **#**: Significant difference compared to PE
- **##**: Significant difference compared to CC
- **###**: Significant difference compared to G6
Figure 4
A  Lean mouse

B  ob/ob mouse

Figure 5
Figure 6
Figure 7

A  PKCδ

B  Rho kinase

Lean mouse  ob/ob mouse

Relative values
Illustration