Augmented Contractility to Noradrenaline in Femoral Arteries from the Otsuka Long-Evans Tokushima Fatty Rat, a Model of Type 2 Diabetes

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Although vasculopathies may occur systemically, there are few reports regarding femoral arteries of type 2 diabetes. Here, we investigated whether contractile response to noradrenaline in femoral arteries would change in type 2 diabetic male Otsuka Long-Evans Tokushima Fatty (OLETF) rat at the chronic stage of disease (1 year old) versus age-matched control Long-Evans Tokushima Otsuka (LETO) rat. OLETF rat exhibited hyperglycemia, hypertension, hyperlipidemia, and hypoinsulinemia compared to age-matched LETO rat. Noradrenaline-induced contraction was increased in femoral arteries in OLETF rats compared with LETO rats whereas serotonin- or phenylephrine-induced contractions were similar between these two animals. Acetylcholine- and sodium nitroprusside-induced relaxations were similar between the two groups. Very small relaxations in femoral arteries induced by clonidine and isoprenaline were obtained in LETO but not OLETF group. Noradrenaline-induced contraction was enhanced by treatment with \( \text{N}^{\text{G}} \)-nitro-\( \text{L} \)-arginine (L-NNA), a nitric oxide synthase (NOS) inhibitor, and the between-group difference of contraction was eliminated by such treatment. Indomethacin, a non-selective cyclooxygenase (COX) inhibitor, reduced noradrenaline-induced contraction in both groups, whereas the contraction was greater in OLETF group versus LETO. Femoral arterial protein expression of endothelial NOS, COX-1, and superoxide dismutases were similar between the two groups, whereas reduction of COX-2 expression was seen in OLETF group compared with LETO. Increased contractile responsiveness to noradrenaline is seen in OLETF rat femoral artery and this may be due to reduction of suppressive effect of NO.

Key words cyclooxygenase (COX); diabetes; endothelial nitric oxide synthase (NOS); femoral artery; contraction

1. INTRODUCTION

The prevention and management of type 2 diabetes are now global issues.\(^{1,2}\) Vascular dysfunction is an important hallmark of type 2 diabetes, occurs systemically, and frequently heralds the development of various complications.\(^{3,4}\) Among systemic diabetic complications, diabetic foot is a chronic and highly disabling complication that affects patients with peripheral vascular disease.\(^{3-6}\) Traditionally, diabetic foot was believed to result from peripheral neuropathy; however, epidemiological evidence suggests that the prevalence of peripheral arterial disease is high in diabetic patients and, regardless of the presence of peripheral neuropathy, can be found in 50% of cases with lower limb lesions.\(^{6-9}\) In general, diabetic foot affects patients with long-term duration of the disease and, because they may also have co-morbidities, they may be particularly fragile and difficult to manage clinically.\(^{6-9}\) Although femoral artery is the main artery to supply oxygenated blood to the lower limb, there are few reports regarding the function of femoral artery in type 2 diabetes\(^{10-14}\) compared with other vessels such as aorta and mesenteric arteries.\(^{15-17}\)

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Otsuka Long-Evans Tokushima fatty (OLETF) rats exhibit stable experimental and pathological characteristics resembling human type 2 diabetes mellitus.\(^{18,19}\) We and others demonstrated that OLETF rats exhibit dysfunction in various arteries including basilar, renal, and mesenteric artery and aorta at various stages of the disease.\(^{16,20-23}\) However, to date, no study has investigated vascular function in femoral arteries of OLETF rats with long-term duration of disease.

The present study was conducted to test the hypothesis that reactivity to noradrenaline would be altered in femoral arteries of OLETF at age 1 year, when they are at the stage of later-phase of the disease\(^{8,24}\) and to evaluate some of the molecular mechanisms related to contractile responses.

2. MATERIALS AND METHODS

2.1. Animals and Assessment of Blood Glucose, Insulin, Lipids, and Blood Pressure

Four-week-old male OLETF (\(n=17\)) and control Long-Evans Tokushima Otsuka (LETO) (\(n=16\)) rats were obtained from Hoshino Laboratory Animals (Ibaraki, Japan). Water and food were given \textit{ad libitum} in a controlled environment (room temperature: 24±1°C, humidity: 50±5%, 12-h light/dark cycle) until the rats were...
1 year old. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the U.S. National Institutes of Health and “Guide for the Care and Use of Laboratory Animals” adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan). The parameters of blood glucose, insulin, lipids (total cholesterol, triglyceride, high-density lipoprotein [HDL] cholesterol, non-esterified fatty acid [NEFA]), and systolic blood pressure were assessed as previously reported. In brief, glucose of non-fasting blood taken from the tail vein under anesthesia was detected by commercially available glucose meter (OneTouch Ultra, LifeScan, a Johnson & Johnson, Milpitas, CA, U.S.A.). Plasma was prepared from arterial blood of non-fasting rats. Plasma insulin was detected by an enzyme immunoassay (Shibayagi Co., Ltd., Gunma, Japan). Plasma levels of total cholesterol, triglyceride, HDL cholesterol, and NEFA were detected by each enzyme kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Systolic blood pressure in rat that had been in a constant-temperature box at 37°C for at least 5 min was measured by tail cuff method (blood pressure analyzer, BP-98A; Softron, Tokyo, Japan).

2.2. Functional Experiments In all experiments, non-fasted rats were anesthetized with isoflurane via a nose cone for surgical procedures and euthanized by thoracotomy and exsanguination. The isometric force of femoral arteries and data analysis were essentially conducted as described previously. The concentration–response curves for noradrenaline (10⁻⁷–10⁻⁴ M), serotonin (10⁻⁹–10⁻⁵ M), and phenylephrine (10⁻⁴–10⁻² M) were obtained in femoral arterial rings.

To investigate the effects of nitric oxide synthase (NOS) or cyclooxygenase (COX) inhibitors on noradrenaline-induced contraction, concentration–response curves for noradrenaline were created in the presence of Nω-nitro-L-arginine (L-NNA) (a NOS inhibitor, 10⁻⁵ M), indomethacin (a non-selective COX inhibitor, 10⁻⁵ M), or NS398 (a selective COX-2 inhibitor, 10⁻⁶ M) before application of noradrenaline for 30 min and thereafter. Femoral arterial contractions are presented as a percentage of the high-K⁺ (80 ms)-induced contraction.

To investigate endothelium-dependent and -independent relaxations, α₁ agonist- and β agonist-induced relaxation, concentration–response curves for acetylcholine (ACH; 10⁻⁹–10⁻⁵ M), sodium nitroprusside (SNP; 10⁻¹⁰–10⁻⁶ M), clonidine (10⁻⁹–10⁻⁵ M), and isoprenaline (10⁻⁶–10⁻³ M) were performed in femoral artery precontracted with serotonin (10⁻⁷–10⁻⁵ M). Femoral arterial relaxation responses are presented as a percentage of the serotonin-induced contraction.

2.3. Immunoblotting and Protein Expression Immunoblotting and data analysis of protein expression were essentially performed as previously reported. Femoral arteries were cleaned, snap-frozen in liquid nitrogen, then homogenized in ice-cold RIPA buffer (ThermoScientific, Rockford, IL, U.S.A.) with protease- and phosphatase-inhibitor cocktails (Roche Diagnostics, Indianapolis, IN, U.S.A.). Equal amounts of protein (30 µg) were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (10%) for Western blotting. Polyvinylidene difluoride membranes were probed for expression of proteins such as phosphorylated endothelial NOS (eNOS) (Ser1177) (1 : 500) (#9571, Cell Signaling Technology, Beverly, CA, USA), eNOS (1 : 1000) (#BD610296, BD Biosciences, San Jose, CA, USA), COX-1 (1 : 1000) (#61019, Cayman Chemical, Ann Arbor, MI), COX-2 (1 : 1000) (#610126, Cayman chemical), EC-superoxide dismutase (SOD) (1 : 1000) (#707-704, Millipore, Billerica, MA, U.S.A.), Cu/Zn SOD (1 : 2000) (#ADI-SOD-101, Enzo Life Sciences, Famingdale, NY, U.S.A.), Mn-SOD (1 : 1000) (#BD615880, BD Biosciences), and β-actin (1 : 5000) (#A5316, Sigma Chemical Co., St. Louis, MO, U.S.A.). Phosphorylated eNOS protein expression was normalized to total eNOS protein expression; all other proteins were normalized to β-actin. The resulting bands were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

2.4. Statistical Analysis Data are expressed as mean±standard error (S.E.). For the functional studies, individual concentration–response curves were analyzed by nonlinear regression curve fitting using Graph Pad Prism (v. 5.0; GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical evaluations were performed using Student’s t-test between two groups. Statistical analyses of concentration–response curves were conducted using two-way ANOVA with repeated measures followed by Bonferroni’s post hoc test. The significance level of all tests was set at α=0.05.

3. RESULTS

3.1. Animal Characteristics (Body Weight, Blood Pressure, and Blood Metabolic Parameters) As shown in Table 1, OLETF rats manifested hypertension, hyperglycemia, hyperinsulinemia, and hyperlipidemia (i.e., high levels of total cholesterol, triglyceride, HDL cholesterol, and NEFA) compared to age-matched LETO rats. Body weight was similar between the two groups (Table 1).

3.2. Femoral Arterial Contraction Induced by Noradrenaline, Serotonin, and Phenylephrine The cumulative application of noradrenaline (10⁻⁹–10⁻⁴ M) (Fig. 1A), serotonin (10⁻⁹–10⁻⁴ M) (Fig. 1B), or phenylephrine (10⁻⁹–10⁻⁴ M) (Fig. 1C) led to concentration-dependent contraction of femoral arteries isolated from OLETF and LETO rats. The contractile response induced by noradrenaline was increased further in OLETF group than in LETO group (Fig. 1A). On the other hand, serotonin (Fig. 1B) and phenylephrine (Fig. 1C)-induced contractions were similar between OLETF and LETO groups.

3.3. Effect of NOS or COX Inhibitors on Noradrenaline Table 1. Values of Body Weight, Blood Pressure, and Blood Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LETO</th>
<th>OLETF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>562.6±10.7 (10)</td>
<td>577.5±32.1 (11)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>115±6 (10)</td>
<td>153±5 (11)*</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>109.6±2.4 (10)</td>
<td>388.6±43.6 (11)*</td>
</tr>
<tr>
<td>Insulin (µg/mL)</td>
<td>2.35±0.19 (10)</td>
<td>1.54±0.16 (10)*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>121.4±4.6 (10)</td>
<td>256.6±24.9 (10)*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>87.0±7.2 (10)</td>
<td>520.0±80.7 (10)*</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>68.5±2.6 (10)</td>
<td>120.5±8.6 (10)*</td>
</tr>
<tr>
<td>NEFA (meq/L)</td>
<td>0.68±0.06 (10)</td>
<td>1.96±0.22 (10)*</td>
</tr>
</tbody>
</table>

Values are means±S.E. Number of determinations within parentheses. *p<0.05 vs. LETO. The value of blood pressure presents at 10 months old and other parameters present at 1 year old. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; SBP, systolic blood pressure; HDL, high-density lipoprotein; NEFA, non-esterified fatty acid.
Fig. 1. Concentration–Response Curves for Noradrenaline (10^{−10}−10^{−4} M) (A), Serotonin (10^{−9}−10^{−4.5} M) (B), and Phenylephrine (10^{−10}−10^{−4} M) (C) in Femoral Arteries of OLETF and LETO Rats; (D–F) Effect of Nitric Oxide Synthase Inhibitor \( \text{N}^\text{G}-\text{Nitro-L-arginine (L-NNA, 10}^{−4} \text{ M)} \) (D), Non-specific Cyclooxygenase (COX) Inhibitor Indomethacin (10^{−5} M) (E), and Selective COX-2 Inhibitor NS398 (10^{−6} M) (F) on Noradrenaline-Induced Contraction in Femoral Arteries

Data are mean±S.E.; \( n=6–11 \). * \( p<0.05 \) vs. corresponding LETO.

Fig. 2. Concentration–Response Curves for Acetylcholine (ACh; 10^{−9}−10^{−5} M) (A), Sodium Nitroprusside (SNP; 10^{−10}−10^{−7} M) (B), Clonidine (10^{−9}−10^{−5} M) (C), and Isoprenaline (10^{−9}−10^{−5} M) (D) in Femoral Arteries of OLETF and LETO

Data are mean±S.E.; \( n=6–11 \). * \( p<0.05 \) vs. LETO.
Since NO and/or prostanoids play important roles in the control of vascular tone on stimulation with endogenous contractile ligands, we performed measurements of concentration–response curve for noradrenaline in the presence of L-NNA (an inhibitor of NOS, $10^{-4}$ M) (Fig. 1D), indomethacin (an inhibitor of COX, $10^{-5}$ M) (Fig. 1E), or NS398 (an inhibitor of COX-2, $10^{-6}$ M) (Fig. 1F) to evaluate the contribution of NO or prostanoids to the increased noradrenaline-induced contraction observed in femoral arteries from OLETF rats. On incubation with L-NNA, noradrenaline-induced contraction was similar between OLETF and LETO groups (Fig. 1D), namely, the difference of the contraction was abolished by L-NNA. On the other hand, in incubation with indomethacin or NS398, the difference of the noradrenaline-induced contraction was not abolished and such contraction was greater in the arteries.

**Fig. 3.** Protein Expression of Phospho- and Total-eNOS and COXs in Femoral Arteries of OLETF and LETO Rats

*Upper:* Representative immunoblot bands. *Lower:* Corresponding densitometric analysis presenting expression of phospho-eNOS (Ser1177)/eNOS (A), COX-1/β-actin (B), and COX-2/β-actin (C). Data are mean±S.E.; $n=6$. *p<0.05, OLETF vs. LETO.*

**Fig. 4.** Protein Expression of SODs in Femoral Arteries of OLETF and LETO Rats

*Upper:* Representative immunoblot bands. *Lower:* Corresponding densitometric analysis presenting the expression of EC-SOD/β-actin (A), Cu/Zn-SOD/β-actin (B), and Mn-SOD/β-actin (C). Data are mean±S.E.; $n=6$. *p<0.05, OLETF vs. LETO.*
from OLETF group than in those from LETO group (Figs. 1E, 1F).

3.4. Femoral Arterial Relaxation Induced by ACh, SNP, Clonidine, and Isoprenaline
The cumulative application of ACh (10^{-9}–10^{-3}M) (Fig. 2A) led to a concentration-dependent relaxation of femoral arteries isolated from OLETF and LETO rats whereas higher concentrations (i.e., >10^{-6}M) tended to induce contraction. The response induced by ACh was similar between OLETF and LETO groups. SNP (10^{-10}–10^{-5}M)-induced relaxation was similar between OLETF and LETO groups (Fig. 2B). In some vasculatures, clonidine (α2 agonist) or isoprenaline (β1 agonist) led to relaxation as shown in Figs. 2C and D, very small relaxations were induced by clonidine (Fig. 2C) or isoprenaline (Fig. 2D) in LETO group but not OLETF group.

3.5. Protein Expression in Femoral Arteries
As shown in Fig. 3, no differences existed between OLETF and LETO femoral arteries in terms of protein levels of the phosphorylated eNOS (p-eNOS/total eNOS, p>0.05; Fig. 3A) and COX-1 (p>0.05; Fig. 3B). OLETF femoral arteries had reduced protein levels of COX-2 (p<0.05; Fig. 3C).

We hypothesized that SODs expression may reduce in OLETF femoral arteries because SODs or their modulators could modulate noradrenaline-induced contractile responses under some conditions. As shown in Fig. 4, no differences existed between OLETF and LETO femoral arteries in terms of protein levels of EC-SOD (p>0.05; Fig. 4A), Cu/Zn-SOD (p>0.05; Fig. 4B), and Mn-SOD (p>0.05; Fig. 4C).

4. DISCUSSION
OLETF rats manifest stable clinical and pathological features reflecting human type 2 diabetes. Reportedly, OLETF rats are characterized by: 1) increasing body weight just after weaning; 2) late onset of hyperglycemia after approximately 18 weeks of age and diagnosable diabetes after 24 weeks of age; and 3) hyperinsulinemia that is present at 24 weeks of age and declines after approximately 55 weeks of age, with conversion to insulin-dependent diabetes after 40 weeks of age. Using this model, several reports by us and others demonstrated that altered vascular function is seen in various arteries such as aorta and basilar, mesenteric, and renal arteries at various stages of disease progression.

The main finding of the present study is that the increased contractile responsiveness to noradrenaline but not serotonin is seen in femoral artery of type 2 diabetic OLETF rat and this abnormality may be attributable to impairment of counteracting effect of NO rather than COX-derived prostanoids. Noradrenaline and serotonin play pivotal roles in the regulation of vascular tone under (patho)physiological conditions. In femoral arteries, some reports have suggested abnormal responsiveness to noradrenaline or serotonin in various disease conditions including diabetes and hypertension. In the present study, femoral arteries isolated from OLETF rats at age 1 year exhibit hyper-responsiveness to noradrenaline but not serotonin, another endogenous vasoconstrictor substance, compared to age-matched control LETO rats. These results suggest that adrenergic contractile contraction is augmented in femoral artery of OLETF rat at chronic stage of the disease. In the present study, an agonist of α1 receptor phenylephrine-induced contraction did not differ between OLETF and LETO groups. Moreover, small relaxations induced by α1 agonist clonidine and β1 agonist isoprenaline were observed in arteries of LETO rats but not of OLETF rats. Since noradrenaline is an endogenous ligand for adrenoceptor including α and β receptors, the differences of femoral arterial contraction induced by noradrenaline may be attributable to alterations of components in each adrenoceptor stimulation. Indeed, this is supported by several reports suggesting that β1- and β2-adrenoceptors contribute to noradrenaline-mediated relaxation in the femoral artery and aorta, respectively, of young rats, and noradrenaline-mediated rat aortic relaxation depends on endothelial β receptor and this was impaired by ageing and hypertension.

Vascular contractile responses are modulated by various endogenous substances such as NO and prostanoids. Indeed, NO could suppress noradrenaline-induced contraction and inhibition of NOS could augment noradrenaline-induced contraction in various arteries. Judging from the effects of 1-NNa, NO suppressed noradrenaline-induced femoral arterial contraction, and this suppressive effect of NO is diminished in OLETF femoral artery. In endothelial cells, activities of eNOS are tightly regulated by various pathways including phosphorylation of various sites, interactions with co-factors, and dimerization. In the present study, no alterations of the expression of total eNOS and phosphorylated eNOS (Ser1177), which is one of activated form of eNOS under basal condition, were noted. However, future investigations are required including above eNOS regulations and levels of NO metabolites under basal and stimulation with noradrenaline. Prostanoids also affect contractile responses. For example, inhibition of COX suppressed contractile responses induced by adrenergic agonists in various arteries and we very recently found that prostanooids could contribute to increased noradrenaline-induced contraction in femoral arteries from spontaneously hypertensive rats.

Moreover, Shi and Vanhoutte found that enhanced phenylephrine-induced contraction in streptozotocin-induced diabetic femoral artery was attributable to increased oxidative stress and COX-derived vasoconstrictor prostanoids. In the present study, however, we found that: 1) treatment with indomethacin did not abolish the difference of noradrenaline-induced contraction between the two groups; 2) COX-1 expression was similar; and unexpectedly, 3) COX-2 expression was decreased in OLETF group. Since the effect of indomethacin on noradrenaline-induced contractile response seems larger in LETO rats than in OLETF rats, the possibility that the inhibitory effect of a COX-2 derived factor on contraction is reduced in OLETF rats may not be excluded. COX-2-derived prostanoids may largely affect noradrenaline-induced contraction and this underlies the increased contraction in the OLETF group. In the present study, a specific COX-2 inhibitor NS398 did not affect noradrenaline-induced contraction, and contraction was still increased in the arteries of OLETF rats than in those of LETO rats. Therefore there results imply that the component of COX-2 under noradrenaline stimulation was not determinant for the difference in femoral arterial contractions induced by noradrenaline between OLETF and LETO rats. Moreover, femoral arterial expression of eNOS and SODs was similar between OLETF and LETO groups. These results suggest that prostanooids were not contributory factors to augment noradrenaline-induced contraction in diabetic OLETF femoral artery. These discrepancies
between previous evidence and the present study might be due to differences in the models of diabetes, duration and severity of disease, and in the artery studied. However, further investigation is required on time-dependent changes in femoral arterial contraction and direct relationship among adrenocorticotropin subtypes, NO, prostanooids, and oxidative stress in femoral artery of this model following noradrenaline stimulation.

In the present study, we found no difference of ACh- and SNP-induced femoral arterial relaxation between OLETF and LETO rats. Apparent similar response to ACh might be attributable to compensation among NO, endothelium-derived hyperpolarization factor EDHF, and endothelium-derived contracting factor (EDCF) in OLETF femoral arteries because the arteries could release not only NO but also EDHF(F) or EDCF. Although NO could affect noradrenaline-induced femoral arterial contraction, future investigation is required to determine as to what extent of NO, EDHF(F), and EDCF signaling were activated by noradrenaline stimulation and contribute to contraction induced by noradrenaline in arteries from both rats.

In conclusion, an enhanced noradrenaline-induced contraction is seen in femoral artery of type 2 diabetic OLETF rat. Evidence of vascular responsiveness to various endogenous ligands in femoral artery in type 2 diabetes is lacking. In the present study, we first demonstrated that augmented femoral arterial contraction is induced by noradrenaline but not serotonin in type 2 diabetic OLETF femoral arteries. Although further investigations are required to elucidate the detailed molecular mechanisms and causative factor(s) underlying augmented noradrenaline-induced contraction, we believe that this finding could help in the management of peripheral arterial diseases associated with chronic type 2 diabetes.

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Conflict of Interest The authors declare no conflict of interest.

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