Role of Glyceraldehyde-Derived AGEs and Mitochondria in Superoxide Production in Femoral Artery of OLETF Rat and Effects of Pravastatin

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A complication of diabetes mellitus is the over-production of vascular superoxides, which contribute to the development of arteriosclerosis and peripheral arterial disease (PAD). Hyperglycemia induces the formation and accumulation of advanced glycation end-products (AGEs), which in turn stimulate vascular superoxide production. The mechanism underlying AGE-mediated vascular superoxide production remains to be clarified in lower limb complications associated with diabetes. In the present study, we investigated the role of AGEs and the mitochondrial respiratory complex in superoxide production in femoral arteries using the type 2 diabetes model Otsuka Long-Evans Tokushima Fatty (OLETF) rats [vs. non-diabetic Long-Evans Tokushima Otsuka (LETO) rats]. The effects of in vivo administration of pravastatin on superoxide production in femoral arteries were also examined. Using chemiluminescent assays, luminescence microscopy, and competitive enzyme-linked immunosorbent assay (ELISA), we determined that vascular superoxide production and serum glyceraldehyde-derived AGEs (Glycer-AGEs) increased in OLETF rats. Pravastatin inhibited these responses without changing serum total cholesterol concentrations. The mitochondrial complex II inhibitor thenoyltrifluoroacetone (TTFA) also inhibited vascular superoxide production. Application of Glycer-AGEs in situ increased superoxide production in the vascular wall of femoral arteries from pravastatin-treated OLETF rats, which was then inhibited by TTFA. These results suggest that hyperglycemia increases serum Glycer-AGEs, which subsequently induce superoxide production in the femoral artery of OLETF rats in a mitochondrial complex II-dependent manner. Collectively, our results have partially elucidated the pathological mechanisms leading to diabetes-related PAD, and indicate dual beneficial actions of pravastatin for the prevention of oxidative damage to the vascular wall.

Key words type 2 diabetes; superoxide; mitochondria; advanced glycation end-product; Otsuka Long-Evans Tokushima Fatty rat; pravastatin

Diabetes is a major independent risk factor for peripheral arterial disease (PAD), in part due to oxidative stress induced by chronically elevated blood glucose levels. Excessive generation of reactive superoxide anions in vascular cells can arise from defects in the enzymatic processes of the mitochondrial respiratory chain and nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, as well as uncoupled endothelial nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (eNOS). Hyperglycemia propagates the accumulation of advanced glycation end-products (AGEs), which increases superoxide production and the susceptibility for vascular endothelial dysfunction and arteriosclerosis. The binding of toxic glyceraldehyde-derived AGEs (Glycer-AGEs) to their receptor (RAGE) induces vascular superoxide production via protein kinase C (PKC)-activated NAD(P)H oxidase, contributing to diabetic complications such as atherosclerosis, nephropathy, and retinopathy. Interestingly, in bovine aortic endothelial cells, it was found that hyperglycemia increased AGE production, sorbitol production, PKC activity, and intracellular superoxide production; however, these could be reversed by the mitochondrial complex II inhibitor thenoyltrifluoroacetone (TTFA), suggesting that the mitochondrial respiratory chain mediates AGE-induced vascular superoxide production. The present study aimed, in part, to further clarify the mechanism underlying hyperglycemia-induced intracellular superoxide production in vascular cells.

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an established model of human type 2 diabetes. We previously found that superoxide production increased in the aorta and left anterior descending coronary artery (LAD) of OLETF rats in comparison to the non-diabetic Long-Evans Tokushima Otsuka (LETO) rats, which was attributed to heightened NAD(P)H oxidase activity and elevated levels of uncoupled eNOS in the vascular endothelium. Similarly, superoxide production was shown to increase by activation of the NAD(P)H oxidase and uncoupled eNOS in the femoral artery of streptozotocin (STZ)-treated diabetic rats, which lack insulin and, thus, experience chronically elevated blood glucose levels (that being considered a type 1 diabetes model).

Hyperlipidemia due to the metabolic impairment associated with obesity and type 2 diabetes is another major risk factor for PAD. Statin drugs, which inhibit 3-hydroxy-3-methylglutaryl (HMG)-CoA and reduce plasma cholesterol, are commonly prescribed to reduce cardiovascular complications in susceptible individuals. Statins are used to prevent and treat intermittent claudication in hypercholesterolemic patients with prior myocardial infarction or angina pectoris, and to reduce

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regional arterial stiffness in type 2 diabetes. Statins were found to reduce superoxide production in the femoral arteries of atherosclerotic swine through inhibition of NAD(P)H oxidase.

To further elucidate the effects of statins on vascular superoxide production in diabetes-induced PAD, the following parameters were investigated in hyperglycemic OLETF vs. age-matched LETO rats: (i) superoxide production within vascular wall of the femoral artery, (ii) the effect of Glycer-AGEs on mitochondrial respiration and superoxide production, and (iii) pravastatin-mediated effects on serum concentrations of Glycer-AGE and vascular superoxide production.

MATERIALS AND METHODS

Experimental Animals The present study was performed in accordance with the Guidelines for the Conduction of Animal Experiments issued by Nagoya City University and was approved by the Committee on the Ethics of Animal Experiments within that institution. Male OLETF (n=40) and LETO (n=20) rats were obtained from the Tokushima Research Institute, Otsuka Pharmaceutical Co. (Tokushima, Japan). The rats were fed standard laboratory chow (CE-2;CLEA Japan, Inc., Tokyo, Japan) and given free access to drinking water. They were housed in pairs in a specific pathogen-free facility with controlled temperature (23±2°C) and a 12-h light/dark cycle. From 20 weeks of age, half of the OLETF rats received pravastatin (Daichi-Sankyo Co., Tokyo, Japan) at a dose of 100 mg/kg/d for 8 weeks in their drinking water.

Prior to tissue collection, rats were fasted overnight, anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Osaka, Japan), and then euthanized via exsanguination. The femoral artery was immediately excised, placed in Krebs solution, and the connective tissue was removed. Then, the artery was stored in Krebs solution at 4°C, until analyzed.

Blood samples were collected from fasted rats to determine the concentrations of glucose and glycated hemoglobin (HbA1c), as well as serum insulin, triglyceride, total cholesterol, high-density lipoprotein (HDL)-cholesterol, and low-density lipoprotein (LDL)-cholesterol.

Biochemical Analysis

Measurement of Blood Parameters

Glucose and HbA1c concentrations were quantified in whole blood. Blood glucose levels were determined using a glucose test meter (Sanwa Kagaku Kenkusho Co., Ltd., Nagoya, Japan), while HbA1c was measured using a DCA Vantage analyzer (Siemens Healthcare Diagnostics Inc., Camberley, U.K.). Serum insulin concentrations were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (AKRIN-010T, Shibayagi, Gunma, Japan), and the homeostasis model assessment of the insulin resistance (HOMA-IR) score was calculated as fasting serum insulin (mIU/mL)×fasting serum glucose (mm) being divided by 22.5. The serum triglyceride content was measured via colorimetric assay. The total plasma cholesterol and LDL-cholesterol were determined using an enzymatic assay while the HDL-cholesterol was measured using a direct method.

Measurements of Glycer-AGEs Serum Glycer-AGEs were measured using a competitive ELISA using an immunopurified anti-Glycer-AGE antibody as previously described. Briefly, 96-well microtiter plates were coated with 1 µg/mL Glycer-AGE-bovine serum albumin (BSA) and incubated overnight at 4°C. Wells were washed three times with 0.3 mL of phosphate-buffered saline (PBS)-Tween-20 (PBS-Tween-20). After washing with PBS-Tween-20, test samples (50 µL) were added to each well as a competitor for 50 µL of anti-Glycer-AGE antibody (1:1,000), followed by incubation for 2 h at room temperature (approximately 22°C) with gentle shaking on a horizontal rotary shaker. Then, the wells were washed with PBS-Tween-20 and developed with an alkaline phosphatase-linked anti-rabbit immunoglobulin G utilizing p-nitrophenyl phosphate as the colorimetric substrate. Results are expressed as Glycer-AGE units (U) per milliliter of serum, where 1 U corresponds to 1 µg of Glycer-AGE-BSA standard. Sensitivity and intra- and inter-assay coefficients of variation were 0.01 U/mL, 6.2, and 8.8%, respectively.

Investigation of Superoxide Production and Modulation

Superoxide Measurement Using Chemiluminescence

Superoxide production in femoral arteries (6-mm-long ring segments) was examined using a superoxide-sensitive chemiluminescent dye (L-012; 100 µM, Wako Pure Chemical Industries Ltd., Osaka, Japan), as previously described. Briefly, the arteries were first equilibrated in Krebs solution gassed with 95% O2/5% CO2 for 30 min at 37°C. A scintillation vial containing modified Krebs–2-[4-(2-hydroxy ethyl)-1-piperaziny]ethane sulfonic acid (HEPES) buffer solution with L-012 dye was placed into a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany) to determine the background signal. Arterial segments were then separately placed into scintillation vials, and chemiluminescent signals were traced continuously for 30 min at 37°C. The average luminescence intensity for each sample was calculated from 5-min intervals where the signal was most stable. Arterial segments were then retrieved, and the protein content of each was measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The chemiluminescence signal was subsequently normalized to protein concentration [relative light unit (RLU)µg protein].

Superoxide Measurement Using Fluorescence Dye

Superoxide production in femoral artery segments stored in optimal cutting temperature (OCT) compound was additionally determined using dihydroethidium oxidative-fluorescence dye (Molecular Probes, OR, U.S.A.), as described previously. Images were obtained using a confocal-laser-scanning microscope system (LSM 510, Carl Zeiss, Jena, Germany). The fluorescence intensity in each section was measured from 10 randomly selected regions (8×8 pixels) and averaged using a digital image analyzer software (ImageJ, National Institutes of Health [NIH], Bethesda, MD, U.S.A.).

Modulation of Superoxide Production by Thenoyltrifluoroacetone (TTFA)

Segments of the femoral artery from OLETF and pravastatin-treated OLETF rats stored in OCT compound were used for these experiments. To evaluate the effect of the specific mitochondrial complex II inhibitor thenoyltrifluoroacetone (TTFA, Sigma-Aldrich Chemical Corp., MO, U.S.A.), arterial sections were pre-incubated with Krebs–Henseleit buffer solution in a CO2 incubator for 40 min at 37°C in the presence or absence of TTFA (10 µM). Dihydroethidium fluorescence intensity in the segment was then measured using a confocal-laser-scanning microscope system as described above.

Assessment of TTFA Activity with and without Pravastatin
118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, to obtain pH 7.3–7.4. The Krebs–Henseleit buffer contained 4.7 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgSO₄, 20 mM HEPES, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose (pH 7.4).

The modified Krebs–HEPES buffer contained 99 mM NaCl, 4.7 mM KCl, 19 mM CaCl₂, 1.2 mM MgSO₄, 20 mM Hepes, 1.03 mM KH₂PO₄, 25 mM NaHCO₃, and 11.1 mM glucose (pH 7.4).

Pre-treatment

Segments of the femoral artery from pravastatin-treated OLETF rats stored in OCT compound were used for these experiments. Artery segments were pre-incubated with Krebs–Henseleit buffer solution containing Glycer-AGE-BSA (1 mg/mL), non-glycated control BSA (vehicle, 1 mg/mL), or Glycer-AGE-BSA (1 mg/mL) plus TTFA (10 µM) in a CO₂ incubator at 37°C for 60 min. Dihydroethidium fluorescence intensity in the segment was then measured using a confocal-laser-scanning microscope system (as described above).

**Solutions** The composition of the Krebs solution was as follows: 137.4 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 15.5 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11.5 mM glucose. It was bubbled with 95% oxygen and 5% carbon dioxide to obtain pH 7.3–7.4. The Krebs–Henseleit buffer contained 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose (pH 7.4). The modified Krebs–HEPES buffer contained 99 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgSO₄, 20 mM Hepes, 1.03 mM KH₂PO₄, 25 mM NaHCO₃, and 11.1 mM glucose (pH 7.4).

**Preparation of Experimental Reagents** Dihydroethidium and TTFA were dissolved in dimethyl sulfoxide and ethanol, respectively to prepare stock solutions. All other drugs and chemical reagents were dissolved in ultrapure Milli-Q water (Japan Millipore Corp., Tokyo, Japan). The stock solutions were stored at −80°C and daily working solutions were diluted in Krebs–Henseleit buffer or modified HEPES-buffered solution.

**Statistical Analysis** All results were reported as the mean±standard error of the mean (S.E.M.), and n values represent the number of rats used (each rat provided only one arterial segment for a given experiment). Multiple comparisons were analyzed using a one-way ANOVA followed by Bonferroni’s correction. Two-group comparisons were analyzed using paired t-tests, and the statistical significance was defined as p<0.05.

**RESULTS**

**Metabolic Characteristics of LETO, OLETF, and Pravastatin-Treated OLETF Rats** The body weight of the OLETF rats at 28 weeks of age was greater than that of the age-matched LETO rats (Table 1). Similarly, fasting blood glu-
slightly raised LDL-cholesterol concentrations, were not modified by pravastatin administration in OLETF rats.

**Superoxide Production** Superoxide production was significantly higher in the OLETF rats than it was in the LETO and pravastatin-treated OLETF rats (Fig. 1A). Vascular wall superoxide production was significantly greater in OLETF rats than that in LETO rats; however, this was significantly reduced by pravastatin administration (Fig. 1B). TTFA administration attenuated vascular wall superoxide production in OLETF rats but had no effect on pravastatin-treated OLETF rats (Fig. 2).

**Serum Glycer-AGEs** Serum Glycer-AGEs was significantly higher in OLETF rats than that in LETO rats was. Administration of pravastatin to OLETF rats normalized serum Glycer-AGEs to the equivalent levels of LETO rats (Fig. 3).

**Glycer-AGE-Derived Superoxide Production and Effect of TTFA in Pravastatin-Treated OLETF Rats** Acute application of Glycer-AGEs increased superoxide production in the vascular rings from the pravastatin-treated OLETF rats, in comparison to those administered the vehicle (Fig. 4). Co-application of TTFA inhibited Glycer-AGE-stimulated superoxide production by approximately 35%.

DISCUSSION

The male OLETF rat, which is an established model of diabetes mellitus, displays characteristics of type 2 diabetes including obesity, hyperlipidemia, hyperglycemia, insulinis, and glycosuria. We found that body weight, blood HbA1c, blood glucose, serum insulin, and HOMA-IR scores were greater in male OLETF rats at 28 weeks of age than those in age-matched LETO rats were. Furthermore, serum triglyceride and total cholesterol levels were higher in OLETF rats than in LETO rats, indicating that dyslipidemia contributed to insulin resistance and impaired glucose homeostasis. Under these conditions, superoxide production in the femoral artery was three times higher in OLETF rats than it was in age-matched LETO rats. These results are consistent with the previous findings in the aorta and LAD of OLETF rats at a similar age.
Hyperglycemia induces the formation and accumulation of serum Glycer-AGEs that bind to RAGE, which subsequently activates PKC and NAD(P)H oxidase, increasing intracellular superoxide production.\(^3,4,14,23\) It was previously established that OLETF rats at 34 weeks of age show elevated levels of AGEs, RAGE mRNA and protein, 8-hydroxydeoxyguanosine (an oxidative stress marker), and two membrane components of NADPH oxidase in the thoracic aorta, compared to age-matched LETO control rats.\(^3\) This study confirmed that the serum Glyc-AGE concentration increased in 28-week-old OLETF compared to that in LETO controls and that acute Glyc-AGE treatment increased superoxide production in the femoral artery of pravastatin-treated rats. These results suggest that in hyperglycemia, the accumulation of highly toxic Glyc-AGEs may be responsible for intracellular superoxide production in the femoral artery.

Intracellular superoxide is generated in mitochondrial complexes, especially in complex I and at the interface between ubiquinone and complex III where electrons are transferred.\(^2,24–26\) High glucose increases the amount of glucose that flows into cells, which facilitates glycolysis and increases pyruvate production. The tricarboxylic acid cycle is accelerated by increased pyruvate and enhanced production of NADH and 1,5-dihydro-FAD (FADH\(_2\)). Consequently, the increased supply of electrons to complex I and II by NADH and FADH\(_2\), respectively forms a proton electrochemical gradient generated by the enhancement of the electron transport chain system, which increases superoxide production.\(^3\) It was found that in bovine aortic endothelial cells, high glucose increases superoxide production and this was inhibited by TTFA (a specific inhibitor of mitochondrial complex II)\(^27,28\) and carbonyl cyanide \(m\)-chlorophenylhydrazone (an uncoupling agent) but not by rotenone (a specific inhibitor of mitochondrial complex I).\(^3\) Another study investigated the effects of rotenone on OLETF rat aorta and confirmed that inhibition of mitochondrial complex I did not attenuate superoxide production.\(^4\) However, mitochondrial reactive oxygen species (ROS) were reported to increase in the aortic and cardiac preparations from OLETF rats at 42 weeks of age.\(^29\) Our study confirmed that inhibition of complex II by TTFA reduced superoxide production in the femoral artery of OLETF rats. We also found that Glyc-AGEs increased superoxide production in the femoral artery from pravastatin-treated rats and this effect was inhibited by TTFA. Taken together, these results suggest that mitochondrial complex II plays a prominent role in the increased superoxide production observed in femoral arteries of OLETF rats. It was previously noted that superoxide production in the aorta of OLETF rats was enhanced by the PKC activator, phorbol 12,13-butyrate and that this effect could be inhibited by the NAD(P)H oxidase inhibitor apocynin,\(^14\) suggesting that NAD(P)H oxidase activity may also contribute to increased superoxide production in femoral artery in OLETF rats. In the present study, administration of pravastatin to OLETF rats reduced serum concentrations of Glyc-AGEs as well as superoxide production in the femoral artery, without significant modulation of blood lipid or glycemic parameters. Taken together, these results indicate that pravastatin inhibited vascular superoxide production independent of its cholesterol-lowering activity. We previously found that pravastatin reduced superoxide production by both normalizing eNOS uncoupling and inhibiting NAD(P)H oxidase in LAD of OLETF rats.\(^12,14\) Thus, it remains to be clarified whether these mechanisms also contribute to the pravastatin-sensitive inhibition of superoxide production in the femoral artery of OLETF rats.

CONCLUSION

In conclusion, we found that the hyperglycemia-associated increase in serum Glyc-AGEs enhanced superoxide production in the femoral artery of OLETF rats, via a mitochondrial complex II-dependent mechanism. We also suggest that the cholesterol-lowering drug pravastatin may have a novel use in the treatment of PAD due to its protective effects against vascular oxidative damage, possibly by the inhibition of Glyc-AGE production.

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Conflict of Interest The authors declare no conflict of interest.

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