Aortic Superoxide Production at the Early Hyperglycemic Stage in a Rat Type 2 Diabetes Model and the Effects of Pravastatin

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Endothelium-derived superoxide induces vascular dysfunctions. The aim of this study was to examine the activity of protein kinase C (PKC) isoforms and endothelial nitric oxide synthase (eNOS), which leads to vascular superoxide production in type 2 diabetes, in addition to the effects of pravastatin. We studied these mechanisms in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (type 2 diabetes model) at the early hyperglycemic stage (vs. non-diabetic Long-Evans Tokushima Otsuka [LETO] rats). Superoxide production and catalase activity were measured in aortas, as were the protein expressions of PKC_δ_ and phospho-Ser^{1177} eNOS. Superoxide production was increased in OLETF rats, and this increase was inhibited by the selective conventional PKC (ePKC) inhibitor Go6976 and by the non-selective cPKC and novel PKC inhibitor GF109203X. Phospho-Ser^{1177} eNOS was significantly increased in OLETF rats, whereas the protein expressions of PKC_δ_ and phospho-Thr^{385} PKC_δ_ and catalase activity were all greatly reduced. Pravastatin administration to OLETF rats in vivo had normalizing effects on all of these variables. The increment in superoxide production seen in OLETF rats (but not the production in pravastatin-treated OLETF rats) was abolished by high concentration of N^{ω}-nitro-L-arginine methyl ester (electron transport inhibitor of eNOS), by sepiapterin (precursor of tetrahydrobiopterin), and by LY294002 (phosphatidylinositol 3-kinase [PI3-kinase] inhibitor). In OLETF rats at the early hyperglycemic stage, aortic superoxide production is increased owing to activation of uncoupled eNOS through phosphorylation by PI3-kinase/Akt. This may be related to the observed reduction in PKC/catalase activities. Pravastatin inhibited endothelial superoxide production via normalization of PKC/catalase activities.

Key words hyperglycemia; superoxide; protein kinase C; endothelial nitric oxide synthase; Otsuka Long-Evans Tokushima Fatty rat; pravastatin

Diabetes is an important risk factor for the development of coronary artery disease. Endothelial dysfunction is an independent predictor of adverse cardiovascular events, and an increased vascular superoxide production has been suggested to play a pivotal role in the development of endothelial dysfunction in diabetes in humans. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase, mitochondrial electron transport chains, and endothelial nitric oxide synthase (eNOS) have been proposed as possible sources of the superoxide generated in vascular cells in diabetes. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an established model of human type 2 diabetes, and previous comparisons with its normal counterpart (the non-diabetic Long-Evans Tokushima Otsuka [LETO] rat) have revealed increased superoxide production in the aorta at ca. 30 weeks of age. However, the source of this superoxide production and the underlying mechanism remain to be clarified in diabetic humans and animals.

The members of the protein kinase C (PKC) family of signal transducers are characterized by a dependence upon lipids for their activity. Specifically, the conventional PKCs (cPKCs, ePKC_ε_, and ePKC_β_), and the novel PKCs (nPKCs) bind to and are activated by diacylglycerol (DAG) and Ca^{2+}, while the novel PKC isoforms (nPKC_δ_, nPKC_ζ_, and nPKC_θ_) are all capable of binding to DAG but are independent of Ca^{2+}. Hyperglycemia activates PKCs through an increase in the de novo synthesis of DAG, and recent the presence of 2 cPKCs isoforms (PKC_α_ and PKC_β_δ_) and 2 nPKCs isoforms (PKC_ζ_ and PKC_θ_) has been reported in rat aorta. PKCs increase vascular superoxide production through the phosphorylation of p47^{phox} (a component of NADPH oxidase). Raising the possibility that, in vascular cells in diabetes, superoxide may be generated through an activation of PKC-NADPH oxidase. However, accumulated evidence also suggests that "uncoupled eNOS" may be a more likely source for such superoxide generation; indeed, eNOS produces superoxide rather than nitric oxide (NO) under tetrahydrobiopterin (BH_4)-depleted conditions (referred to as "uncoupled eNOS"). Thus, to understand vascular superoxide production in type 2 diabetes, we need to understand the roles played by PKC isoforms and "uncoupled eNOS." Clinical use of pravastatin, an inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (statin), has reduced the risk of coronary heart disease in Japan. The usefulness of HMG-CoA reductase inhibitors for the secondary prevention of cardiovascular diseases has also been demonstrated in diabetic patients. Thus, it seems likely that in type 2 diabetes, an inhibition of vascular superoxide production by pravastatin makes a significant contribution to the normalization of endothelial functions that is seen with this agent and that this inhibition may thereby help to reduce the adverse cardiovascular events associated with this disease state. However, the mechanism underlying the pravastatin-induced...
inhibition of vascular superoxide production in type 2 diabetes remains to be fully clarified.

To address the above issues, we first explored pharmacologically which type of PKC isoform (cPKCs or nPKCs) might be responsible for increased vascular superoxide production in OLETF rats. Then, we examined the effects of agents that modulate the action of “uncoupled eNOS”—such as N^\text{\textregistered}-nitro-L-arginine methyl ester (L-NAME, electron transport inhibitor of eNOS), sepiapterin (a precursor of BH4), and LY294002 (phosphatidylinositol 3-kinase [PI3-kinase] inhibitor)—on aortic superoxide production. The effects of these agents were also examined in OLETF rats that had received long-term (8 weeks) treatment with pravastatin in vivo. Finally, changes in the protein expression of PKC\_\text{\textdelta} and catalase activity were measured in LETO, OLETF, and pravastatin-treated OLETF rats (to clarify the roles of H\_2O\_2 in the relationship between eNOS phosphorylation at Ser\_\text{\textsuperscript{1177}} and superoxide production).

**MATERIALS AND METHODS**

**Animals** All animal care and experimental procedures performed in this study conformed to Guidelines on the Conduct of Animal Experiments issued by Nagoya City University and were approved by the Committee on the Ethics of Animal Experiments in that institution. Male OLETF rats and LETO rats (genetic control for OLETF rats) were obtained from Tokushima Research Institute, Otsuka Pharmaceutical Co. (Tokushima, Japan) (total number used being 58). The rats were fed standard laboratory chow (MF; Oriental Yeast Co., Tokyo, Japan) and were housed individually in a temperature- and light-controlled room (around 23°C, 12-h light/dark cycles) with free access to water. From 20 weeks of age, some of the OLETF rats were given pravastatin (100 mg·kg\_1·d\_1) with free access to water. From 20 weeks of age, some of the OLETF rats that had received long-term (8 weeks) treatment with pravastatin in vivo. Finally, changes in the protein expression of PKC\_\text{\textdelta} and catalase activity were measured in LETO, OLETF, and pravastatin-treated OLETF rats (to clarify the roles of H\_2O\_2 in the relationship between eNOS phosphorylation at Ser\_\text{\textsuperscript{1177}} and superoxide production).

**Western Blot Analysis** Western blotting was carried out as described elsewhere. 18–20 Briefly, aortic strips were quick-frozen with 10% trichloroacetic acid (TCA) in acetone-dry ice, then allowed to reach room temperature. They were then washed with acetone to remove residual TCA. The strips were homogenized in sample buffer (62.5 m\text{m} Tris–HCl [pH 6.8], 10% glycerol, and 2% sodium dodecyl sulfate [SDS]) containing a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, U.S.A.) and phosphatase inhibitor (PhosSTOP; Roche Diagnostics, Mannheim, Germany). Protein samples were electrophoresed on SDS-gradient polyacrylamide gel (4–20% for both PKC\_\text{\textdelta} and phospho-Thr\_\text{\textsuperscript{505}} PKC\_\text{\textdelta} and 6% for phospho-Ser\_\text{\textsuperscript{1177}} eNOS; Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) as described by Zou et al. 21) Polyclonal antibodies against PKC\_\text{\textdelta} (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and phospho-Thr\_\text{\textsuperscript{505}} PKC\_\text{\textdelta} (1:500 dilution; Cell Signaling Technology Inc., Danvers, MA, U.S.A.), or phospho-Ser\_\text{\textsuperscript{1177}} eNOS (1:1000 dilution; Cell Signaling Technology Inc.) was used as the primary antibody. The signals from the immunoreactive bands were detected by means of an enhanced chemiluminescence detection system. The density of the protein was measured by densitometry and quantified by digital image-analyzer software (Image J; National Institute of Mental Health Bethesda, Bethesda, MD, U.S.A.), and the result was expressed as the ratio of the density in OLETF rats or pravastatin-treated OLETF rats to that in LETO rats.

**Assay for Catalase Activity** Aortic homogenates were prepared in 10 m\text{m} N-(2-hydroxyethyl)pyperazine-N\_2\text{-ethanesulfonic acid (HEPES), 320 m\text{m} sucrose, 1 m\text{m} ethylene-diaminetetraacetic acid, and protease inhibitor cocktail on ice.} 22) After centrifugation at 3000 rpm for 10 min at 4°C, the supernatants were used for measuring catalase activity. Total protein extracts (20 \mu g) were diluted to 600 \mu L in 50 m\text{m} phosphate buffer (pH 7.4). Then, 1 \mu L of 10 m\text{m} hydrogen peroxide (H\_2O\_2) solution was added, and the decomposition of the substrate was recorded by measuring the decrease in absorbance at 240 nm over a 30-s period. Catalase activity is expressed as degradation of H\_2O\_2 \mu g\_protein\_1·min\_1. 23

**Solutions** The composition of the Krebs solution was as follows (m\text{m}): 137.4 Na\_2, 5.9 K\_2, 1.2 Mg\_2, 2.5 Ca\_2, 15.5 HCO\_3, 1.2 H\_2PO\_4\_2, 134 Cl\_\text{\textsuperscript{-}}, and 11.5 glucose. It was bubbled with 95% oxygen and 5% carbon dioxide (pH 7.3–7.4). The modified Krebs-HEPES buffer contained 99 mm NaCl, 4.7 mm KCl, 1.9 mm CaCl\_2, 1.2 mm MgSO\_4, 20 mm Hepes, 1.03 mm K\_2HPO\_4, 25 mm NaHCO\_3, and 11.1 mm glucose (pH 7.4).

**Drugs and Materials** The drugs and reagents were as follows: sevoflurane (Maruishi Pharmaceutical Co., Osaka, Japan); L-012, SOD, acetone, TCA, and H\_2O\_2 (Wako Pure
RESULTS

General Features of LETO, OLETF, and Pravastatin-Treated OLETF Rats At 28 weeks of age, body weight was much greater in OLETF rats than in LETO rats (Table 1). Likewise, OLETF rats exhibited higher values than LETO for the following: (a) concentrations of blood glucose, serum HbA1c, and insulin; (b) HOMA-IR score; and (c) plasma concentrations of total cholesterol and triglyceride (but not LDL-cholesterol or HDL-cholesterol). The 8-week administration of pravastatin did not modify any of these parameters in OLETF rats (Table 1).

Superoxide Production Superoxide production in the aorta (estimated from the chemiluminescence intensity of L-012) was ca. 3 times higher in OLETF than that in LETO (Fig. 1). Eight weeks’ administration of pravastatin to OLETF rats in vivo normalized this production. The increased superoxide production in OLETF rats was attenuated both by Gô6976 (a conventional PKC inhibitor) and by GF109203X (a non-selective PKC inhibitor). In pravastatin-treated OLETF rats, Gô6976 did not alter superoxide production, while GF109203X tended to increase it (Fig. 2).

\( \text{Gô6976} \) (an inhibitor of “eNOS uncoupling,” at a high concentration), sepiapterin (a precursor of BH 4), and LY294002 (a phosphatidylinositol 3-kinase [PI3-kinase] inhibitor) each inhibited the elevated superoxide production seen in OLETF

Table 1. General Features of LETO, OLETF, and Pravastatin-Treated OLETF (OLETF+PRV) Rats

|                  | LETO (n)               | OLETF (n)               | OLETF+PRV (n)             |
|------------------|------------------------|-------------------------|--------------------------|
| Body weight (g)  | 493.2±14.2 (10)        | 655.2±10.6*** (23)     | 658.6±7.9*** (25)        |
| Blood glucose (mm) | 4.8±0.2 (10)          | 8.8±0.6*** (23)        | 6.9±0.5* (25)            |
| HbA1c (%)        | 3.3±0.1 (10)          | 4.8±0.3*** (23)        | 4.2±0.1*** (25)          |
| Insulin (ng mL\(^{-1}\)) | 0.7±0.1 (10)      | 1.6±0.2** (23)         | 1.7±0.2** (25)           |
| HOMA-IR score    | 3.9±0.8 (10)          | 16.01±2.2*** (23)      | 14.1±1.6*** (25)         |
| Total cholesterol (mg·dL\(^{-1}\)) | 98±3 (10)            | 147±6*** (23)         | 150±6*** (25)            |
| Triglyceride (mg·dL\(^{-1}\)) | 39±3 (10)            | 271±22*** (23)        | 251±17*** (25)           |
| HDL-cholesterol (mg·dL\(^{-1}\)) | 65±2 (10)           | 76±2 (23)             | 78±7 (25)               |
| LDL-cholesterol (mg·dL\(^{-1}\)) | 23±1 (10)           | 23±2 (23)             | 27±3 (25)               |

\( n \) indicates the number of animals used. Values are mean±S.E.M. *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \) vs. LETO. LETO, Long-Evans Tokushima Otsuka rats; OLETF, Otsuka Long-Evans Tokushima Fatty rats; OLETF+PRV, pravastatin-treated OLETF rats; HbA1c, glycosylated haemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-cholesterol, high-density lipoprotein-cholesterol; LDL-cholesterol, low-density lipoprotein-cholesterol.

Fig. 1. Superoxide Production Levels (Estimated from Chemiluminescence Intensity of L-012) in Rat Aortas

LETO, Long-Evans Tokushima Otsuka rats, open bars; OLETF, Otsuka Long-Evans Tokushima Fatty rats, solid bars; OLETF+PRV, pravastatin-treated OLETF rats, shaded bars. Each column is the mean of data from 6 preparations (each from a different animal) with S.E.M. **\( p<0.01 \) vs. LETO, †\( p<0.05 \) vs. OLETF

Fig. 2. Effects of Gô6976 and GF109203X on Superoxide Production in Aortas Isolated from OLETF Rats Treated or Not Treated with Pravastatin

OLETF, Otsuka Long-Evans Tokushima Fatty rats, solid bars; OLETF+PRV, pravastatin-treated OLETF rats, open bars. Each column is the mean of data from 9 preparations (each from a different animal) with S.E.M. **\( p<0.01 \), ***\( p<0.001 \) vs. vehicle treatment in OLETF
rats ("OLETF," solid bars), but none of these agents modified superoxide production in pravastatin-treated OLETF rats ("OLETF + PRV," open bars, Fig. 3).

Changes in eNOS Phosphorylation

The aortic level of phospho-Ser1177 eNOS was significantly higher in OLETF than in LETO (Fig. 4). Eight-weeks’ administration of pravastatin to OLETF rats in vivo had a normalizing effect on the increased eNOS phosphorylation.

Changes in PKC δ Protein Expression

The expression levels of the unphosphorylated and phosphorylated forms of PKC δ protein in the aorta were each significantly lower in OLETF ("OLETF," solid bars) than in LETO ("LETO," open bars), and 8-weeks’ administration of pravastatin to OLETF rats in vivo led to partial recovery of each protein expression ("OLETF + PRV," shaded bars, Fig. 5).

Changes in Catalase Activity

Catalase activity in the aorta was lower in OLETF ("OLETF," solid bars) than in LETO ("LETO," open bars), and 8-weeks’ administration of pravastatin to OLETF rats in vivo normalized this activity ("OLETF + PRV," shaded bars, Fig. 6).
DISCUSSION

Similar to the findings reported by Kawano et al., we found at ca. 28 weeks of age, the OLETF rats were obese and mildly hyperglycemic with a high serum insulin concentration, indicating the presence of an insulin-resistant diabetic state in these rats. We found here that superoxide production (estimated from L-012 chemiluminescence intensity) in the aorta was greatly increased in OLETF rats (vs. age-matched LETO rats). Since endothelium removal abolishes the L-012 signals in the OLETF aorta, we suggest that L-012 detects superoxide production from endothelial cells. The increment in endothelial superoxide production seen in the OLETF aorta was suppressed by acute in vitro application of either a BH4 precursor or an inhibitor of electron transport in eNOS, suggesting that "eNOS uncoupling" is responsible for the increased superoxide production in the aorta of OLETF rats. Pravastatin administration in vivo for 8 weeks from 20 weeks of age normalized the elevated superoxide production seen in aortas from OLETF rats.

**Effects of Pravastatin on Blood Glucose and Dyslipidemia** Blood glucose was not affected by pravastatin in the present study. However, we previously reported a reduction in HbA1c concentrations with pravastatin use. The variations in the blood glucose concentrations of the OLETF rats may explain the present results. In addition, dyslipidemia was not affected by pravastatin. Because the LDL/HDL cholesterol ratio is generally low in rats, including OLETF rats, the LDL cholesterol-lowering and HDL cholesterol-increasing effects of pravastatin might have resulted in the inconsistent effects observed in the total cholesterol levels in the treated rats.

**Effects of PKC Isoforms on Vascular Superoxide Production** We previously found that, in coronary arteries obtained from OLETF rats at the early hyperglycemic stage: (i) superoxide production was increased in endothelial cells (but not in smooth muscle cells), (ii) the protein expressions of the NADPH oxidase components p47phox and gp91phox were increased, and (iii) the activity of NADPH oxidase itself was increased. Earlier, we reported that the selective NADPH oxidase inhibitor apocynin completely blocked the elevated superoxide production observed in aortas isolated from OLETF rats at the same stage. Here, we found that the selective cPKC inhibitor Gö6976 and the nonselective inhibitor GF109203X each blocked the increment in superoxide production seen in aortas from OLETF rats (Fig. 2). These results suggest that pravastatin inhibits endothelial superoxide production under conditions in which the BH4 for eNOS is reduced. In this way, a dependent relationship exists between PKCδ and "uncoupled eNOS."  

**Effect of Pravastatin on the Action of PKCδ on Vascular Superoxide Production** Pravastatin had no effect on the body weight of OLETF rats or on their measured concentrations of blood glucose, serum HbA1c and insulin, and plasma lipid (total cholesterol and triglyceride). Under these conditions, pravastatin abolished the increase in endothelial superoxide production seen in the aorta OLETF, suggesting that this agent normalizes vascular superoxide production through an action independent of its lipid-lowering effects. Similar results have also been reported in small mesenteric arteries.

We previously found that pravastatin reduces endothelial superoxide production in the coronary arteries of OLETF rats, together with reductions not only in the protein expressions of NADPH oxidase components (p22phox, p47phox and gp91phox) but also in NADPH oxidase activity. Earlier, we found that the increase in superoxide in aortic endothelial cells induced by phorbol 12,13-butyrate (a potent PKC activator) was similar between OLETF rats and 8-week pravastatin-treated OLETF rats. These results suggest that pravastatin inhibits the activation mechanism for NADPH oxidase, rather than the expressions of its components. Here, we found that either...
one of a high concentration of l-NAME, the BH₄ precursor sepiapterin, or the selective cPKC inhibitor Gö6976 modified endothelial superoxide production in aortas from pravastatin-treated OLETF rats. These results suggest that at the early hyperglycemic stage, pravastatin normalizes NADPH oxidase activity by inhibiting PKC and thereby inhibiting the formation of “uncoupled eNOS” in the OLETF aorta, thus reducing endothelial superoxide production.

A potentially interesting observation (Fig. 2) was that GFI09203X, but not Gö6976, tended to increase superoxide production in pravastatin-treated OLETF rats. Furthermore, aortas from OLETF rats treated with pravastatin exhibited partially normalized expressions of both PKCᵦ protein and the phosphorylated form of PKCᵦ protein (Fig. 5). Under these conditions, catalase activity was normalized, εNOS phosphorylation at Ser¹¹⁷7 was reduced, and the PI3-kinase inhibitor LY294002 did not modify the endothelial superoxide production. These results suggest that, in pravastatin-treated OLETF rats, the partially restored levels of PKCᵦ protein may induce a reduction in endothelial superoxide production, possibly through an inhibition of H₂O₂ production. Confirmation of this unique action of PKCᵦ must, however, await future experiments.

In conclusion, in OLETF rats at the early hyperglycemic stage, phosphorylation of “uncoupled eNOS” at Ser¹¹⁷7 (owing to decreases in PKCᵦ protein expression/catalase activity) under the condition of BH₄ deficiency leads to a greater increase in superoxide production. Pravastatin partially normalizes PKCᵦ protein expression, catalase activity, and the phosphorylation of eNOS at Ser¹¹⁷7, all of which contribute to its vascular protective activity. It is possible that administration of pravastatin immediately after the onset of dyslipidemia may prevent subsequent cardiovascular disease.

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