Chronic treatment with losartan (angiotensin II type 1 receptor antagonist) normalizes enhanced acetylcholine-induced coronary vasoconstriction in isolated perfused hearts of type 2 diabetic OLETF rats

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Abstract

We previously reported that isolated perfused hearts from streptozotocin (STZ)-induced diabetic rats exhibited increases in the sensitivity of the coronary vasoconstriction induced by acetylcholine (ACh) infusion (versus age-matched controls) (Kamata et al., 2008). Here, we examined the ACh-induced coronary vasoconstriction in perfused hearts taken from Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a type 2 diabetes model, at the chronic stage of diabetes (38–40 weeks old). The ACh-induced vasoconstriction was greatly enhanced in such rats [versus age-matched control Long-Evans Tokushima Otsuka (LETO) rats]. This enhancement was improved by the chronic administration of the angiotensin II type I receptor (AT₁-receptor) antagonist losartan (25 mg kg⁻¹, p.o., for 4 weeks). Further, the enhancement of the ACh-induced vasoconstriction seen in the OLETF group was suppressed by tempol, a superoxide dismutase mimetic. These results suggest that the coronary artery contractile response to ACh is enhanced in type 2 diabetic OLETF rats, and that this enhancement may be attributable to increased AT₁ receptor-mediating signaling and/or to increased oxidative stress.

Key words: ACh, contraction, coronary artery, diabetes, perfusion pressure

Introduction

Cardiovascular complications are the leading causes of morbidity and mortality in patients with type 1 or type 2 diabetes (Grundy et al., 1999; Brownlee, 2001; Zimmet et al., 2001; Lee et al., 2004). Vascular dysfunction (e.g., alterations in the reactivity of blood vessels to neurotransmitters and hormones) is a well-established complication of diabetes mellitus (Kamata et al., 1989; Hattori et al., 1991; Poston and Taylor, 1995; De Vriese et al., 2000; Kobayashi et al., 2000, 2004, 2005; Cohen, 2005; Matsumoto et al., 2003a, b, 2004, 2005, 2008a).
Despite angiographically normal epicardial coronary arteries, the coronary vasodilator reserve is frequently impaired in diabetes patients (Nahser et al., 1995; Strauer et al., 1997). Moreover, a failure of the adaptive coronary flow response to cardiac hyperactivity may, in part, be responsible for the higher incidence of ischemic heart disease in the diabetic population (Durante et al., 1989).

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a model of spontaneous type 2 diabetes, develops hyperglycemic obesity with hyperinsulinemia and insulin resistance after the age of 25 weeks, a pattern of changes similar to that seen in patients with noninsulin-dependent diabetes mellitus (Kawano et al., 1992). In aortas isolated from OLETF rats, endothelial NADH oxidase is enhanced after the onset of the hyperglycemia, resulting in an increased vascular production of superoxide (Kim et al., 2002). Moreover, we (Matsumoto et al., 2006a, b, 2007a, b, 2008b, c, 2009) and others (Kagota et al., 2000; Minami et al., 2002) have reported abnormal vasomotor responsiveness in a variety of vascular beds in OLETF rats. However, it is unclear whether these are alterations in coronary vasomotor responses in this animal model.

It has been found both by us (Kamata et al., 2008, 2009) and by others (Sakai, 1980; Kawamura et al., 1989; Hoover and Neely, 1997; Zhang and Hoover, 2000) that muscarinic-receptor agonists such as acetylcholine (ACh) induce vasoconstriction in the coronary arteries of experimental animals. Although human isolated coronary arteries have been variously reported to display relaxation or contraction upon muscarinic-receptor activation, vasoconstrictor responses become increasingly predominant with the progression of coronary artery diseases (Hodgson and Marshall, 1989; Egashira et al., 1995). In view of the anatomical and functional evidence that the coronary vasculature is innervated by cholinergic neurons (Kalsner, 1989; Feigl, 1998), it is possible that the parasympathetic nervous system plays a role in coronary vasospasm. In research on chronic type 2 diabetic states, the responsiveness of coronary arteries to muscarinic-receptor stimulation is therefore an important issue, yet it has received little attention so far.

The role of the renin-angiotensin system (RAS), particularly of angiotensin II (Ang II), is of considerable interest in vascular physiology and pathology (Kim and Iwao, 2000; Kobayashi et al., 2006; Paul et al., 2006; Taguchi et al., 2007; Toda et al., 2007). Numerous clinical trials have shown that both angiotensin converting enzyme (ACE) inhibitors and Ang II type 1 (AT1)-receptor antagonists have multiple beneficial effects in hypertension and other cardiovascular diseases, including improvements in morbidity and mortality (The SOLVD investigators, 1991; Cohn et al., 2001; Dahlof et al., 2002). Likewise, a growing body of evidence indicates that regulation of the RAS is important for the prevention of adverse cardiovascular events, both in hypertensive patients and in diabetic patients (Sowers, 2004). To judge from the above, both ACE inhibitors and AT1-receptor antagonists might be expected to have beneficial effects on the coronary circulation, and perhaps to reduce the incidence and/or severity of adverse cardiovascular events, in diabetic patients.

For the present study, we designed experiments to characterize the coronary vasoconstrictor response to ACh in perfused hearts isolated from OLETF rats [versus age-matched control Long-Evans Tokushima Otsuka (LETO) rats]. We also investigated whether the reactivity to ACh shown by coronary arteries from established diabetic rats might be
normalized by chronic administration of the AT1-receptor antagonist losartan.

Methods

Animals and experimental design

Five-week-old male rats [OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats, a genetic control for OLETF] were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). Food and water were given ad libitum in a controlled environment (room temperature 21–22°C, room humidity 50 ± 5%, 12:12-h light-dark cycle) until the rats were 38–40 weeks old. Some OLETF and LETO rats were given losartan (25 mg/kg/day, p.o.) for 4 weeks starting at 34–36 weeks old. Thus, we studied four groups of rats at 38–40 weeks old: losartan-untreated LETO and OLETF groups and losartan-treated LETO and OLETF groups. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health, and with “Guide for the Care and Use of Laboratory Animals” adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

Materials

The superoxide dismutase (SOD) mimetic 4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl (Tempol) was from Calbiochem (La Jolla, CA, U.S.A.), while acetylcholine chloride (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan), and losartan was from Banyu Pharmaceutical (Tokyo, Japan). All concentrations are expressed as the final molar concentration of the base in the perfusate.

Measurement of blood glucose and blood pressure

At 38–40 weeks of age, rats were anesthetized with diethyl ether and euthanized by decapitation each morning (at 0900 hours). Then, blood samples were collected. Plasma glucose and systemic blood pressure were measured as described previously (Kamata et al., 2008; Matsumoto et al., 2007a, 2008b, 2009), the former by the use of a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its blood-pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan).

Preparation of the perfused heart

Perfusion pressure was recorded as in our previous papers (Kamata et al., 2008, 2009). At 38–40 weeks of age, rats were anesthetized with diethyl ether and euthanized by decapitation each morning (at 0900 hours). Each animal’s heart was rapidly removed and placed into a Petri dish containing ice-cold oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO3, 1.8 CaCl2, 1.2 NaH2PO4, 1.2 MgSO4, and 11.0 dextrose. After washing with ice-cold KHS, the heart was prepared for cannulation of the
ascending aorta, then immediately transferred to an isolated heart apparatus for perfusion by the Langendorff technique. The perfusion buffer (KHS) in the system reservoir was continuously gassed with 95% O2–5% CO2. A peristaltic pump (PST-100; Iwaki, Tokyo, Japan) was used to perfuse hearts at a constant flow rate of 4 ml/min. Since the flow through the coronary vasculature was kept constant, the recorded changes in perfusion pressure directly reflect alterations in coronary vascular resistance (an increase signifying vasoconstriction and a decrease vasodilation). To maintain its temperature at 37°C, the buffer was passed through a water-heated glass coil. Perfusion pressure was measured by means of a pressure transducer (TP-400T; Nihon-Kohden, Tokyo, Japan) attached to the sidearm of a three-way stopcock located at the proximal end of the aortic cannula. The output from the pressure transducer was sent to a recorder for the monitoring of perfusion pressure.

Following a 40-min equilibration period, the perfusion circuit was transformed into a closed system by collecting the perfusate in a second bath and from thence recirculating it through the heart. The total volume of the closed system was 30 ml, and agents were administered via the bath. Concentration-response curves for ACh (10^-7–10^-4 M) were obtained by cumulatively increasing the total concentration of the agonist in the bath. In some of these experiments, tempol (1 mM) was applied 30 min before the ACh application and was present thereafter.

### Statistical analysis

Data are expressed as means ± S.E.M. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one-way analysis of variance (ANOVA), with a probability level of \( P < 0.05 \) being regarded as significant. Statistical comparisons between concentration-response curves were made using a two-way ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc (\( P < 0.05 \) again being considered significant).

### Results

#### General parameters

As shown in Table 1, at the time of the experiment, (a) both body weight and heart weight (wet weight) were greater in OLETF rats than in the control LETO rats, (b) all OLETF rats (non-fasted) exhibited hyperglycemia, their blood glucose concentrations being significantly higher than those of the age-matched LETO rats (also non-fasted), (c) systolic blood pressure was higher in OLETF rats than in LETO rats, and (d) basal perfusion pressure (at a constant flow rate) did not differ significantly between OLETF rats and LETO rats. In OLETF rats, chronic losartan treatment did not alter plasma glucose, body weight, heart weight, or basal perfusion pressure, but it significantly lowered systolic blood pressure (vs. nontreated OLETF rats). In the control LETO rats, treatment with losartan significantly lowered both systolic blood pressure and body weight (vs. losartan-untreated LETO rats).

### Effects of ACh on perfusion pressure in coronary arteries from diabetic and age-matched control rats

ACh caused a dose-dependent rise in perfusion pressure in coronary arteries from both
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LETO and OLETF rats, but this response was significantly greater in the latter group at $3 \times 10^{-7}$–$10^{-4}$ M ACh (Fig. 1). Comparison of EC$_{50}$ values revealed that for ACh, vasoconstrictor sensitivity did not differ significantly between the LETO [3.49 ± 0.32 μM (n=9)] and OLETF [2.96 ± 0.51 μM (n=9)] groups.

**Effect of chronic losartan treatment on ACh-induced coronary vasoconstriction**

We next examined whether chronic losartan treatment might alter the enhanced ACh-induced increase in perfusion pressure seen in coronary arteries from type 2 diabetes rats. To this end, OLETF rats and LETO rats (34–36 weeks old) were treated daily for 4 weeks with...
losartan (25 mg/kg/day, p.o.), and then experiments were conducted on isolated perfused hearts. Interestingly, in the OLETF group (Fig. 2A), but not in the LETO group (Fig. 2B), such losartan treatment significantly reduced the vasoconstriction induced by ACh (10⁻⁶ and 3 × 10⁻⁶ M).

Effect of tempol on ACh-induced vasoconstriction in coronary arteries

To investigate whether the enhanced ACh-induced vasoconstriction seen in the OLETF group might be associated with increased oxidative stress, we examined the effect of tempol, a SOD mimetic, on this response (Fig. 3). Tempol caused minimal increased perfusion pressure in both OLETF (2.09 ± 1.24 mmHg, n=6) and LETO (0.48 ± 0.39 mmHg, n=6, P>0.05 vs. OLETF) groups. When perfused hearts isolated from OLETF rats were pretreated with 1 mM tempol, the augmented ACh-induced coronary vasoconstriction was significantly suppressed (Fig. 3A). However, the ACh-induced vasoconstriction seen in perfused hearts isolated from LETO rats was not significantly altered by such pretreatment (Fig. 3B).

Discussion

The main conclusions to be drawn from the present study are that an enhanced vasoconstrictor response to ACh is present in perfused hearts isolated from type 2 diabetic OLETF rats at 38–40 weeks of age, and that this enhancement can be suppressed by chronic treatment with the AT₁-receptor antagonist losartan or by pretreatment of the isolated preparation with tempol.
OLETF rats are characterized by an early increase in serum insulin and also by late-onset hyperglycemia, mild obesity, and mild type 2 diabetes (Kawano et al., 1992). Numerous reports by us and others have demonstrated that in this diabetic model, abnormalities of vascular function are present in various blood vessels, such as the aorta (Sakamoto et al., 1998; Kagota et al., 2000; Kim et al., 2002; Matsumoto et al., 2006a), basilar artery (Matsumoto et al., 2007b), renal artery (Kagota et al., 2000), and mesenteric arteries (Minami et al., 2002; Matsumoto et al., 2006b, 2007a, 2008b,c, 2009). Moreover, Yu et al. (2002) found that the coronary flow reserve [an integrating parameter reflecting endothelial function and vascular smooth muscle relaxation (Strauer, 1992; Dayanikli et al., 1994)] was decreased while minimal coronary vascular resistance was increased in OLETF rats at 15 weeks of age or older. They suggested that the degree of functional deterioration in the coronary circulation correlated directly with the severity of coronary arteriolar structural remodeling occurring during the development of microangiopathy in OLETF rats. The above evidence suggests that the OLETF rat is a suitable model for the investigation of cardiovascular complications in type 2 diabetes. Consequently, and since coronary artery disease is frequently encountered, and is a very important issue, in diabetic states (Bax et al., 2007; BARI 2D Study Group, 2009), we designed the present study to characterize coronary vasoconstrictor responses in OLETF rats at the chronic stage of type 2 diabetes (using perfused hearts).

Isolated human coronary arteries have been variously reported to contract or relax upon muscarinic-receptor stimulation, although vasoconstriction increasingly predominates with the progression of coronary artery diseases (Hodgson and Marshall, 1989; Egashira et al., 1995). In our present and previous (Kamata et al., 2008, 2009) studies on isolated rat hearts, coronary

![Fig. 3. Effect of 1 mM tempol on ACh-induced contraction in perfused coronary arteries obtained from LETO and OLETF rats. Each data-point represents the mean ± S.E.M. from 6 (tempol-treated groups) or 9 (tempol-untreated groups) preparations; the S.E.M. is included only when it exceeds the dimension of the symbol used. The curves shown here for tempol-untreated rats are taken from Fig. 1. * P<0.05 vs. OLETF group.](image-url)
vasodilator responses to ACh were not detected at all, and they were reported to be absent or minor under basal conditions in an earlier study of the isolated, buffer-perfused rat heart (Weselcouch et al., 1995). Moreover, here we found that ACh-induced coronary vasoconstriction was significantly greater in the type 2 diabetic OLETF group than in the age-matched control LETO group. This, together with our previous reports of vasoconstrictor hyperreactivity to ACh in perfused coronary arteries from rats with long-term STZ-induced type 1 diabetes (Kamata et al., 2008, 2009), leads us towards the general conclusion that ACh-induced coronary vasoconstriction may be enhanced in long-term diabetic disease states.

There is a broad body of evidence indicating that increased AT₁-receptor signaling promotes cardiovascular complications in patients with diabetes, and indeed is probably involved in the pathogenesis of diabetic cardiomyopathy and coronary artery disease (Beckman et al., 2002). In the present study, the enhancement of ACh-induced vasoconstriction seen in the perfused OLETF rat heart was suppressed by chronic treatment with losartan, suggesting that it resulted from a sustained increase in AT₁-receptor activation. This idea is consistent with the finding by Jesmin et al. (2002, 2003) that both Ang II labeling and AT₁-receptor expression in coronary vessels were profoundly increased in OLETF rats (versus LETO rats).

It is thought that elevated levels of reactive oxygen species (ROS), including superoxide, may play an important role in the abnormal vascular tone seen in diseases affecting the cardiovascular system, such as diabetes (Hattori et al., 1991; Kamata and Kobayashi, 1996; Matsumoto et al., 2007c, 2008a; Taguchi et al., 2007). Indeed, such abnormalities can be improved by SOD or by the SOD-mimetic tempol (Kamata and Kobayashi, 1996, Kamata et al., 2009; Shastri et al., 2002; Matsumoto et al., 2006c). In the present study, the augmentation of ACh-induced vasoconstriction seen in the OLETF group was effectively suppressed by tempol pretreatment, suggesting that in such rat coronary arteries, ACh-induced vasoconstriction might be enhanced by increased superoxide production. Since Ang II can lead to increases in the vascular levels of superoxide (Kim and Iwao, 2000; Toda et al., 2007), the suppressive effect of losartan on the enhanced ACh-induced vasoconstriction seen in the OLETF rat perfused heart may be mediated via a reduction in oxidative stress.

In conclusion, the present study has demonstrated that the vasoconstrictor response to ACh is enhanced in perfused coronary arteries isolated from type 2 diabetic OLETF rats. This enhancement may be due, at least in part, to a sustained increase in AT₁-receptor stimulation and/or to increased oxidative stress.

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