Effect of N-epsilon-(carboxymethyl)lysine on coronary vasoconstriction in isolated perfused hearts from control and streptozotocin-induced diabetic rats

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Abstract

Advanced glycation end products (AGEs) derived from glucose are implicated in the pathogenesis of diabetic vascular disease. However, their direct modulatory effects on coronary vascular tone remain unclear. We previously reported that coronary vasoconstriction was induced by acetylcholine (ACh) infusion of the isolated perfused rat heart and that sensitivity was greater in perfused hearts from streptozotocin (STZ)-induced diabetic rats than in those from age-matched controls (Kamata et al., 2008). Here, we investigated the effect of N-epsilon-(carboxymethyl)lysine (CML), which has one of the main AGE structures, on ACh-induced vasoconstriction in perfused hearts isolated from control and diabetic rats. ACh-induced vasoconstriction was significantly greater in the STZ-induced diabetic group than in the age-matched controls. CML enhanced the ACh-induced vasoconstriction in coronary arteries from control rats, but not in those from STZ-induced diabetic rats. In the controls, the vasoconstriction induced by the calcium-channel activator Bay K 8644 was also enhanced by CML. These CML-mediated enhancements of the vasoconstrictions induced by ACh and Bay K 8644 were significantly suppressed by tempol, a superoxide dismutase mimetic. The plasma CML and glucose levels were each significantly elevated in STZ-induced diabetic rats. These findings suggest (a) that CML augments ACh-induced coronary vasoconstriction, an effect that may be attributable to increased superoxide and to activation of voltage-gated Ca2+ channels and (b) that this modulating effect may be desensitized in the STZ-induced diabetic heart.

Key words: AGE, CML, coronary artery, diabetes, perfusion pressure

Introduction

Vascular complications are largely responsible for the mortality and morbidity associated with human diabetes mellitus (Brownlee, 2001; Zimmet et al., 2001). Depending on the specific
beds affected, vascular complications may be manifested as atherosclerosis, retinopathy, nephropathy, hypertension, distal vasculopathy, or ischemic heart disease, etc. (Brownlee, 2001; Zimmet et al., 2001). Of these complications, ischemic heart disease is a leading cause of morbidity and mortality within the diabetic population, despite insulin therapy (Dubrey et al., 1994). Several factors, such as atherosclerosis of the coronary arteries and autonomic neuropathy, may play important roles in the increased occurrence of cardiovascular disease in diabetes (Fein and Sonnenblick, 1985). Vascular dysfunction (e.g., alterations in the reactivity of blood vessels to neurotransmitters and hormones) is a well-established complication of diabetes mellitus (Oyama et al., 1986; Kamata et al., 1989; Poston and Taylor, 1995; Pieper, 1999; De Vriese et al., 2000; Matsumoto et al., 2003a, b, 2006a, 2008; Kobayashi et al., 2000, 2004, 2005), and a failure of the adaptive coronary flow response to cardiac hyperactivity may contribute to the higher incidence of ischemic heart disease in the diabetic population (Durante et al., 1989).

Although major advances in the diagnosis and treatment of diabetes and the related vasculopathy were made in the last century, it remains a serious clinical and public health problem. There is increasing evidence of a causal role for the formation of advanced glycation end products (AGEs) in the development of diabetic complications, including nephropathy and vascular disease (Brownlee, 2001; Wautier and Schmidt, 2004; Smit and Lutgers, 2004; Goldin et al., 2006; Soldatos and Cooper, 2006). Further, AGEs normally accumulate during aging (Mitsuhashi et al., 1993; Nakayama et al., 1993; Brownlee, 1995), and accumulate at an accelerated rate in diabetes (Brownlee, 1995; Smit and Lutgers, 2004). AGEs are also found in atherosclerotic lesions within human blood vessels, suggesting a possible link between their deposition and atherogenesis (Nakamura et al., 1993). AGEs, which are advanced products of the Maillard reaction, include pentosidine, N-\((\text{carboxyethyl})\)lysine, and N-\((\text{carboxymethyl})\)lysine (CML). CML, the most commonly encountered AGE in vivo (Reddy et al., 1995; Ikeda et al., 1996), increases with aging and is found at elevated levels both in human atherosclerotic lesions (Nerlich and Schleicher, 1999; Baidoshvili et al., 2004) and in vascular tissue in diabetic patients (Schleicher et al., 1997). It is known that CML formation may occur via a number of processes: namely glycation, auto-oxidative glycosylation, reaction of proteins with nonglucose carbohydrates, or lipoxidation, or via the reaction of proteins with the products of myeloperoxidase or chronic inflammation (Fu et al., 1996; Anderson et al., 1999).

We (Kamata et al., 2008) and others (Sakai, 1980; Kawamura et al., 1989; Hoover and Neely, 1997; Zhang and Hoover, 2000) have suggested that in coronary arteries, muscarinic-receptor agonists such as acetylcholine (ACh) induce vasoconstriction. Actually, human isolated coronary arteries have variously reported to relax or contract upon muscarinic-receptor activation, with vasoconstrictor responses becoming increasingly predominant with the progression of coronary artery disease (Hodgson and Marshall, 1989; Egashira et al., 1995). Since both anatomical and functional evidence exist 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. The responsiveness of coronary arteries to muscarinic-receptor stimulation is therefore an important issue in research on chronic diabetic states, in which AGEs accumulate within various tissues. However, few studies have examined this issue, and no study has yet investigated the direct relationship between CML and
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vasoconstrictor responses in coronary arteries. We therefore investigated the effect of CML on the coronary vasoconstrictor response to ACh using perfused hearts from STZ-induced diabetic and age-matched control rats.

**Methods**

**General**

The experimental design 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 (accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

**Materials**

Bay K 8644 and streptozotocin (STZ) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CML was purchased from Funakoshi (Tokyo, Japan). 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). All concentrations are expressed as the final molar concentration of the base in the perfusate.

**Animal model of diabetes**

Male Wistar rats (8-weeks old and 180–230 g body weight) received a single injection via the tail vein of STZ 65 mg/kg dissolved in a citrate buffer, as reported previously (Kobayashi *et al.*, 2000; Matsumoto *et al.*, 2003a, 2004, 2005, 2007). Age-matched control rats were injected with the buffer alone. Food and water were given *ad libitum*. The experiments described here were performed 4–6 months after the injection.

**Assessment of plasma glucose and CML**

The plasma glucose level was determined by the use of a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan) as reported previously (Kobayashi *et al.*, 2000; Matsumoto *et al.*, 2006b, 2007). Plasma CML was measured by the use of a commercially available CircuLex™ anti-CML rat autoantibody ELISA kit (CycLex Co., Ltd., Ina, Nagano, Japan). One hundred-time diluted plasma samples were used for the measurement of CML, and the assay was performed as described in the manufacturer’s procedure booklet.

**Preparation of the perfused heart**

Perfusion pressure was recorded as in our previous papers (Kamata *et al.*, 2008). Briefly, rats were anesthetized with diethyl ether and euthanized by decapitation 4–6 months after treatment with STZ or buffer. 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 Na₂HPO₄, 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. The basal perfusion pressure (at a constant flow rate of 4 ml/min) was not different between hearts from age-matched control [25.7 ± 1.6 mmHg (n=40)] and STZ-induced diabetic [25.6 ± 1.2 mmHg (n=21)] rats. Concentration-response curves for ACh (10⁻⁷ – 10⁻⁴ M) and Bay K8644 (10⁻¹⁰ – 10⁻⁵ M) were obtained by cumulatively increasing the total concentration of the agonist in the bath. In some of these experiments, 1 µM CML or 1 mM tempol was applied 30 min before the ACh or Bay K8644 application and was present thereafter. In some other experiments, the perfused heart was treated with 1 mM tempol for 10 min, then incubated with 1 µM CML for 30 min. After this incubation period, ACh was cumulatively applied.

Statistical analysis

Data are expressed as the mean ± S.E.M. Multiple comparisons between treatment groups were made using an analysis of variance (ANOVA) followed by a Bonferroni test.

Results

General parameters

As shown in Fig. 1, at the time of the experiment: (a) the body weight of the STZ rats was lower than that of the control rats (Fig. 1A), (b) all STZ rats (non-fasted) exhibited hyperglycemia, their blood glucose concentrations being significantly higher than those of the age-matched nondiabetic control rats (also non-fasted) (Fig. 1B), and (c) the plasma CML concentration was significantly higher in STZ rats than in control rats (Fig. 1C). In addition, the heart weight of the STZ rats (1.34 ± 0.05 g, n=21) was also lower than that of the control rats (1.88 ± 0.04 g, n=40; P<0.001).

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
diabetic and control rats (Fig. 2, Table 1). The maximum response to ACh was not different between the STZ-induced diabetic group and the age-matched controls (Fig. 2A). However, at certain lower concentrations of this agent (viz. 0.3 and 1 μM ACh), the vasoconstriction was significantly greater in the STZ-induced diabetic group than in the age-matched controls. Comparison of the EC50 values obtained for ACh revealed that the vasoconstrictor sensitivity was significantly increased in STZ-induced diabetic rats (vs. the age-matched controls) (Table 1).

**Effect of CML on ACh-induced vasoconstriction in coronary arteries**

When CML (1 μM) was applied to the perfused heart, it did not itself cause tension development in the coronary arteries, but it did significantly potentiate the ACh-induced coronary vasoconstriction in the nondiabetic control group (Fig. 2B). Unexpectedly, in STZ-induced diabetic rats, CML did not affect the ACh-induced vasoconstriction (Fig. 2C). To investigate whether the CML-induced augmentation of the ACh-induced vasoconstriction seen in the control group was associated with oxidative stress, we examined the effect of tempol, a SOD mimetic (Fig. 2B). When perfused hearts isolated from control rats were pretreated with 1 mM tempol, which did not itself alter the ACh-induced vasoconstriction (Fig. 2D), the augmentation of the ACh-induced vasoconstriction seen in the presence of CML was significantly suppressed (Fig. 2B). When the STZ-induced diabetic perfused heart was pretreated with 1 mM tempol, this reagent did not alter the ACh-induced vasoconstriction either in the absence or in the presence of CML (Fig. 2C, 2E).
Effect of CML on Bay K8644-induced vasoconstriction in coronary arteries

We previously reported that the vasoconstriction induced by the voltage-gated Ca\(^{2+}\)-channel agonist Bay K8644 was increased in coronary arteries from long-term STZ-induced diabetic rats (Kamata et al., 2008). Here, we investigated whether the Bay K 8644-induced coronary
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Table 1. Sensitivity (EC50) values for the contractions induced by ACh (with or without drugs) in coronary arteries from control and STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>EC50 (µM)</th>
<th>Control</th>
<th>Diabetic</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>4.5 ± 0.8 (6)</td>
<td>1.9 ± 0.2 (6)*</td>
</tr>
<tr>
<td>CML (10^-6 M)</td>
<td>4.6 ± 0.5 (6)</td>
<td>2.2 ± 0.3 (5)</td>
</tr>
<tr>
<td>Tempol (10^-3 M)</td>
<td>7.2 ± 1.4 (5)</td>
<td>3.5 ± 1.7 (5)</td>
</tr>
<tr>
<td>CML (10^-6 M) + Tempol (10^-3 M)</td>
<td>3.3 ± 0.4 (6)</td>
<td>2.6 ± 1.0 (5)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. ACh-induced contraction in the absence (vehicle) or presence of drugs (CML and/or tempol). Number of determinations is shown within parentheses. *P<0.05 vs. vehicle-treated control group.

Fig. 3. Effects of 1 mM tempol on Bay K 8644-induced contraction, in the absence or presence of 1 µM CML, in perfused coronary arteries from control rats. Ordinate shows peak increase in perfusion pressure at each concentration. Details are given under Methods. Each data-point represents the mean ± S.E.M. from 5 or 6 preparations; the S.E.M. is included only when it exceeds the dimension of the symbol used. *, P<0.05, **, P<0.01, ***, P<0.001, Vehicle-treated group vs. CML-treated group. †, P<0.05; ‡, P<0.01, Tempol + CML-treated group vs. CML-treated group.

Vasoconstriction in the controls might be modulated by CML (Fig. 3). When CML (1 µM) was applied to perfused hearts from control rats, it significantly potentiated the Bay K8644-induced coronary vasoconstriction (Fig. 3). When perfused hearts isolated from control rats were pretreated with 1 mM tempol, the augmentation of the Bay K8644-induced vasoconstriction seen in the presence of CML was significantly suppressed (Fig. 3), a result similar to that described above for the vasoconstrictor response to ACh.

Discussion

In the present study on perfused hearts, CML enhanced the ACh-induced contraction in the coronary arteries of the controls, but not in those of the STZ-induced diabetic rats. This enhancement by CML of the ACh-induced vasoconstriction was suppressed by pretreatment
with tempol. Likewise, the Bay K8644-induced contraction was enhanced by CML in the control group and this effect of CML was also suppressed by tempol pretreatment.

Isolated human coronary arteries have been variously reported to relax or contract upon muscarinic-receptor activation, although vasoconstriction increasingly predominates with the progression of coronary artery disease (Hodgson and Marshall, 1989; Egashira et al., 1995). In our present and previous (Kamata et al., 2008) studies, vasodilator responses to ACh were not detected at all, and they were reported to be absent or minor under basal conditions in an other study of the isolated, buffer-perfused rat heart (Weselcouch et al., 1995). It is known that ACh can produce vasoconstriction either directly, by stimulating muscarinic receptors on vascular smooth muscle (Kalsner, 1989), or indirectly through muscarinic receptors located on endothelial cells (Luscher et al., 1992). Moreover, Zhang and Hoover (2000) demonstrated that voltage-independent receptor-operated Ca\(^{2+}\) channels, voltage-operated Ca\(^{2+}\) channels, and protein kinase C (PKC) are major signaling components for muscarinic receptor-mediated contraction in rat coronary resistance arteries. In view of these earlier findings, and because we previously reported vasoconstrictor hyperreactivity to ACh in the perfused coronary arteries from rats with long-term STZ-induced diabetes (Kamata et al., 2008), we hypothesized that factor(s) favoring enhanced ACh-induced coronary vasoconstriction might exist in long-term diabetic disease states.

AGEs, proteins that accumulate in the plasma of diabetics as a result of increased glucose concentrations, are closely linked with vascular disease (Brownlee, 2001; Wautier and Schmidt, 2004; Smit and Lutgers, 2004; Goldin et al., 2006; Soldatos and Cooper, 2006). So far, only a few molecular structures have been identified for AGEs \textit{in vivo} (e.g., CML, pentosidine, imidazolones, and oxalic acid monolysinylamide). CML-modified proteins are important AGEs \textit{in vivo}. They have been found to accumulate especially within vascular tissue and atherosclerotic lesions, and within the glomerular tissue isolated from diabetic rodents (Horie et al., 1997; Berg et al., 1998; Sakata et al., 1999). Excessive accumulation of AGE-modified proteins can lead to tissue damage through a variety of mechanisms. When we began our study, little was known about the direct relationship between CML and coronary vasoconstriction. We found that while CML enhanced ACh-induced vasoconstriction in coronary arteries from control rats, it had no such effect in those from STZ-induced diabetic rats.

For some time, it has been thought that elevated levels of reactive oxygen species (ROS), including superoxide, might play an important role in the abnormal vascular tone seen in diseases affecting the cardiovascular system, such as diabetes (Kamata and Kobayashi, 1996; Matsumoto et al., 2006b, 2007, 2008; Thakali et al., 2006). Indeed, such abnormalities can be improved by SOD or by the SOD mimetic tempol (Kamata and Kobayashi, 1996; Shastri et al., 2002; Matsumoto et al., 2006b). In the present study, the augmentation of ACh-induced vasoconstriction seen in the presence of CML was effectively suppressed by tempol pretreatment, suggesting that in rat coronary arteries, ACh-induced vasoconstriction might be enhanced by CML-induced superoxide production. This conclusion is supported by reports that both overproduction of ROS and increased oxidative stress can be induced by AGEs (Wautier et al., 2001; Urata et al., 2002; Gao et al., 2008; Su et al., 2008). Moreover, the vasoconstriction induced by the voltage-gated Ca\(^{2+}\)-channel activator Bay K8644 (Kanmura et al., 1984; Karaki et
Coronary vasoconstriction and AGE (Anderson, M.M., Requena, J.R., Crowley, J.R., Thorpe, S.R. and Heinecke, J.W. (1999). The myeloperoxidase system of human phagocytes generates Nebsilony-(carboxymethyl)lysine on proteins: a mechanism for producing advanced glycation end products at sites of inflammation. *J. Clin. Invest.* **104**: 103–113. Baidoshvili, A., Niessen, H.W., Stooker, W., Huybregts, R.A., Hack, C.E., Rauwerda, J.A., Meijer, C.J., Eijsman, L., van Hinsbergh, V.W. and Schalkwijk, C.G. (2004). N(omega)-(carboxymethyl)lysine depositions in human aortic heart valves: similarities with atherosclerotic blood vessels. *Atherosclerosis* **174**: 287–292.) was also augmented by CML treatment in the control perfused heart, and this effect of CML was completely suppressed by pretreatment with tempol. Changes in vascular smooth muscle membrane potential and Ca$^{2+}$ handling have been proposed as mediators of the augmentation of vasoconstriction observed in certain disease states (Silva et al., 1994; Lee et al., 2004), and our previous study (Kamata et al., 2008) suggested that the vasoconstrictor hyperreactivity to ACh seen in the perfused coronary arteries of rats with long-term STZ-induced diabetes may be due at least in part to alterations in the activity of voltage-gated Ca$^{2+}$ channels. On the basis of these pieces of evidence and the present data, we suggest that the augmenting effect of CML on coronary vasoconstriction may be attributable to increased superoxide levels, with consequent alterations in Ca$^{2+}$ signaling.

In contrast to its effect in control rats, CML did not enhance the ACh-induced coronary vasoconstriction in perfused hearts from STZ-induced diabetic rats. Several pieces of evidence suggest that in diabetic states, there is both accumulation of AGEs within tissues and increased levels of circulating AGEs (Horie et al., 1997; Schleicher et al., 1997; Berg et al., 1998; Schalkwijk et al., 2004; Smit and Lutgers, 2004; Goldin et al., 2006). Indeed, in the present study we found that the plasma CML level was elevated in STZ-induced diabetic rats. Moreover, activities related to vasoconstriction, such as those of L-type voltage dependent Ca$^{2+}$ channels (Wang et al., 2000), are altered in diabetic states. Taken together, our results and the relevant previous evidence tempt us to speculate that in perfused hearts from STZ-induced diabetic rats, the augmenting effect of CML on ACh-induced coronary vasoconstriction might be desensitized as a consequence of a constitutive attack on the vasculature by the increased levels of CML.

In conclusion, our study suggests that CML potentiates ACh-induced coronary vasoconstriction in the isolated perfused rat heart, and that this effect might be partly due to increased oxidative stress. Future examinations of the roles played by AGEs in cardiovascular disease may lead both to the discovery of new pathways and to the design of new treatment modalities aimed at preventing the development of the vascular complications associated with long-term diabetes.

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**References**


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