Mechanism underlying enhanced endothelium-dependent vasodilatation in thoracic aorta of early stage streptozotocin-induced diabetic mice

SHEN Bing, YE Chun-Ling, YE Kai-He, LIU Jian-Jun, SUN Peng, JIANG Jia-Hua

Department of Pharmacology, Pharmacy College of Jinan University, Guangzhou 510632, China

KEY WORDS diabetes mellitus; streptozotocin; thoracic aorta; endothelium; nitric oxide; cyclic GMP

ABSTRACT

AIM: To investigate the mechanism of the enhanced endothelium-dependent vasodilatation in thoracic aorta of the early stage streptozotocin (STZ)-induced diabetic C57BL/6j mice. METHODS: Radioimmunity was used to detect the metabolite of prostaglandin I\(_2\) (PGI\(_2\)), 6-keto-prostaglandin F\(_1\alpha\) (6-keto-PGF\(_1\alpha\)), in the blood serum. Vascular muscle tension and phenylephrine (PE)-induced rhythmic activity in the isolated thoracic aorta of mice were also compared. RESULTS: 6-Keto-PGF\(_1\alpha\) in the serum was significantly higher in STZ-induced diabetic mice than age-matched controls [(1.8±1.0) µg/L vs (0.5±0.3) µg/L, P<0.01]. PE induced rhythmic activity in both diabetic and control mouse aorta but the amplitude was markedly higher in diabetic mice than in controls [(4.9±1.7) % vs (12±5) %, P<0.01]. PE, high K\(^+\) solution-induced contraction, and acetylcholine (ACh)-induced relaxation [(56±10) % vs (81±8) %, P<0.01] were notably enhanced in diabetic mice than those in controls. Alone \(L\)-nitro-\(L\)-arginine methyl ester (\(L\)-NAME) or 6-(phenylamino)-5,8-quinolinedione (LY-83583) abolished the rhythmic activity and ACh-induced relaxation in controls but only partially inhibited them in diabetic mice. Indomethacin did not affect rhythmic activity but depressed ACh-induced relaxation. \(L\)-NAME plus indomethacin significantly depressed the rhythmic activity and ACh-induced relaxation than \(L\)-NAME alone (P<0.01). Furthermore tetraethylammonium plus \(L\)-NAME abolished them in diabetic mice. CONCLUSION: The mechanism that enhanced endothelium-dependent vasodilatation in STZ-induced diabetic mice is due to enhanced production of PGI\(_2\) and endothelium-derived hyperpolarizing factor (EDHF). The phenomena maybe only take place in early stage of diabetic mice.

INTRODUCTION

Microvascular disease is the main cause of morbidity and mortality in patients with diabetes mellitus (DM). Endothelial dysfunction may be a critical and initiating factor to develop diabetic vascular disease\(^1\). The endothelial cells play an important role to regulate basal vascular tone via the release of endothelium-derived relaxing factors (EDRF) that include nitro oxide (NO), prostaglandin I\(_2\) (PGI\(_2\)), and endothelium-derived hyperpolarizing factor (EDHF)\(^2\). NO, PGI\(_2\), and EDHF can induce relaxation of underlying vascular muscle through activation of potassium channels. In the case of PGI\(_2\) and NO, this may involve the intracellular accumulation of second messengers: cyclic AMP (cAMP)
and cyclic GMP (cGMP), respectively. But EDHF and NO may also activate potassium channels directly. Acetylcholine (ACh), an agonist of endothelial cell receptors can cause release of NO, PGI₂, and EDHF. Furthermore, NO is a main factor to execute endothelium-dependent relaxation in normal state and many studies have demonstrated that diabetic endothelial dysfunction is due to oxidative stress and decreased bioavailability of NO. However, few studies have focused on the contribution of EDHF and PGI₂ to endothelial function in diabetes mellitus. In the present study, the experiments were designed to investigate the mechanism underlying the diabetic-related enhancement of ACh-induced endothelium-dependent vasodilatation.

**MATERIALS AND METHODS**

**Drugs** Phenylephrine (PE), ACh, tetraethylammonium (TEA), and N⁰-nitro-L-arginine methyl ester (L-NAME) (Sigma Chemical, St Louis, MO, USA) were dissolved in distilled water. 6-(Phenylamino)-5,8-quinolinedione (LY-83583, Sigma) was dissolved in ethanol. Indomethacin (Sigma) was dissolved in Me₂SO. All subsequent dilutions were made with Krebs Henseleit solution. Similar dilutions of the solvents into Krebs Henseleit solution were used as controls and had no effect on either the basal tension or the evoked tension of thoracic aorta (TA) rings. All concentrations given are final molar concentrations in the organ chambers. Streptozotocin (STZ, Sigma), 6-keto-prostaglandin F₁₅ (6-keto-PGF₁₅) radioimmunoassay kit was bought in Dongya Institute of Immunological Technology, Beijing, China.

**Animals, induction of diabetes mellitus, and tissue preparation** Twenty male C57BL mice were obtained at the age of 4 weeks from Experimental Animal Center of Peking University Health Science Center (SPF, Certificate No SCXK11-00-0004). Diabetic group daily received five ip injections of STZ (40 mg/kg) dissolved in a citrate buffer, pH 4.2, just before injection. Age-matched control mice were injected with buffer alone. All blood was sampled from the tail vein after an 8-h fast at every four weeks and at the time to study (17-18 weeks) after making model successfully. The plasma glucose level was determined with One Touch Blood Glucose Monitoring System (Lifescan Inc, USA) and the mice with fasting plasma glucose level higher than 11.1 mol/L were selected to study. TA of the mice were quickly dissected free and placed in Krebs Henseleit solution at room temperature (22-23 °C) after the mice were killed by cervical dislocation. By a dissecting microscope, adhering perivascular tissue was carefully removed and the descending TA was cut into 2-mm long rings.

**Tension measurement** The vessels were mounted onto two thin stainless steel holders, one of which was connected to a force displacement transducer and the other to a movable device that allowed the application of a passive tension of 500-550 mg, which were determined to be the optimal resting tension for obtaining the maximal active tension induced by K⁺ solution 60 mmol/L. The mounted rings were kept in 2-mL organ baths containing Krebs Henseleit solution at 37 °C and continuously bubbled with a gas mixture of 95 % O₂ and 5 % CO₂ to maintain a pH of 7.4. The isometric tension was recorded on a polygraph (Biolap 310). After an equilibration period of 1 h, the contractile function of vessel was tested twice by replacing the Krebs Henseleit solution with K⁺ solution 60 mmol/L, and the second contraction was taken as the reference contraction. After washout, the vessel was contracted once with PE 10 µmol/L for 10 min and then relaxed with ACh 10 µmol/L for 4 min. After another washout period, cumulative dose-response curves for PE, ACh were created. ACh-induced vasorelaxation was tested after precontractions evoked by PE 10 µmol/L to determine endothelium-dependent and endothelium-independent relaxation, respectively. Prolonged exposure to PE 10 µmol/L evoked spontaneous rhythmic activity which was recorded for 15 min. The amplitude was measured as the mean value of the last five oscillations. Each drug was investigated on TA segments from at least four mice.

**Solutions** The Krebs Henseleit solution consisted of (in mmol/L): NaCl 118, KCl 2.5, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄(7H₂O) 1.2, NaHCO₃ 25.2, and glucose 11.1 (pH 7.4). It was titrated to the appropriate temperature-corrected pH with NaOH 10 mol/L. The high K⁺ solution (60 mmol/L) was prepared by exchanging NaCl with an equimolar amount of KCl.

**Data analysis** Results were expressed as means±SD. The number of observations indicated the number of vessel segments studied. Since several segments were taken from the same animal, these segments were used to investigate different experiments, except PE-induced rhythmic activity and response to high K⁺ solution, PE, and ACh experiments. But statistical analyses were always performed using both the number of vessels and the number of animals. Differ-
ences were regarded statistically significant only if significant differences were found using both methods. The two-tailed paired-samples t-test was used to compare results in treated and untreated aortas from each strain and two-tailed independent samples t-test was used to compare results in different groups. Differences were considered significant when $P<0.05$.

RESULTS

General characteristics  Weight and fasting plasma glucose levels of mice were measured at every four weeks after the fasting plasma glucose level was higher than 11.1 mol/L. By comparison with the age-matched controls, fasting plasma glucose levels were significantly higher in STZ-induced diabetic mice but the weight was significantly lower (Fig 1A, 1B).

PGI$_2$ content in blood serum  Radioimmunity was used to detect PGI$_2$ metabolite, 6-keto-PGF$_{1\alpha}$ in the blood serum. 6-keto-PGF$_{1\alpha}$ was markedly higher in STZ-induced diabetic mice than in age-matched controls [(1.8±1.0) µg/L vs (0.49±0.25) µg/L] (Fig 2).

PE-induced rhythmic activity  The contractions triggered by PE 10 µmol/L were observed for 15 min without any intervention and were repeated five times and the fifth value was recorded. The amplitude of rhythmic activity and ACh-induced relaxation were strikingly larger in diabetic mice than in age-matched controls (Tab 1).

![Fig 1. Weight (A) and fasting plasma glucose levels (B) in diabetic (n=10) and control group (n=9). Mean±SD. *P<0.05 vs control.](image)

![Fig 2. The bars show the content of 6-keto-PGF$_{1\alpha}$ in the serum of STZ-induced diabetic and age-matched control mice. n=9. Mean±SD. *P<0.01 vs control.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MT/mg</th>
<th>Amplitude of RA/%</th>
<th>ACh-induced relaxation/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>598±117</td>
<td>4.9±1.7</td>
<td>56±10</td>
</tr>
<tr>
<td>DM</td>
<td>17</td>
<td>631±149</td>
<td>12±5$^c$</td>
<td>81±8$^c$</td>
</tr>
</tbody>
</table>

MT: maximal tension; RA: rhythmic activity.

Response to high K$^+$ solution, PE, and ACh  The contractile force of TA rings induced by K$^+$ solution 60 mmol/L was significantly higher in STZ-induced diabetic mice than that in age-matched controls [(621±100) mg vs (416±127) mg] (Fig 3). The sensitivity to PE and ACh-induced relaxations of TA rings of diabetic mice were also increased notably (Fig 4A, 4B, and 5).

Effects of L-NAME, indomethacin, and LY-83583  Pretreatment of TA rings of age-matched controls with L-NAME 1 mmol/L, an NOS inhibitor, for 10 min before administration of PE 10 µmol/L abolished PE-induced rhythmic activity and ACh-induced
vasorelaxation (Fig 6A, 6B, and Tab 3). But L-NAME induced partial inhibition of PE-induced rhythmic activity and ACh-induced vasorelaxation in TA rings of STZ-induced diabetic mice (Fig 7A, 7B, and Tab 2). Furthermore, pretreatment of TA rings of age-matched controls with LY-83583 1 µmol/L, a guanylate cyclase

![Image](image_url)
inhibitor\(^{[6]}\), for 10 min abolished PE-induced rhythmic activity and strikingly decreased ACh-induced vasorelaxation (Fig 6C, and Tab 3). But it also induced partial inhibition of rhythmic activity in diabetic mice. Meanwhile, ACh-induced relaxation was larger in diabetic mice than in age-matched controls after application of LY-83583 (Fig 7B, 7C, and Tab 2).

**Tab 2. Effects of L-NAME (1 mmol/L), LY-83583 (1 µmol/L), indomethacin (10 µmol/L), and TEA (1 mmol/L) on rhythmic activity and ACh-induced vasorelaxation in STZ-induced diabetic mice.** \(n=6 - 8\). Mean±SD. \(^aP<0.05, \ ^bP<0.01\) vs control; \(^cP<0.05, \ ^dP<0.01\) vs L-NAME; \(^eP<0.05, \ ^fP<0.01\) vs indomethacin.

<table>
<thead>
<tr>
<th>Group</th>
<th>MT/mg</th>
<th>Amplitude of RA/%</th>
<th>ACh-induced relaxation/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>681±121</td>
<td>16±4</td>
<td>80±7</td>
</tr>
<tr>
<td>L-NAME</td>
<td>947±55(^c)</td>
<td>10±3(^b)</td>
<td>10±5(^c)</td>
</tr>
<tr>
<td>Control</td>
<td>644±155</td>
<td>10±4</td>
<td>81±7</td>
</tr>
<tr>
<td>LY-83583</td>
<td>1233±170(^r)</td>
<td>4.5±2.0(^r)</td>
<td>18±5(^c)</td>
</tr>
<tr>
<td>Control</td>
<td>693±132</td>
<td>13±3</td>
<td>77±5</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>725±50</td>
<td>12±10</td>
<td>69±7(^b)</td>
</tr>
<tr>
<td>Control</td>
<td>638±137</td>
<td>12±6</td>
<td>85±8</td>
</tr>
<tr>
<td>Indomethacin+ L-NAME</td>
<td>933±60(^c)</td>
<td>4.2±1.0(^b)</td>
<td>3.6±1.7(^c)</td>
</tr>
<tr>
<td>Control</td>
<td>628±94</td>
<td>13±4</td>
<td>78±9</td>
</tr>
<tr>
<td>TEA+ L-NAME</td>
<td>1020±97(^c)</td>
<td>0±0(^c)</td>
<td>0±0(^c)</td>
</tr>
</tbody>
</table>

MT: maximal tension; RA: rhythmic activity.

**Fig 7.** (A) Original registration showing the contractile effect and rhythmic activity induced by PE 10 µmol/L and relaxation induced by ACh 10 µmol/L in TA rings of STZ-induced diabetic mice. Pretreatment of the vessels with (B) L-NAME 1 mmol/L and (C) LY-83583 1 µmol/L for 10 min before addition of PE caused basal tension increase, reduced the relaxation to ACh but did not abolish rhythmic activity.

**Contribution of PGJ\(_2\) and EDHF-induced hyperpolarizations to relaxation** Indomethacin plus L-NAME depressed PE-induced rhythmic activity and ACh-induced vasorelaxation remarkably than L-NAME or indomethacin alone in TA rings of STZ-induced diabetic mice (Fig 8A, 8B, and Tab 2). TEA 1 mmol/L, an inhibitor of Ca\(^{2+}\)-activated potassium channels\(^{[7]}\), plus L-NAME abolished PE-induced rhythmic activity and ACh-induced vasorelaxation in diabetic mice (Fig 8C, and Tab 2). Indomethacin plus L-NAME and TEA plus L-NAME both increased basal and maximal tension in diabetic mice.

**DISCUSSION**

In the present study, the vasomotion and endothelial function were investigated in a murine experiment.
model of STZ-induced diabetes. So far few articles have reported enhanced endothelial function but lots of studies have showed endothelial dysfunction in diabetic experimental model such as rats, rabbits, dogs, and so on[4,8]. Furthermore we also identified clearly that PGI$_2$ was increased in the serum of early stage of STZ-induced diabetic mice. The results are also consistent with the idea that aorta of diabetic mice shows enhanced contractile responses to high K$^+$ and alpha-adrenoceptor agonists[9].

Vasoconstrictor-induced rhythmic activity in vessels has previously been investigated in both physiological and pathological states[10,11]. Three studies have demonstrated that rhythmic activity in vascular smooth muscle comes from cGMP-dependent activation of K$^+$ channels activated by endothelium-derived NO and is inhibited by L-NAME, LY-83583, and removal of the endothelium[12]. Then in normal condition, the output of endothelium-derived NO is a main factor to reflect endothelial function of vessels. Thus the change of rhythmic activity relates to endothelial function of vessels. In this study, we used a PE-induced rhythmic activity to observe endothelial function of TA rings. The amplitude of rhythmic activity in diabetic mice was considerably larger than that in age-matched controls. This shows enhanced endothelial function in TA rings of STZ-induced diabetic mice in early stage indirectly. However, Jiang et al have found that PE-induced rhythmic activity was enhanced while endothelial function was decreased in the mouse aorta lacking apolipoprotein E and low density lipoprotein receptor genes in vitro[12]. In my opinion, not only pathological mechanism and animal species are different but the mechanism of the change of rhythmic activity is also different in both studies. Furthermore, the enhanced rhythmic activity resulted an increase in opening of K$_Ca$ channels and rhythmic activity was abolished by L-NAME and LY-83583 in Jiang et al study, but L-NAME and LY-83583 could not abolish rhythmic activity in this study. So the change of rhythmic activity can reflect the endothelial function of vessels in my study.

DM is recognized as a condition in which there are increased oxidant stress, decreased bioavailability of NO, and depressed expression and structural modifications of G-proteins[13]. In the present study, rhythmic activity and ACh-induced relaxation were only partially inhibited by L-NAME or LY-83583 in STZ-induced diabetic mice but were abolished in age-matched controls. So the results indicate that there are other endothelium-derived relaxing factors (EDRF) to compensate NO-cGMP signal transduction in diabetic mice. Then PGI$_2$ and EDHF were investigated in diabetic mice, respectively. Firstly, indomethacin alone depressed ACh-induced relaxation significantly. Also indomethacin plus L-NAME depressed rhythmic activity and ACh-induced relaxation markedly than L-NAME alone but could not abolished them both. These results show that (1) PGI$_2$ is a parter of compensation to relax vessel in diabetic mice; (2) There is another factor to compensate to induce vasorelaxation except for NO and PGI$_2$ signal transduction. Then TEA, which can inhibit the effects of EDHF, was used to investigate EDHF. In the results, TEA plus L-NAME abolished rhythmic activity in TA rings of diabetic mice[14]. Taken together, PGI$_2$ and EDHF were all involved in compensates or substitutes NO to activate K$^+$ channels and induce vasorelaxation in these diabetic mice. As for whether there is increased release of NO responsible for the enhanced relaxant potency of ACh in diabetes or not, we will make it clear in future. However, indomethacin not affecting rhythmic activity strikingly indicates that NO and PGI$_2$ signal
transduction can compensate to activate rhythmic activity each other and maybe is mainly NO in vitro.

PGI\textsubscript{2}, as one of EDRF is produced by endothelial cells. While its biotic half life in the serum is only about 3 min and it can quickly change into stable metabolite: 6-keto-PGF\textsubscript{1α}. So the content of 6-keto-PGF\textsubscript{1α} shows the content of PGI\textsubscript{2} in the serum. In the present study, the content of 6-keto-PGF\textsubscript{1α} is evidently higher in the serum of STZ-induced diabetic mice than age-matched controls. This also coincides with our results of isolated TA rings experiments. So we provided two identifications of increased PGI\textsubscript{2} in the function and content.

Hyperglycaemia is a characteristic in DM. In this condition, the production of oxygen-derived radical is increased and NO is destroyed easily. NO has been generally considered as the principal mediator of endothelium-dependent relaxation in normal state\textsuperscript{[15]}. But PGI\textsubscript{2} and EDHF may also be an important regulator of vascular tone and reactivity in DM\textsuperscript{[16,17]}. Lots of studies indicate impaired ACh-induced relaxation in diabetic experimental model, but we have observed an enhanced ACh-induced relaxation. The possibility is that (1) the species of experimental animal model is different; (2) the plasma glucose of the STZ-induced diabetic mice is not so high by this method to induce diabetic animal experimental model; (3) the courses of these diabetic mice are maladjusted and then impaired endothelial function then to mediate basal vascular tone. However, in later stage of DM endothelial cells are maladjusted and then impaired ACh-induced relaxation can be observed. These may be dependent on the duration of disease\textsuperscript{[18]}

In conclusion, the mechanism that enhanced endothelium-dependent vasodilatation in STZ-induced diabetic mice is due to increased production of PGI\textsubscript{2} and EDHF. The phenomenon maybe only takes place in diabetic early period.

REFERENCES

3 Bolz SS, de Wit C, Pohl U. Endothelium-derived hyperpolarizing factor but not NO reduces smooth muscle Ca\textsuperscript{2+} during acetylcholine-induced dilation of microvessels. Br J Pharmacol 1999; 128: 124-34.
6 Summers BA, Overholt JL, Prabhakar NR. Nitric oxide inhibits L-type Ca\textsuperscript{2+} current in glomus cells of the rabbit carotid body via a cGMP-independent mechanism. J Neurophysiol 1999; 81: 1449-57.
10 Gustafsson H, Nilsson H. Rhythmic contractions in isolated small arteries of rat: role of K\textsuperscript{+} channels and the Na\textsuperscript{+}, K\textsuperscript{+}-pump. Acta Physiol Scand 1994; 150: 161-70.