

Full-length article

Protective effects of cariporide on endothelial dysfunction induced by high glucose¹Shuang-xi WANG, Xiao-ming XIONG, Tao SONG, Li-ying LIU²*Department of Pharmacology, Pharmaceutical College, Central South University, Changsha 410078, China***Key words**

sodium-hydrogen antiporter; endothelium; glucose; nitric oxide; malondialdehyde; superoxide dismutase

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Received 2004-04-23

Accepted 2004-09-27

doi: 10.1111/j.1745-7254.2005.00042.x

Abstract

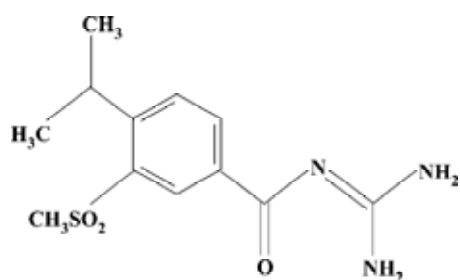
Aim: To explore the effects of cariporide, a selective sodium-hydrogen antiporter inhibitor, on endothelial dysfunction induced by high glucose. **Methods:** Acetylcholine (ACh)-induced endothelium-dependent relaxation (EDR), sodium nitroprusside (SNP)-induced endothelium-independent relaxation and biochemical parameters including malondialdehyde (MDA), superoxide dismutase (SOD), and nitric oxide (NO) were measured in rat isolated aorta. **Results:** A 6-h incubation of aortic rings with high glucose (44 mmol/L) resulted in a significant inhibition of EDR, but had no effects on endothelium-independent relaxation. After the 6-h incubation of aortic rings in the co-presence of cariporide (0.01, 0.1, and 1 μ mol/L) with high glucose, cariporide prevented the inhibition of EDR caused by high glucose in concentration-dependent manners. Similarly, high glucose decreased SOD activity and contents of NO, and increased MDA concentration in aortic tissue. Cariporide (1 μ mol/L) significantly resisted the decrease of NO content and SOD activity, and elevation of MDA concentration caused by high glucose in aortic tissues. Mannitol (44 mmol/L) or cariporide (1 μ mol/L) alone had no effect on EDR, endothelium-independent relaxation and biochemical parameters. **Conclusion:** Cariporide significantly prevented endothelial dysfunction induced by high glucose. The mechanisms of endothelial dysfunction induced by high glucose may involve the activation of sodium-hydrogen antiporter and the generation of oxygen-free radicals, but it is not related to the change of osmolarity.

Introduction

The relation between diabetes and premature vascular disease has been well established^[1]. One of the defects involves endothelial dysfunction characterized by impaired endothelium-dependent relaxation responses. Many metabolic disturbances of diabetes, hyperglycemia have been suggested to be the main cause of endothelial dysfunction. High glucose *in vitro* or *in vivo* has been reported to inhibit acetylcholine (ACh)-induced endothelium-dependent relaxation^[2] responses, to impair the biological synthesis pathway of nitric oxide (NO)^[3], and to generate reactive oxygen species^[4].

It has been demonstrated that hyperactivity of sodium-hydrogen exchange subtype 1 (NHE-1) has been implicated

in the vascular injury in diabetes mellitus^[5]. Ganz *et al*^[6] found that the activity and expression of NHE-1 significantly increased in mesangial cells after exposure to high glucose. Our previous experiments have demonstrated that the benzoylguanidine compound cariporide (4-isopropyl-3-methylsulfonyl-benzoylguanidine methanesulfonate), which is a selective NHE-1 inhibitor, protected against injuries of endothelial functions induced by high lipid diet in rabbits^[7]. However, it is not known whether NHE-1 inhibitor protects against endothelial function affected by high glucose. The aim of this study is to explore the effect of cariporide against endothelial dysfunction of isolated rat aortic rings induced by high glucose and to investigate its mechanisms.



Chemical structure of cariporide

Materials and methods

Drugs and chemicals Cariporide was obtained from Hoechst Company (Frankfurt, Germany). SNP, ACh, and phenylephrine (Phe) were purchased from Sigma Chemical Co (Saint Louis, Mo, USA). The kits for measurement of nitrite/nitrate (NO), MDA, and SOD activity were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

Animal Male Sprague-Dawley rats (No. SCXK2003-0003; Grade II) weighing 180–200 g, supplied by the Animal Center of Xiangya Medical College (Central South University, China) were used.

Preparation of rat thoracic aorta rings The rats were killed by exsanguinations after an intraperitoneal anesthesia with pentobarbital sodium 30 mg/kg and intravenous anticoagulation with heparin sodium 150 U/kg. The descending thoracic aorta was rapidly dissected from the rats and immersed in Krebs' solution, composed of (mmol/L): NaCl, 118.3; KCl, 4.7; MgSO₄·7H₂O, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 24.0; glucose, 11; and Na₂-EDTA, 0.026; and bubbled with 95% O₂+5% CO₂ (pH 7.4). After the perivascular tissue was carefully removed, the aortic rings (which were approximately 4 mm in length), were prepared.

Bioassay of vasoreactivity^[8] For isometric force recording, the aortic rings were mounted between two stainless steel hooks and suspended in a 10 mL organ bath containing above Krebs' solution at 37 °C bubbled with 95% O₂+5% CO₂ gas mixture (pH 7.4). An initial load of 2.0 g was applied, and the tension of the aortic ring was monitored by a force transducer and recorded on a polygraph (Model YL-1, Chengdu Instruments, China). After a 90-min equilibration period, the ring was precontracted by Phe 1 μmol/L. When the development tension attained its peak value, the ring was relaxed by ACh or SNP, respectively. Accumulative concentration-response curves to ACh (0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 μmol/L) or SNP (0.001–1 μmol/L) were recorded.

Determination of MDA concentration After a 6-h incubation of aortic segments, the aortic segments were blot-

ted dry and weighed, then made into 5% tissue homogenate in ice-cold 0.9% NaCl solution. A supernatant was obtained from tissue homogenate by centrifugation (1000×g, 4 °C, 10 min). The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured. Briefly, 1.0 mL of 20% trichloroacetic acid and 1.0 mL of 1% TBARS reagent were added to 100 μL supernatant, then mixed and incubated at 100 °C for 80 min. After cooling on ice, samples were centrifuged at 1000×g for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

Assay of SOD activity in aortic rings The supernatant of tissue homogenate of the aortic rings were obtained as described earlier. A competitive inhibition assay was performed by using xanthine/xanthine oxidase reaction-generated superoxide radicals to reduce nitro blue tetrazolium (NBT) quantitatively to blue formazan. Conversion of superoxide radicals to hydrogen peroxide by superoxide dismutase inhibited dye formation and served as a measure of superoxide dismutase activity. Briefly, the supernatant of 0.5 mL with xanthine 50 μmol/L and xanthine oxidase 2.5 μmol/L in potassium phosphate buffer 50 mmol/L (pH 7.8, 37 °C) were incubated for 40 min and NBT was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity.

Assay of NO concentration of incubation medium The incubation medium of the aortic artery was centrifuged (1000×g, 15 min, 4 °C) and the supernatant was used for NO measurement. NO was assayed by the Griess method. Because NO is a compound with a short half life and is rapidly converted to the stable end products nitrate (NO₃⁻) and nitrite (NO₂⁻), the principle of the assay is the conversion of nitrate into nitrite by cadmium and followed by color development with Griess reagent (sulfanilamide and *N*-naphthyl ethylenediamine) in acidic medium. The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer.

Protocol of experiment The first series of experiments were designed in order to evaluate the protective effects of cariporide against ACh-induced endothelium-dependent and SNP-induced endothelium-independent relaxing response of isolating rat aortic rings affected by high glucose. The experiment was divided into 7 groups with 8 aortic rings from 8 rats in each group. First, a normal control bioassay of vasoreactivity was formed in normal Krebs' solution. The rings, of which a percentage of relaxation induced by ACh

3 $\mu\text{mol/L}$ to the contraction elicited by Phe 1 $\mu\text{mol/L}$ is more than 80%, were considered as intact endothelium and used in the study. The aortic rings of each group were then continually incubated for 6 h in the following medium: (1) control group: glucose 11 mmol/L in Krebs' solution^[9]; (2) high glucose group: glucose 44 mmol/L in Krebs' solution^[9]; (3)–(5) cariporide-treated groups: cariporide 0.01, 0.1, and 1 $\mu\text{mol/L}$ in Krebs' solution with glucose 44 mmol/L, respectively; (6) cariporide alone group: cariporide 1 $\mu\text{mol/L}$ in Krebs' solution; (7) mannitol group: mannitol 44 mmol/L in Krebs' solution. The incubation mediums were changed every 30 min and cariporide was present throughout the incubation. After a 6-h incubation of aortic rings, the perfusion solution was changed to Krebs' solution, and bioassay of vasoreactivity was performed.

The second series of experiments were designed to assay the effects of high glucose on SOD activity, MDA concentration, NO and the effects of cariporide on the biochemical parameters in rat aortic rings. The experiment was divided into 5 groups with 8 aortic segments of 2 cm from 8 rats in each group; control group, high glucose group, mannitol group, cariporide-treated group, and cariporide-alone group. The components of incubation medium were the same as described earlier except that cariporide only had a dose of 1 $\mu\text{mol/L}$. The incubations media were changed every 30 min. Cariporide was added throughout incubation. After a 6-h incubation, the segments were transferred to 1 mL normal Krebs' solution which contained ACh 1 $\mu\text{mol/L}$ for 30 min. The aortic segments and the medium were then collected and frozen at $-70\text{ }^{\circ}\text{C}$ until analyzed.

Data analysis The ACh (3 $\mu\text{mol/L}$) or SNP (1 $\mu\text{mol/L}$)-induced maximal relaxation (E_{max}) in aortic rings was calculated as a percentage of the contraction to Phe (1 $\mu\text{mol/L}$). The half maximum effective concentration (EC_{50}) was defined as a concentration of the ACh that induced 50% of maximum relaxation response to contraction elicited by Phe (1 $\mu\text{mol/L}$) and calculated from the concentration-response curve generated by linear regression analysis. All data were expressed as mean \pm SD. Statistical comparisons were made using one-way ANOVA followed by Newman–Keuls test. $P < 0.05$ was statistically significant.

Results

Effects of high glucose on EDR and endothelium-independent relaxation in aortic rings There were no significant differences in ACh-induced relaxation responses of rat isolated aortic rings before the 6-h incubation among various groups (data not shown). After a 6-h incubation of aortic rings in control glucose (11 mmol/L) buffer, ACh (0.003–3

$\mu\text{mol/L}$) still evoked a normal concentration-dependent relaxation (Figure 1), the E_{max} of aortic rings reached $88.4\% \pm 12.3\%$, and the EC_{50} value was $94.5 \pm 10.8\text{ nmol/L}$. After the 6-h incubation of the aortic rings and exposure to high glucose (44 mmol/L), the E_{max} fell to $43.7\% \pm 16.1\%$ and the EC_{50} value increased to $154.8 \pm 22.9\text{ nmol/L}$ ($P < 0.01$ vs control group, $n=8$). However, no significant changes of E_{max} and EC_{50} were shown in the rings incubated in the cariporide (1 $\mu\text{mol/L}$)-alone group or mannitol (44 mmol/L) group, compared with the control group (Figure 1).

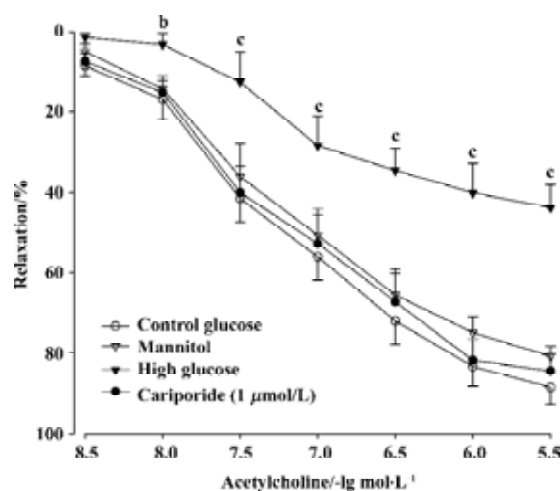


Figure 1. EDR induced by ACh in rat isolated aortic rings. After a 6-h incubation of aortic rings in control glucose (11 mmol/L), high glucose (44 mmol/L), mannitol (44 mmol/L), or cariporide (1 $\mu\text{mol/L}$) in Krebs' solution, the ACh (0.003–3 $\mu\text{mol/L}$)-induced EDR was measured and calculated as a percentage of the contraction to Phe (1 $\mu\text{mol/L}$). $n=8$. Mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs control group.

The endothelium-independent relaxation induced by SNP (0.001–1 $\mu\text{mol/L}$) was not significantly different between the different treated groups (data not shown).

Effects of cariporide on the impairment of EDR induced by high glucose Treatment with cariporide in different concentration (0.01, 0.1, and 1 $\mu\text{mol/L}$, $n=8$, respectively) significantly prevented inhibition of EDR induced by high glucose (Figure 2). The E_{max} were $61.7\% \pm 10.5\%$, $76.0\% \pm 10.5\%$, and $83.4\% \pm 10.1\%$, and the EC_{50} value was $131.5 \pm 15.9\text{ nmol/L}$, $117.1 \pm 13.7\text{ nmol/L}$, and $109.6 \pm 10.5\text{ nmol/L}$, respectively. There was a significant difference ($P < 0.05$ or $P < 0.01$), compared with those in the high glucose group (Figure 2).

Effects of cariporide on biochemical index in aortic segments A 6-h incubation of isolated aortic segments in high glucose resulted in an elevation of MDA content, decrease of SOD activity in aortic tissue, and reduction of NO

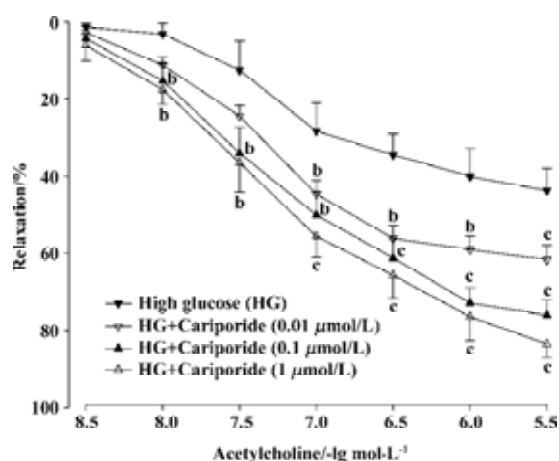


Figure 2. Effects of cariporide (0.01, 0.1, and 1 $\mu\text{mol/L}$) on EDR impaired by high glucose (44 mmol/L). After a 6-h incubation, ACh (0.003–3 $\mu\text{mol/L}$)-induced EDR were measured and calculated as a percentage of the contraction to Phe (1 $\mu\text{mol/L}$). $n=8$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs HG group.

releasing from aortic segments. Treatment with cariporide (1 $\mu\text{mol/L}$) in the high glucose (44 mmol/L) group significantly prevented the increase of MDA content, and protected the activity of SOD and release of NO in aortic segments (Table 1). Mannitol (44 mmol/L) or cariporide (1 $\mu\text{mol/L}$) alone had no effects on MDA, SOD, and NO, compared with the control group (Table 1).

Discussion

Diabetes mellitus is characterized by chronic hyperglycemia and associated with significant morbidity as a result of long-term complications, including diabetic nephropathy, atherosclerosis, and hypertension. High glucose had a lot of toxicity effects in endothelial cells, such as impairment of endothelial dependent relaxation^[10], decrease of NO release, generation of free radicals, and increase in apoptosis^[11]. The endothelial dysfunction has been thought to be the major cause of vascular disease due to hyperglycemia condition.

It was reported that hyperglycemia resulted in an increase of NHE-1 activity in diabetes^[12,13]. In addition, high glucose may induce the activation of NHE-1 and increase NHE-1 mRNA expression *in vitro* smooth muscle cells or myocytes^[14,15]. However, there are no reports as to whether NHE-1 inhibitor is able to protect against the impairment of endothelial functions caused by high glucose. The present study explores the effects of cariporide against high glucose-induced endothelial dysfunction of rat isolated aorta and discusses its mechanisms.

In the present study, we employed a mimic pronounced hyperglycemia model in which the rat isolated aortic rings were exposed to high glucose for 6 h. High glucose significantly inhibited EDR and the release of NO in rat isolated aortic rings, but did not affect vasodilatation induced by SNP, a NO donor. It has been demonstrated that endothelium dysfunction induced by high glucose related to the release of NO from endothelial cells. The same concentration of mannitol had no effect on EDR of aortic rings, which showed that the damage of EDR of aortic rings induced by high glucose was not due to a hyperosmotic effect. These results in the present study were consistent with a previous study^[2]. We also found that cariporide in dose-dependent manners prevented the inhibition of EDR induced by high glucose in rat isolated thoracic aorta (Figure 1), and simultaneously maintained SOD activity and NO release and decreased MDA concentration caused by high glucose in rat aortic segments (Table 1). It was reported that cariporide significantly inhibited the injuries of mitochondrial and pulmonary endothelial cells, and protected cardiac ischaemia and reperfusion *in vitro* by reducing intracellular pH and inhibiting NHE-1 activation, which simultaneously attenuated oxidant production^[16–18]. The Na^+/H^+ exchanger inhibitor, amiloride, significantly reduced oxidant production of hepatic stellate cells including intracellular hydroperoxides and MDA induced by oxidant^[19]. Accordingly, it is believed that the protective effects of cariporide against endothelial dysfunctions induced by high glucose may partly be due to anti-oxidation and pro-

Table 1. MDA concentration, SOD activity and NO content of isolated aortic segments in the different treated groups. $n=8$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control glucose group. ^e $P<0.05$, ^f $P<0.01$ vs high glucose group.

Groups	MDA/ $\mu\text{mol}\cdot\text{g}^{-1}$ protein	SOD activity/ $\text{kNU}\cdot\text{g}^{-1}$ protein	NO/ $\text{mmol}\cdot\text{g}^{-1}$ protein
Control glucose (11 mmol/L)	4.5 \pm 2.5	166 \pm 43	0.9 \pm 0.6
Mannitol (44 mmol/L)	4.3 \pm 1.7	167 \pm 73	0.8 \pm 0.5
Cariporide (1 $\mu\text{mol/L}$)	4.5 \pm 3.9	147 \pm 72	0.8 \pm 0.3
High glucose (44 mmol/L)	7.2 \pm 1.4 ^b	86 \pm 30 ^c	0.38 \pm 0.28 ^b
HG + Cariporide (1 $\mu\text{mol/L}$)	4.6 \pm 2.4 ^e	146 \pm 19 ^f	1.0 \pm 0.5 ^e

tective activity of anti-oxidative enzymes.

In the resting state, NHE-1 is relatively quiescent. When intracellular pH falls, the NHE-1 is activated, and the rate of H⁺ efflux mediated by NHE-1 increases and causes intracellular alkalinization and Na⁺ overload, which leads to intracellular Ca²⁺ overload through a mechanism of reverse Na⁺-Ca²⁺ exchange. It is presumed that during high glucose condition, there is a metabolic mismatch between glycolysis and glucose oxidation that results in the accumulation of hydrogen ions, which, in turn, activates NHE-1, leading to intracellular alkalinization and Ca²⁺ overload. Therefore, we hypothesized that, in the present study, the mechanisms of cariporide against high glucose-induced endothelial dysfunction of rat isolated aortas might be also related to decreasing intracellular alkalinization and Ca²⁺ overload. Although intracellular pH and intracellular Ca²⁺ concentration were not assayed in the study, it has been reported that cariporide could protect from releasing NO in vascular endothelium through inhibiting activity of NHE-1, decreasing intracellular alkalinization and Ca²⁺ overload^[17,20].

In conclusion, this study demonstrates that cariporide significantly prevents endothelial dysfunction, decreases NO release, elevates MDA concentration and reduces SOD activity induced by high glucose in rat isolated thoracic aorta. The mechanisms of protective effects of cariporide may be related to the inhibition of NHE-1 and the decrease of oxidative stress injury. These results provide a potential new target for intervention in the prevention of diabetic complications.

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