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Original Article

Arecoline improves vascular endothelial function in high fructose-fed rats via increasing cystathionine-γ-lyase expression and activating K_{ATP} channels

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Aim: To investigate the effect of arecoline, a major component of betel nut, on vascular endothelial function in high fructose-fed rats and the potential mechanisms underlying the effect.

Methods: Male Wistar rats were fed a high-fructose or control diet for 16 weeks. At the beginning of week 13, the rats were injected ip with low (0.5 mg·kg^{-1}·d^{-1}), medium (1.0 mg·kg^{-1}·d^{-1}) or high (5.0 mg·kg^{-1}·d^{-1}) doses of arecoline for 4 weeks. At the termination of the treatments, blood was collected, fasting blood glucose (FBG) and serum insulin (FSI) levels were measured, and insulin sensitivity index (ISI) was calculated. The thoracic aortas were isolated and aortic rings were prepared for studying ACh-induced endothelium-dependent vasorelaxation (EDVR). The mRNA and protein expression of cystathionine-γ-lyase (CSE) in the thoracic aortas was analyzed using RT-PCR and Western blot analysis, respectively.

Results: In high fructose-fed rats, the levels of FBG and FSI were remarkably increased, whereas the ISI and the mRNA and protein expression of CSE were significantly decreased. ACh-induced EDVR in the aortic rings from high fructose-fed rats was remarkably reduced. These changes were reversed by treatment with high dose arecoline. Pretreatment of the aortic rings rings from high fructose-fed rats with the CSE inhibitor propargylglycine (10 mmol/L) or the ATP-sensitive potassium (K_{ATP}) channel blocker glibenclamide (10 mmol/L) abolished the restoration of ACh-induced EDVR by high dose arecoline. On the contrary, treatment with high dose arecoline significantly impaired ACh-induced EDVR in the aortic rings from control rats, and pretreatment with propargylglycine or glibenclamide did not cause further changes.

Conclusion: Arecoline treatment improves ACh-induced EDVR in high fructose-fed rats, and the potential mechanism of action might be associated with increase of CSE expression and activation of K_{ATP} channels by arecoline.

Keywords: arecoline; betel-quid chewing; propargylglycine; glibenclamide; high fructose-fed rat; endothelium-dependent vasorelaxation; cystathionine-γ-lyase; K_{ATP} channel; diabetes mellitus; vascular endothelial dysfunction

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Introduction

Insulin resistance (IR), defined as the decreased ability of cells or tissues to respond to physiological levels of insulin, is thought to be a pathogenic hallmark of type 2 diabetes mellitus (T2DM). Epidemiological evidence has demonstrated that IR is frequently associated with a number of other health disorders, including obesity, hypertension and cardiovascular disease (CVD)[1–3]. Recent research indicates that vascular endothelial dysfunction (VED) plays a key role in linking the pathogenesis of vascular complications and IR[4], and macro- and micro-vascular complications are still the main causes of morbidity and mortality in patients with diabetes mellitus (DM)[5]. Our laboratory has previously demonstrated that fructose-fed rats had attenuated endothelial relaxation in response to ACh compared with controls[6]. Therefore, improvement of VED may be useful for inhibiting the development of vascular complications in IR or DM patients.

Arecoline is a major component of the betel nut and has many important physiological activities. Some studies have indicated that betel-quid chewing is associated with an increased risk of oral cancer and esophageal carcinoma[7, 8]. Epidemiological studies have shown that betel-quid chewing is associated with the risk of T2DM and metabolic syndrome in men[9, 10]. Hsu and Hsieh *et al* reported that arecoline inhibited preadipocyte differentiation and locked insulin signaling

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in 3T3-L1 adipocytes[11, 12]. Our previous study found that arecoline improved glucose and lipid metabolism in type 2 diabetic rats and prevented high fructose–induced pancreatic β-cell dysfunction[13, 14]. We also found that arecoline regulated the expression of cystathionine-γ-lyase (CSE), which is suggested to be the only H_2S-generating enzyme in the vascular system[15, 16], and reduced inflammatory factor expression induced by oxidized low-density lipoprotein in RAW264.7 cells[17]. Zhao *et al* reported that H_2S relaxed rat aortic tissues *in vitro* in a K_{ATP} channel-dependent manner[18]. Siebert *et al* showed that H_2S mediated vasorelaxation of the hepatic artery by activation of K_{ATP} channels[19]. Goto *et al* reported that areca seed extract led to endothelium-dependent vasodilation in rat aortas[20]. The above studies suggest that arecoline might play an important role in vasorelaxation mediated by the CSE-H_2S-K_{ATP} pathway. However, it is unclear whether arecoline can improve endothelium-dependent vasorelaxation (EDVR) in high fructose–fed rats. Therefore, in the present study, we sought to investigate the effect of arecoline on high fructose–induced EDVR and its mechanism of action.

Materials and methods

Reagents

Arecoline, ACh, *L*-phenylephrine hydrochloride (*L*-PE), glibenclamide and propargylglycine (PAG) were obtained from Sigma Co (St Louis, MO, USA). *D*-fructose was purchased from Sangon Biotech Co (Shanghai, China). TRIzol and AMV reverse transcriptase were purchased from BBI, and affinity-purified anti-CSE polyclonal antibodies were purchased from Santa Cruz (CA, USA).

Animals and groups

Male Wistar rats (200±20 g) were housed in standard animal laboratories with a 12 h light/dark cycle with free access to food and water. The rats were randomly divided into two groups of twenty four animals each. For 16 weeks, the control group (Con) received a normal chow diet and the high-fructose group (HF) received a high-fructose diet containing 75% fructose, 12% fat and 13% protein[21, 22]. At the beginning of week 13, the two groups of rats were treated with low (L-Are, 0.5 $mg\cdot kg^{-1}\cdot d^{-1}$), medium (M-Are, 1.0 $mg\cdot kg^{-1}\cdot d^{-1}$) or high (H-Are 5.0 mg/kg/d) doses of arecoline by intraperitoneal injection or left untreated for 4 weeks.

Measurement of fasting blood glucose (FBG) and fasting serum insulin (FSI)

At the termination of the study, all rats were fasted for 12 h and anesthetized with sodium pentobarbital (40 mg/kg body wt, ip). Blood was collected by carotid puncture into non anticoagulated plastic centrifuge tubes, and the tubes were centrifuged at 3000 r/min for 10 min at 4 °C. FBG was measured with an HI-TACH717 automatic biochemical analyzer. FSI was measured using a radioimmunoassay method, and the insulin sensitivity index (ISI) was calculated using Li's formula [ISI=-ln(FBS×FSI)][23].

Preparation of thoracic aortas and measurement of vascular reactivity

The thoracic aortas from the rats were isolated and immediately placed into a cold Krebs solution of the following composition (mmol/L): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.0. Aortic segments were cleaned of perivascular fat and connective tissue, cut into 2 to 3-mm segments, and mounted on two stainless steel hooks in a 10 mL organ bath containing Krebs solution at 37 °C, aerated with 95% O_2 and 5% CO_2. One hook was connected to an isometric force transducer to measure tension. The rings were equilibrated for 90 min, during which time the bathing fluid was changed every 15 min. The tissue was kept under a constant tension of 2 g throughout the experiment. Endothelium-dependent vessel relaxation was assessed qualitatively in 1 μmol/L phenylephrine-precontracted rings as the degree of relaxation caused by the cumulative addition of increasing concentrations of acetylcholine (ACh; 10^{-9}–10^{-5} mol/L). The effects of the CSE inhibitor propargylglycine (PAG) and the potassium channel blocker glibenclamide (Glib) were also evaluated. In these experiments, the rings were incubated with PAG (10 mmol/L) or Glib (10 mmol/L) for 30 min and then the ACh-induced EDVR was determined.

Determination of CSE mRNA expression by RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously[24]. Briefly, total RNA was extracted from thoracic aortas with TRIzol according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically, and cDNA was prepared by reverse transcription of RNA (1 μg) using oligonucleotide primers. Subsequently, the equivalent of 0.05 μg was amplified by PCR using the following primers: CSE 5′-GTATTGAGGCACCAACAGGT-3′ and 5′-GTTGGGTTTGTGGGTGTTTC-3′ (149 bp); and GAPDH 5′-TCAACGGCACAGTCAAGG-3′ and 5′-GGCTAAGCAGTTGGTGGT-3′ (308 bp). The PCR conditions were 94 °C for 4 min, followed by 32 cycles of 94 °C for 60 s, 58 °C for 30 s, and 72 °C for 60 s, followed by a 72 °C extension for 10 min.

Thoracic aorta Western blot analysis

For CSE analysis by Western blot, thoracic aortas were lysed in a buffer containing 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl and 0.5% NP-40, followed by centrifugation at 13 000×*g* for 15 min at 4 °C. Protein concentrations were determined with the BCA protein assay. Briefly, 30 μg of each sample was separated on a 10% SDS-PAGE gel and then transferred to polyvinyl difluoride (PVDF) membranes. The membranes were blocked for 60 min in a buffer containing 0.1% Tween 20 and 5% milk. Antibodies against CSE or GAPDH were used to identify the specific proteins and visualized by the ECL method described previously[25]. The intensity of the protein band of interest was quantified by densitometry.

Statistical analysis

Data were presented as the mean±SD. Statistical comparisons

between two groups were made with Student's *t*-test, whereas ANOVA was used to test the differences between multiple groups. $P<0.05$ was considered significant.

Results

Effects of arecoline on FBG, FSI, and the ISI

As shown in Table 1, FBG and FSI were significantly increased, whereas the ISI was remarkably decreased in the HF group, compared with the control group ($P<0.05$). Treatment with arecoline reversed the above parameters in the HF group, and this change was significant in the group treated with the highest dose of arecoline (H-Are; 5.0 mg·kg^{-1}·d^{-1}). Arecoline treatment had no significant effect on the control group.

Table 1. Effect of arecoline on the FBG, FSI, and ISI in rats.

Groups	FBG (mmol/L)	FSI (mmol/L)	ISI
Con	5.67±0.10	22.57±0.46	-4.45±0.33
Con+L-Are	5.76±0.10	22.86±0.49	-4.88±0.03
Con+M-Are	5.50±0.10	23.35±0.65	-4.85±0.02
Con+H-Are	5.57±0.10	24.48±0.54	-4.91±0.04
HF	9.02±0.06^{b}	39.46±0.40^{b}	-5.75±0.24^{b}
HF+L-Are	8.50±0.07	38.06±0.92	-5.77±0.03
HF+M-Are	6.70±0.13	29.80±0.45	-5.29±0.09
HF+H-Are	6.00±0.12^{e}	23.65±0.77^{e}	-4.97±0.05^{e}

Normal control and high-fructose fed rats were treated with and without low, medium, and high dose of arecoline (L-Are, 0.5 mg·kg^{-1}·d^{-1}; M-Are, 1.0 mg·kg^{-1}·d^{-1} and H-Are 5.0 mg·kg^{-1}·d^{-1}, intraperitoneal injection) for 4 weeks as the Con group, Con+L-Are group, Con+M-Are group, Con+H-Are group, HF group, HF+L-Are group, HF+M-Are group, and HF+H-Are group, respectively. The fasting blood glucose (FBG) and fasting serum insulin (FSI) were measured and insulin sensitivity index (ISI) was calculated. [ISI= -ln (FBS×FSI)]. Results are expressed as mean±SD (n=6). $^{b}P<0.05$ *vs* the Con group. $^{e}P<0.05$ *vs* the HF group.

Effects of arecoline on EDVR in high fructose-fed rats

As shown in Figure 1A, ACh-induced EDVR was significantly impaired in aortic rings from the high fructose-fed rats compared with the control group. Treatment of high fructose–fed rats with arecoline restored the EDVR in a concentration-dependent manner, and treatment with the highest dose of arecoline (H-Are; 5.0 mg·kg^{-1}·d^{-1}) significantly increased the potency (EC_{50}) and the maximal response (E_{max}) of endothelial relaxation in response to ACh. However, in the Con group, H-Are treatment significantly decreased the ACh-induced EDVR and significantly impaired maximal relaxation in response to ACh (Figure 1B, Table 2). These results suggest that H-Are treatment improved EDVR and prevented vascular endothelial dysfunction (VED) in rats fed a high-fructose diet, whereas H-Are treatment impaired EDVR in rats fed a normal diet.

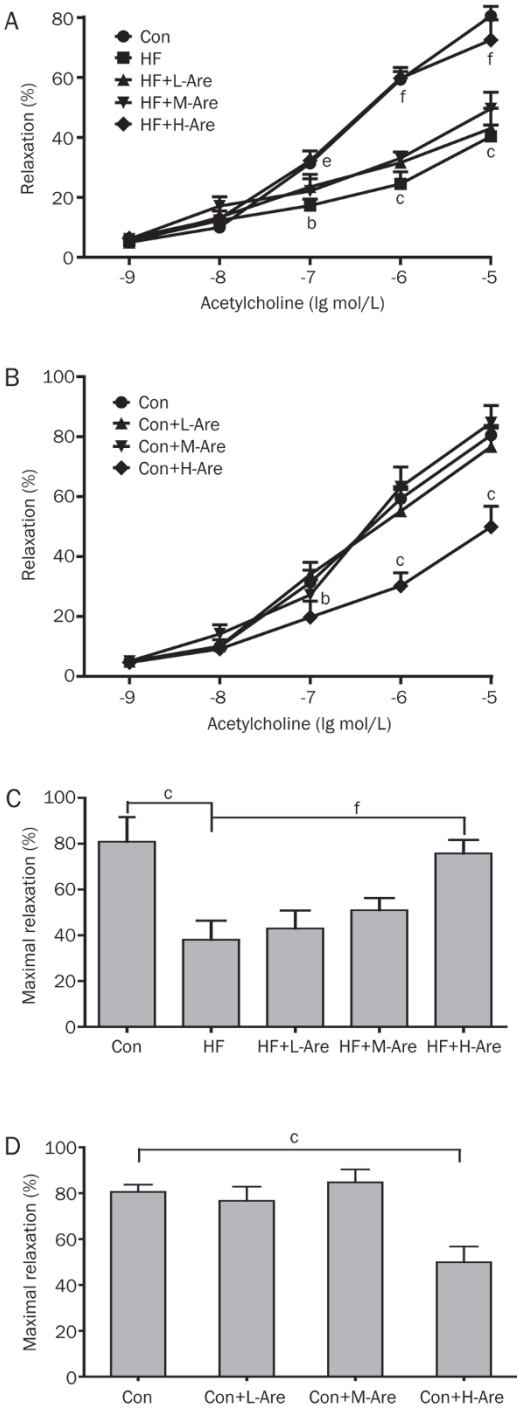

Figure 1. Effects of arecoline on EDVR in high fructose-fed rats. Normal control (Con) and high fructose-fed (HF) rats were treated with low (L-Are, 0.5 mg·kg^{-1}·d^{-1}), medium (M-Are, 1.0 mg·kg^{-1}·d^{-1}) or high (H-Are, 5.0 mg·kg^{-1}·d^{-1}) doses of arecoline by intraperitoneal injection for 4 weeks or were left untreated. Endothelium-dependent vasorelaxation (EDVR) of rings from the thoracic aorta in response to acetylcholine (ACh) was examined. in the high fructose-fed rats (A) and normal control rats (B). Maximal relaxation response to acetylcholine (ACh) was calculated in the high fructose-fed rats (C) and normal control rats (D). Mean±SD (n=6). $^{b}P<0.05$, $^{c}P<0.01$ *vs* the Con group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* the HF group.

Table 2. The potency (EC_{50}; pD_2=-log EC_{50}) and maximum relaxation (E_{max}) values to acetylcholine in rat thoracic aortic rings from Con, Con+PAG, Con+Glib, Con+H-Are, Con+H-Are+PAG, Con+H-Are+Glib, HF, HF+PAG, HF+Glib, HF+H-Are, HF+H-Are+PAG, or HF+H-Are+Glib. Values are expressed as mean±SD, n=6. ^{c}P<0.01 *vs* the Con group; ^{i}P<0.01 *vs* the Con+PAG group or the Con+Glib group; ^{f}P<0.01 *vs* the HF group; ^{l}P<0.01 *vs* the HF+Are group.

Groups	EC_{50}	pD_2	E_{max}
Con	2.44E-7	6.61±0.15	80.52%±3.2%
Con+PAG	4.36E-7	6.36±0.17	74.62%±3.5%
Con+Glib	5.24E-8	7.26±0.19	76.02%±4.5%
Con+H-Are	9.96E-6	5.00±0.14^{c}	49.87%±6.9%^{c}
Con+H-Are+PAG	2.98E-5	4.52±0.15^{i}	46.18%±4.6%^{i}
Con+H-Are+Glib	1.36E-6	5.87±0.12^{i}	52.59%±4.4%^{i}
HF	1.13E-4	3.95±0.11^{c}	40.33%±3.8%^{c}
HF+PAG	5.47E-4	3.26±0.10	37.65%±5.8%
HF+Glib	5.00E-4	3.30±0.11	36.16%±2.2%
HF+H-Are	2.89E-7	6.54±0.16^{f}	72.50%±6.7%^{f}
HF+H-Are+PAG	1.36E-6	5.87±0.17^{l}	52.59%±5.6%^{l}
HF+H-Are+Glib	2.83E-5	4.55±0.14^{l}	44.67%±3.3%^{l}

Effect of the CSE inhibitor PAG or the potassium channel blocker glibenclamide on EDVR in high fructose-fed rats

To examine whether CSE or potassium channels were involved in EDVR, the CSE inhibitor PAG and the potassium channel blocker glibenclamide (Glib) were used. As shown in Figure 2, pretreatment with 10 mmol/L PAG did not significantly change ACh-induced EDVR in the Con group or the HF group. However, endothelial relaxation in response to acetylcholine was partly abolished in aortic rings from rats in the HF group treated with H-Are and PAG compared with those that were not treated with PAG. Similar EDVR results were obtained in high fructose-fed rats after pretreatment with glibenclamide (Figure 3, Table 2). These results indicated that CSE or potassium channels was involved in the arecoline treatment-induced improvement in vessel function in high fructose-fed rats.

Effect of arecoline on CSE mRNA and protein expression in thoracic aorta

Our previous study found that arecoline treatment could regulate the expression of CSE in RAW264.7 cells[17]. To further investigate whether the changes in CSE expression contribute to the arecoline-mediated improvement of EDVR on a high-fructose diet, mRNA and protein levels of CSE were determined. As illustrated in Figure 4, CSE mRNA and protein were expressed and could be detected in rat thoracic aorta. Moreover, a high-fructose diet significantly decreased CSE mRNA and protein expression. However, arecoline treatment resulted in a dose-dependent increase of CSE mRNA and protein expression, which was significant in the H-Are treated

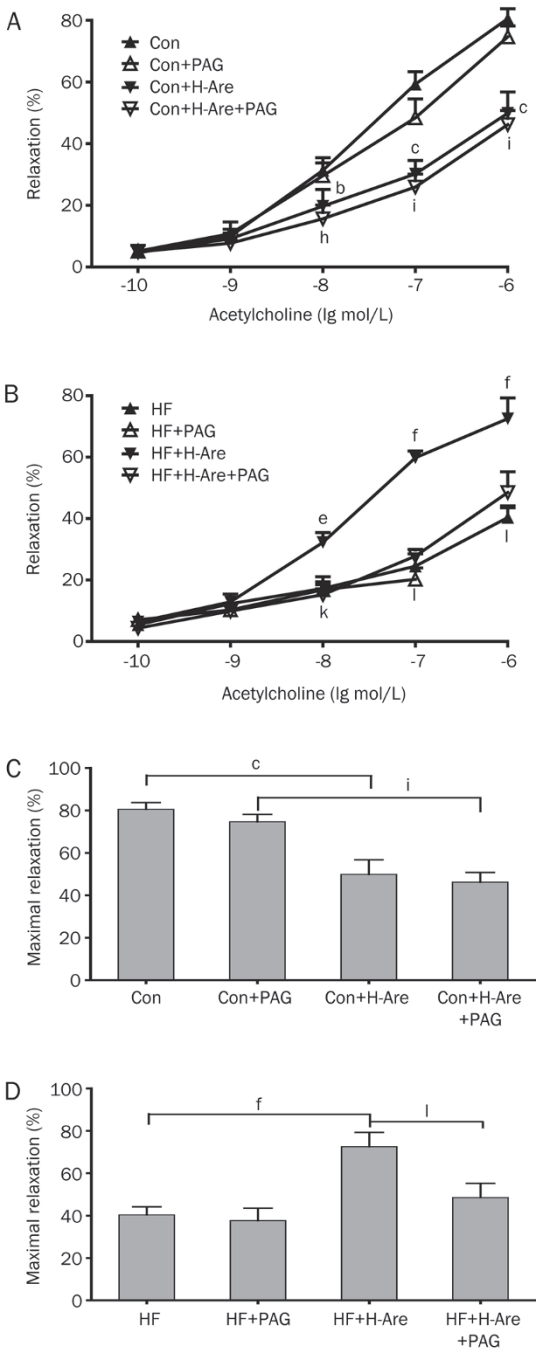

Figure 2. Effect of the cystathionine-γ-lyase (CSE) inhibitor propargylglycine (PAG) on EDVR in high fructose-fed rats. Normal control and high fructose-fed rats were treated with or without high doses of arecoline (H-Are, 5.0 mg·kg^{-1}·d^{-1}) by intraperitoneal injection for 4 weeks. Effects of inhibition of cystathionine-β-synthase (CBS) by 10 mmol/L propargylglycine (PAG) on ACh-induced EDVR were examined. in the normal control rats (A) and high fructose-fed rats(B). Maximal relaxation response to acetylcholine (ACh) was calculated in the normal control rats (C) and high fructose-fed rats(D). Mean±SD(n=6). ^{b}P<0.05, ^{c}P<0.01 *vs* the Con group; ^{e}P<0.05, ^{f}P<0.01 *vs* the HF group; ^{h}P<0.05, ^{i}P<0.01 *vs* the Con+PAG group; ^{k}P<0.05, ^{l}P<0.01 *vs* the HF+Are group.

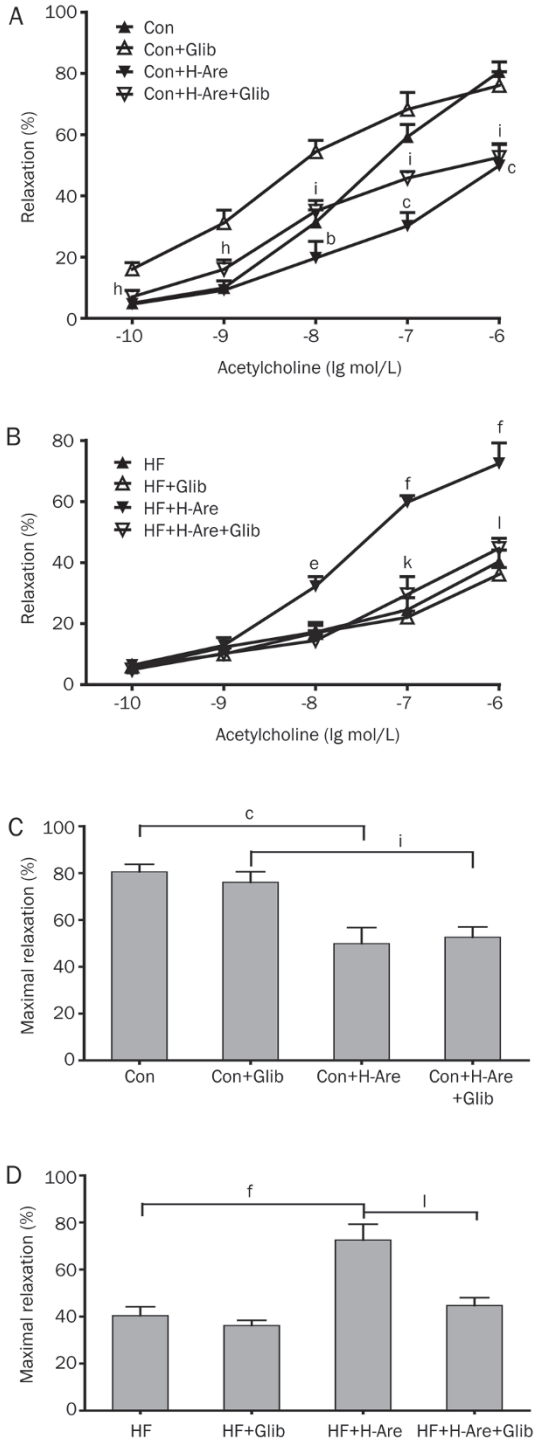

Figure 3. Effect of the potassium channel blocker glibenclamide (Glib) on EDVR in high fructose-fed rats. Normal control and high fructose-fed rats were treated with or without high doses of arecoline (H-Are, 5.0 mg·kg^{-1}·d^{-1}) by intraperitoneal injection for 4 weeks. Effects of inhibition of potassium channels by 10 mmol/L glibenclamide (Glib) on ACh-induced EDVR were examined. in the normal control rats (A) and high fructose-fed rats(B). Maximal relaxation response to acetylcholine (ACh) was calculated in the normal control rats (C) and high fructose-fed rats (D). Mean±SD (n=6). ^{b}P<0.05, ^{c}P<0.01 *vs* the Con group; ^{h}P<0.05, ^{i}P<0.01 *vs* the Con+Glib group; ^{e}P<0.05, ^{f}P<0.01 *vs* the HF group; ^{k}P<0.05, ^{l}P<0.01 *vs* the HF+Are group.

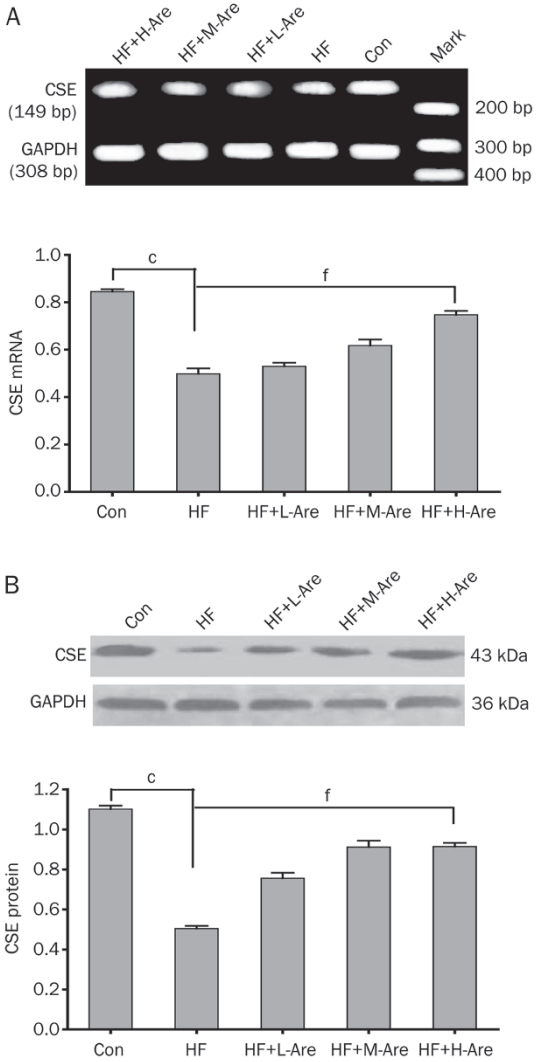

Figure 4. Effect of arecoline on CSE mRNA and protein expression in thoracic aortas. High fructose-fed rats were treated with or without low (L-Are, 0.5 mg·kg^{-1}·d^{-1}), medium (M-Are, 1.0 mg·kg^{-1}·d^{-1}), and high (H-Are 5.0 mg·kg^{-1}·d^{-1}) doses of arecoline by intraperitoneal injection for 4 weeks. Control rats were fed a normal diet. CSE mRNA and protein expression in thoracic aortas was measured by RT-PCR and Western blot, respectively. The results are expressed as the mean±SD (n=6). ^{b}P<0.05 *vs* the control group; ^{e}P<0.05 *vs* the HF group. (A) CSE mRNA expression was measured by RT-PCR. Relative mRNA levels were calculated as the ratio of CSE to GAPDH. (B) CSE protein levels were measured by Western blot. Representative immunoblots (top) and densitometric analyses (bottom) of CSE protein levels normalized to β-actin levels were reported. Values are expressed as the mean±SD (n=6). ^{c}P<0.01 *vs* the Con group; ^{f}P<0.01 *vs* the HF group.

group (mRNA expression 0.48±0.05 *vs* 0.78±0.04, P<0.05; protein expression 0.52±0.04 *vs* 0.73±0.08, P<0.05) (Figure 4).

Discussion

In the present study, FBG was increased, serum insulin was elevated, and the ISI was decreased in high fructose–fed rats compared with controls, suggesting that a high-fructose diet

resulted in insulin resistance (IR), as reported previously[6, 26]. Furthermore, the high-fructose diet impaired EDVR in rat thoracic aortas. The mechanisms involved in the fructose-induced IR and VED are unclear, but some studies have demonstrated that IR and VED may contribute to the development of various cardiovascular diseases[3, 4, 27, 28]. Hence, improvement of VED may reduce the incidence of vascular complications in IR or DM patients.

Arecoline is an alkaloid-type natural product found in betel nuts. It has many important functions such as deworming[29], preventing Alzheimer's disease[30] and inhibiting atherosclerosis[17, 31]. Some studies have reported that arecoline decreased vascular tone and improved EDVR[32, 33]. Our results indicated that high doses of arecoline (H-Are; 5.0 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) significantly impaired EDVR in the Con group, suggesting that long-term use or high doses of arecoline might induce VED or associated diseases. However, we also found that H-Are treatment improved IR and EDVR in the high fructose-fed rats. These results seem to be contradictory because H-Are treatment had opposite effects on EDVR in the Con group and the HF group; we think that this conflicting result might be the result of a difference in conditions between the groups. In the Con group, H-Are treatment might inhibit cell growth and proliferation of endothelial cells (ECs) and lead to VED, which is consistent with the reports from Kuo and Tseng *et al*[33, 34]. Because arecolin treatment impaired vascular endothelium and inhibited KATP channel in the Con group[27], the improvement in EDVR might be a direct or an indirect effect on vascular smooth muscle cells (VSMCs) in high fructose–fed rats. The underlying mechanisms by which arecoline improved EDVR are still not clear.

Recent studies showed that arecoline treatment increased CSE expression and H_2S production in macrophages[17]. Increasing evidence suggests that H_2S might be the third endogenous signaling gasotransmitter, which shares features with nitric oxide (NO) and carbon dioxide (CO_2). Unlike NO, which can be produced by both ECs and VSMCs, H_2S-producing enzymes are not expressed in the vascular endothelium; therefore, H_2S is only generated by VSMCs[18]. H_2S is produced endogenously from cysteine by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) and/or cystathionine-γ-lyase (CSE)[26]. CSE, which is localized to smooth muscle, is thought to be the major H_2S-producing enzyme in the thoracic aorta[16]. Some studies also showed that endogenous H_2S functions to regulate smooth muscle tone in synergy with NO. Furthermore, NO appears to be a physiological modulator of endogenous H_2S production by increasing CSE expression and stimulating its activity[16]. Hence, we detected CSE mRNA and protein expression in the thoracic aorta using RT-PCR and Western blotting, respectively. The data showed that high fructose significantly decreased CSE expression; however, H-Are treatment significantly increased CSE expression. We found that pretreatment with the CSE-specific inhibitor PAG further impaired ACh-mediated relaxation in the HF fed and H-Are treated groups, whereas PAG pretreatment had no significant effect on the Con group and HF fed groups. Taken together, these data demonstrated that the improvement in ACh-induced EDVR after arecoline treatment may be partially caused by increased CSE expression and H_2S generation. However, because of the experimental limitations, endogenous H_2S levels in the thoracic aortas were not determined.

Some studies also showed that H_2S is the only endogenous gaseous K_{ATP} channel opener and that H_2S activated K_{ATP} channels at the whole-cell and single channel levels in VSMCs[16, 18, 19]. Opening of K_{ATP} channels leads to membrane hyperpolarization and relaxation of VSMCs. To elucidate whether K_{ATP} channels promoted the effects of arecoline on EDVR in the thoracic aorta, a specific K_{ATP} channel blocker, Glibenclamide (Glib), was administered. We found that pretreatment with Glib impaired ACh-mediated EDVR in the HF+H-Are group. However, there were no significant differences after Glib treatment in the HF rats that were not treated with H-Are, which confirmed that the arecoline-mediated improvement in ACh-induced EDVR may be partly due to H_2S-induced K_{ATP} channel opening in VSMCs. Our findings were consistent with the results of Zhao *et al*[18, 19]. This study demonstrated that H_2S is different from NO and CO_2 and that H_2S-induced vascular relaxation was mediated mainly by K_{ATP} channel opening in VSMCs.

In summary, the present study showed that arecoline can improve EDVR in high fructose-fed rats, and might exert its function by increasing CSE expression and activation of K_{ATP} channels.

Acknowledgements

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Author contribution

Hong-yan LING, Guang WANG, Shou-hong ZHOU, and Bi HU designed the research; Guang WANG and Wei ZHANG performed the research; Xing LI analyzed the data and images; Hong-yan LING wrote the paper.

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