Original Paper

Chelerythrine Attenuates Renal Ischemia/Reperfusion-induced Myocardial Injury by Activating CSE/H₂S via PKC/NF-κB Pathway in Diabetic Rats

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Key Words

Chelerythrine • Ischemia/reperfusion • Diabetes mellitus • Cystathionine-γ-lyase • Hydrogen disulfide • Protein kinase C • Nuclear factor-κ B

Abstract

Background/Aims: Chelerythrine (CHE), a benzophenanthridine alkaloid, is a potent, selective, and cell-permeable protein kinase C (PKC) inhibitor. The purpose of the present study was to evaluate the effect of CHE on myocardial recovery after renal ischemia/reperfusion (I/R)-induced myocardial injury (RI/RMI) in a streptozocin (STZ)-induced diabetic rat model. **Methods:** Diabetes mellitus (DM) rats preconditioned with CHE and D, L-propargylglycine (PAG) were subjected to renal I/R. The extent of cardiac morphologic lesions and the biochemical markers of cardiorenal function and oxidative stress were detected utilizing hematoxylin-eosin staining, commercial kits, and enzyme-linked immunoassay, respectively. The expressions of cystathionine-γ-lyase (CSE), PKC-α, PKC-β₂, and nuclear factor-kappa B (NF-κB) in the rat myocardial tissue were measured utilizing western blotting. **Results:** Renal I/R treatment resulted in myocardial injury. CHE-preconditioning promoted the recovery from myocardial damage by ameliorating the biochemical parameters of myocardial injury, reducing oxidative stress, increasing the H₂S level, up-regulating the expression of CSE, and down-regulating the expressions of PKC-α, PKC-β₂, and NF-κB. **Conclusion:** These findings suggest that CHE-pretreatment may exert a protective effect on the myocardium against RI/RMI by activating endogenous CSE/H₂S via PKC/NF-κB pathway in STZ-induced diabetic rats. Further studies are needed defining underlying mechanisms.

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Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia over a prolonged period. Serious complications associated with DM may primarily involve the kidney, eye, and nerve due to damage to the microcirculation [1]. Damage to the kidney commonly referred to as diabetic nephropathy, can lead to tissue damage and eventually chronic kidney disease, which may in turn exacerbate renal ischemia/reperfusion (I/R) injury (RI/RI) [2, 3]. The sensitivity of organs to RI/RI and other injuries are increased in DM, resulting in organ dysfunctions [4]. Some diabetic patients may need renal transplantation, in whom I/R injury is one of the most dangerous complications because it may cause distant organ injury such as myocardial injury [5-7]. Renal I/R injury may induce an oxidative stress response to tissue damage in remote organs [8, 9]. In this pathophysiological process of RI/RI, reactive oxygen species (ROS) and nitric oxide (NO) played key roles in inducing myocardial oxidative stress and increasing cardiac lipid peroxidation [10-12].

Endogenous hydrogen disulfide (H₂S) is generated via endogenous cystathionine-γ-lyase (CSE) [13]. As the most abundantly expressed protein in the cardiovascular system, CSE is responsible for most endogenous H₂S production [14]. The physiologic characteristics of endogenously produced H₂S are significantly similar among different gasotransmitters and mainly contributed to cellular proliferation, angiogenesis, inflammation, and regulation of protein activities through S-sulfhydration [15-18]. Chelerythrine (CHE), a natural benzo [c] phenanthridine alkaloid existing in numerous plant species, is known to exert various biological activities, including antimicrobial, antifungal, anti-inflammatory and anticancer activities [19-21]. In addition, CHE can inhibit protein kinase C (PKC) and mitogen-activated protein kinase phosphatase-1 through various signaling pathways, and reduce RI/RI and intestinal I/R injury [22-24].

However, it is not known whether CHE could play a role in protecting the diabetic myocardium and inducing the expression of CSE/H₂S in diabetic rats which in turn can inhibit myocardial damage from renal I/R-induced injury. The main aim of the present study was to investigate whether CHE could attenuate renal ischemia/reperfusion (I/R)-induced myocardial injury (RI/RMI) in a streptozotocin (STZ)-induced diabetic rat model.

Materials and Methods

Establishment of the animal model

Sprague-Dawley (SD) male rats (180-200 g) were obtained from the animal research center of Jiaying University (Jiaying, China). The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Jiaying University Medical College. The study was approved by the Ethical Committee of Jiaying University Medical College (No. JUMC 2015-029). The STZ-induced diabetic rat model was generated through a single intraperitoneal injection of STZ dissolved in citrate buffer (PH 4.5, 50 mg/kg body weight [bw]) [25]. Rats were housed under climate-controlled conditions with a 12-h light/dark cycle and provided with standard food and water for 2 weeks. Rats with the serum glucose level of 16.7-25.6 mmol/L were used in the subsequent experiments.

Forty STZ-induced diabetic rats were equally randomized into four groups: 1) sham-operated group (Sham group), wherein rats were treated only with separating the bilateral renal arteries and veins and then treated with 10% dimethyl sulfoxide (DMSO, 1 ml/kg bw, i.v.); 2) renal I/R group (I/R group), wherein the rats were treated with ischemia by clamping the bilateral renal arteries and veins for 45 min and subsequent 2-h reperfusion with DMSO (1 ml/kg bw, i.v.); 3) renal I/R+CHE group (CHE group), wherein the rats were pretreated with CHE (dissolved in DMSO, 5 mg/kg bw) via a single-dose injection into the abdominal cavity at 30 min prior to the I/R surgery; and 4) renal I/R+CHE+PAG group (PAG group), wherein the rats were administered intraperitoneally with D, L-2-amino-4-pentynoic acid (PAG, an inhibitor of CSE, 20 mg/kg bw) 10 min prior to the I/R surgery, and the way of CHE administration was the same as that in CHE group.

Sample processing

Blood samples were collected from the caudal vein after reperfusion and then centrifuged at 3600× *g* for 10 min at 4 °C to harvest the serum. The animal heart was immediately dissected, and the contaminating blood was washed away with ultrapure water. The myocardial tissues were homogenized in ice-cold normal saline and centrifuged at 3600× *g* for 15 min at 4 °C to harvest the supernatant.

Renal damage assessment

The renal function was evaluated by measurements of the blood urea nitrogen (BUN) and serum creatinine (Scr) levels using an automatic biochemical analyzer (Hitachi, Japan) and Roche Diagnostics (Mannheim, Germany) by the picric acid method according to the manufacturer's instructions [26].

Myocardial histopathological changes

The myocardial tissue was fixed in 4% paraformaldehyde, paraffin-embedded, sliced into 4-μm sections, stained with hematoxylin-eosin (HE) staining. The pathological changes at the cellular level were examined under the microscope (Leica, Germany) and graded according to the degree of damage based on the percentage of involvement of the myocardium. The damage quantification from ten areas corresponding to the myocardial tissue was graded using the following parameters: hemorrhage, myocardial edema (cytoplasmic vacuole formation), cardiomyocyte apoptosis, and myocardial necrosis based on a five-score evaluation system (1, histopathological changes <10%; 2, = 10-25%; 3, =25-50%; 4, =50-75%; and 5, =75-100%) [25]. The mean score for each parameter was calculated and subjected to statistical analysis.

Measurement of antioxidative activity and biochemical markers of myocardial injury

The antioxidative activity was detected with the superoxide dismutase (SOD) activity and the contents of malondialdehyde (MDA) and glutathione (GSH) in the rat myocardial tissue following the commercial kits (Roche). The levels of creatine kinase (CK), lactic dehydrogenase (LDH), nitric oxide synthase (iNOS and eNOS), and NO in the myocardial tissue and the serum levels of H₂S, brain natriuretic peptide (BNT), and cardiac troponin I (cTnI) were measured to assess the myocardial injury with the double antibody sandwich enzyme-linked immunoassay (ELISA) kits (Roche) according to the manufacturer's instructions..

Western blot

Western blot analysis was performed as previously described [25, 27]. Briefly, the myocardial tissue homogenate was resolved on 10% SDS-PAGE gels and then electrophoretically transferred to the nitrocellulose membrane. After blocking with 5% skim milk in Tris-buffered saline containing 0.2-0.4% Tween-20 (TBST) for 1 h, the membrane was incubated with primary antibodies (against active PKC-α, PKC-β₂, NF-κB, and CSE) at 4 °C overnight and subsequently with the alkaline phosphatase-conjugated secondary antibodies. The membrane was developed by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. The protein level was normalized with respect to β-actin band density. The antigen-antibody product was measured by Thermo Scientific Super Signal West Pico Chemiluminescent Substrate (Thermo, USA) and analyzed with a Fluor Chem system (Alpha, USA)..

Statistical analysis

Data were expressed as means ± standard deviation (SD) and analyzed using SPSS 19.0 for Windows. The one-way analysis of variance (ANOVA) was used for data analysis. Difference associated with *P*<0.05 was considered statistically significant.

Results

CHE-pretreatment attenuates I/R-induced damages of renal function

The serum levels of BUN and Scr were significantly increased by 2-3-fold after I/R and reduced by CHE-pretreatment (*P*<0.01) (Fig. 1). These results suggest that renal I/R may develop in the impaired renal function and pretreatment with CHE may attenuate I/R-induced renal damage.

Fig. 1. Alterations of the renal function. Note: (A) BUN and (B) Scr. Data are expressed as mean±SD of three repeat experiments, n=10. I/R treatment increases the serum levels of BUN and Scr, while CHE-pretreatment decreases them. **P*<0.01 vs. Sham group; ***P*<0.01 vs. I/R group; #*P*<0.05 vs. CHE group.

Fig. 2. Pathological changes of the myocardium. Note: Light microscope images (HE, ×400). (A) Sham group, (B) I/R group, (C) CHE group, (D) PAG group, and (E) Injury score. The histopathological alterations (cardiomyocyte edema and apoptosis) were markedly alleviated by CHE-preconditioning. Data are expressed as mean±SD, n=10. **P*<0.01 vs. Sham group; ***P*<0.01 vs. I/R group; #*P*<0.05 vs. CHE group.

Preconditioning with CHE alleviates renal I/R-induced myocardial damages

Several hemorrhagic lesions and myocardial edemas were observed in the intermuscular spaces. There were obvious myocardial cytoplasmic vacuole formation (cardiomyocyte edema) and cardiomyocyte apoptosis in the I/R group rats. These histopathological alterations were markedly alleviated in CHE-preconditioning group and moderately attenuated in PAG-pretreatment group (Fig. 2).

To evaluate the extents of renal I/R-induced myocardial injury and the effects of CHE on RI/RMI, the blood biochemical markers and cardiac enzymes were detected. As compared with the sham group, the serum levels of CK, LDH, BNP, and cTnI and the levels of iNOS, eNOS, and NO in the myocardial tissue were significantly increased, but the serum H₂S level was significantly decreased in I/R group rats (all *P*<0.01). After CHE-preconditioning, the decreased levels of CK, LDH, BNP, cTnI, iNOS, and NO and the increased levels of H₂S and eNOS were significantly different among treated groups (all *P*<0.01). Administration of PAG displayed an inhibitory effect on CHE (Fig. 3). These data indicate that the renal I/R may contribute to the remote myocardial injury. Pretreatment with CHE can effectively protect against renal I/R-induced myocardial injury via regulating the levels or bioactivities of above biochemical parameters.

CHE-preconditioning inhibits the renal I/R-induced oxidative stress via suppression of the PKC/NF-κB pathway and activation of the CSE/H₂S pathway

To evaluate the effects of CHE on renal I/R-induced oxidative stress in the myocardial tissue, SOD activity and the contents of MDA and GSH were detected in the myocardial tissue using the commercial kits. As shown in Fig. 4, as compared with the sham-operation group, MDA content in the myocardial tissue was significantly increased in I/R group ($P < 0.01$), which was accompanied by a marked decrease in SOD activity and GSH content ($P < 0.01$); however, these effects were significantly attenuated by CHE-pretreatment.

The PKC/NF-κB signaling pathway has several roles in physiological activities and pathological changes, including antioxidant, antiinflammation, anti-apoptosis, anticancer, and activation of endogenous CSE/H₂S [28-32]; therefore, to investigate whether it is associated with the protective effects of CHE on renal I/R-induced oxidative damage to myocardium, the expressions of PKC-α, PKC-β₂, NF-κB, and CSE were examined. As expected, in the myocardial tissue of I/R group,

Fig. 3. Alterations of biochemical markers in the myocardial tissue and blood. Note: (A) H₂S, (B) BNP, (C) cTnI, (D) CK, (E) LDH, (F) iNOS, (G) eNOS, and (H) NO. Data are expressed as mean±SD of three repeat experiments, n=10. CHE-preconditioning elevates the serum H₂S level and eNOS level in the myocardial tissue and reduces the serum levels of BNP, cTnI, CK, and LDH and the levels of iNOS and NO in the myocardial tissue. * $P < 0.01$ vs. Sham group; ** $P < 0.01$ vs. I/R group; # $P < 0.05$ vs. CHE group.

Fig. 4. Antioxidative activity in the myocardial tissue. Note: (A) SOD, (B) MDA, and (C) GSH. Data are expressed as mean±SD of three repeat experiments, n=10. CHE-pretreatment enhances the myocardial antioxidative activity. **P*<0.01 vs. Sham group; ***P*<0.01 vs. I/R group; #*P*<0.05 vs. CHE group.

Fig. 5. The protein expressions of PKC-α, PKC-β₂, NF-κB, and CSE in the myocardial tissue. Note: Data are expressed as mean±SD of three repeat experiments, n=10. CHE-pretreatment may up-regulate CSE/H₂S expression by repressing PKC/NF-κB pathway, thus attenuating diabetic RI/RMI. **P*<0.01 vs. Sham group; ***P*<0.01 vs. I/R group; #*P*<0.05 vs. CHE group.

the expression of PKC-α, PKC-β₂, and NF-κB were significantly increased as compared with those in the sham-operation group, and they were all down-regulated significantly by CHE-preconditioning (*P*<0.01). The expression of CSE in the myocardial tissue of I/R group was significantly decreased comparing with the sham-operation group and significantly up-regulated by CHE-preconditioning (*P*<0.01) (Fig. 5). These results clearly indicate that CHE-pretreatment may effectively enhance the antioxidative activation of the myocardial tissue after renal I/R-induced myocardial injury, at least partially via suppression of PKC/NF-κB pathway and activation of CSE pathway.

Discussion

The results of the present study demonstrated that preconditioning with CHE effectively suppressed the myocardial damage in a diabetic renal I/R-induced remote myocardial injury model. CHE-pretreatment also suppressed myocardial oxidative stress in model rats, alongside increased activation of CSE/H₂S and decreased activation of PKC/NF-κB pathway. Taken together, these results suggest that CHE-preconditioning may protect myocardial injury from diabetic renal I/R via the suppression of PKC/NF-κB pathway and activation of CSE/H₂S; therefore, CHE may be considered effective in the control of RI/RMI in DM.

Epidemiological studies and animal model studies have demonstrated that DM complicated with renal I/R frequently results in acute renal failure (ARF) and remote myocardial injury with a substantial morbidity rate [33-37]. Both classic carbohydrate metabolism, lipid metabolism, and immune reaction may contribute to the pathogenesis of renal I/R-induced cardiac injury. In the present study, the pathological changes of renal I/R-induced remote myocardial injury mainly included the hemorrhagic lesions, cardiomyocyte edema, and cardiomyocyte apoptosis as evidenced by increased levels of CK, LDH, BNP, and cTnI in the myocardial tissue, decreased serum level of H₂S, and increased oxidative stress after renal I/R in STZ-induced diabetic rat model. These histopathological alterations were markedly attenuated by CHE-preconditioning.

CHE is a benzophenanthridine alkaloid that is a potent, selective and cell-permeable PKC inhibitor [38]. It was found in this study that CHE was able to protect the function of the remote myocardium after renal I/R-induced injury in diabetic rats in part by up-regulating endogenous CSE/H₂S via inhibiting PKC/NF-κB pathway. This protective effect could be supported by the following observations: (1) the myocardial injury was amplified in the presence of renal I/R as evidenced by impaired renal function, increased serum levels of CK, LDH, BNP, and cTnI and NO level in the myocardial tissue, decreased serum level of H₂S, and increased oxidative stress; (2) the RI/RMI in diabetic rats was further intensified in the absence of remote cardiac endogenous CSE but ameliorated markedly by CHE supplementation, thus providing the initial evidence about the effect of CHE against RI/RMI; and (3) following the up-regulation of PKC/NF-κB pathway, myocardial oxidative stress and nitrate stress were also increased, which may partially explain the mechanism of RI/RMI in DM, and more importantly confirm the protective effect of pretreatment with CHE.

Several studies have demonstrated that the state of hyperglycemia in DM-induced overexpression of PKC in the myocardium could regulate cell growth, affect the contractility of cardiomyocytes and permeability via the two-activated fatty acyl PKC signal transduction systems [39, 40]. The increased expressions of PKC-α and PKC-β₂ mRNA are the primary cause of myocardial injury in DM [41, 42], however, the underlying downstream signal transduction pathway of PKC is still not completely understood. In addition, hyperglycemia can induce the formation of oxygen free radicals, promote the deposition of extracellular matrix, and decrease the diastolic function of the heart [43-46]. Mounting evidence has demonstrated that oxidative stress is involved in increased lipid peroxidation, aggravated myocardial reactivity and overexpression of chemoattractant in the myocardium [35, 36]. The ischemic and anoxic tissue is usually accompanied by oxidative stress, where production of ROS results in the lower expression of glutamate transporters. H₂S can block ROS from activating extracellular signal-regulated kinase1/2 (ERK1/2) and PKC [47]. Thus, H₂S plays an antioxidant role in ischemic injuries. Over the past decades, the perceived image of H₂S has transformed from that of a dangerously toxic molecule to that of an endogenously produced gas that may affect many physiologic processes. There are numerous similarities between H₂S and other gasotransmitters [13, 48, 49]. In the present study, renal I/R in diabetic rats significantly increased the level of MDA, which is the end product of lipid peroxidation, and decreased the activity of antioxidant enzymes, GSH and SOD. However, these changes were markedly attenuated by CHE-pretreatment. These results indicate that CHE-pretreatment may limit the myocardial damage by reducing oxidative stress, which suggests that CHE is a potent antioxidant. In addition, the current study detected repressed PKC/NF-κB pathway activity and enhanced CSE/H₂S activity in the myocardium of CHE group. These results

indicate that PKC may be an important kinase located in the upstream of oxidative stress and CSE/H₂S functions as an endogenous mediator via PKC/NF-κB pathway after diabetic RI/RMI. Therefore, the myocardial preservative effects of CHE in renal I/R-induced myocardial injury may partly depend on activation of CSE/H₂S and suppression of PKC/NF-κB pathway, which is consistent with the highly protective effects of CSE/H₂S in similar models of ischemia [50-53].

Conclusion

The results of the present study reveal a key role for CHE-preconditioning on endogenous CSE/H₂S in the regulation of oxidative stress on RI/RMI in DM. Endogenous H₂S may participate in reducing diabetic RI/RMI by inhibiting PKC/NF-κB pathway. CHE administration could promote the expression of endogenous CSE/H₂S by inhibiting PKC/NF-κB pathway and therefore was considered to be a therapeutic target in diabetic RI/RMI settings. Further detailed studies are needed to better clarify the underlying mechanisms.

Disclosure Statement

The authors declare no conflict of interest.

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