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Caulophine Protects Cardiomyocytes From Oxidative and Ischemic Injury

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Abstract. Caulophine is a new fluorenone alkaloid isolated from the radix of *Caulophyllum robustum* Maxim and identified as 3-(2-(dimethylamino) ethyl)-4,5-dihydroxy-1,6-dimethoxy-9*H*-fluoren-9-one. Due to its new chemical structure, the pharmacological activities of caulophine are not well characterized. The present study evaluated the protective effect and the primary mechanisms of caulophine on cardiomyocyte injury. Viability of cardiomyocytes was assayed with the MTT method, and cell apoptosis was detected by flow cytometry. Myocardial infarction was produced by ligating the coronary artery, and myocardial ischemia was induced by isoproterenol in rats. Myocardial infarction size was estimated with *p*-nitro-blue tetrazolium staining. Lactate dehydrogenase (LDH), creatine kinase (CK), superoxide dismutase (SOD), malondialdehyde (MDA), and free fatty acid (FFA) were spectrophotometrically determined. Histopathological and ultrastructural changes of ischemic myocardium were observed. The results showed that pretreatment with caulophine increased the viability of H_2O_2- and adriamycin-injured cardiomyocytes; decreased CK, LDH, and MDA; increased SOD; and inhibited H_2O_2-induced cellular apoptosis. Caulophine reduced myocardial infarct size and serum CK, LDH, FFA, and MDA; raised serum SOD; and improved histopathological and ultrastructural changes of ischemic myocardium. The results demonstrate that caulophine has the ability to protect cardiomyocytes from oxidative and ischemic injury through an antioxidative mechanism that provides a basis for further study and development of caulophine as a promising agent for treating coronary heart disease.

Keywords: caulophine, cardiomyocyte, oxidative injury, myocardial ischemia, apoptosis

Introduction

Coronary heart disease is one of the most common cardiovascular diseases and one of the leading causes of death in human beings. Myocardial ischemia and subsequent cardiomyocyte injury, even necrosis, are the main pathological change of coronary heart disease. Although pathogenesis of myocardial ischemia has not been clarified fully, a wide range of evidence from experimental and clinical studies suggests that reactive oxygen species (ROS) (1, 2), calcium overload (3), apoptosis (4), and the inflammatory response (5) are mainly involved. So far, drug therapy is the main approach for preventing and treating coronary heart disease, although coronary angioplasty and thrombolytic therapy may be used for myocardial infarction.

Caulophine is a new fluorenone alkaloid isolated from the radix of *Caulophyllum robustum* Maxim, a traditional Chinese herb used for treating external injury and irregular menses (6). Due to its completely new chemical structure, the pharmacological activities of caulophine have been not reported previously. In the present study, we evaluated the protective effect and the primary mechanisms of caulophine on cardiomyocytes in vitro and in vivo.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM) was

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purchased from Invitrogen, Inc. (Frederick, MD, USA). Fetal bovine serum (FBS) was obtained from Hyclone, Inc. (Logan, UT, USA). Dimethyl sulfoxide (DMSO), hydroxyethyl piperazine ethanesulfonic acid (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), trypsin, H_2O_2, and *p*-nitro-blue tetrazolium (NBT) were from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Annexin V-FITC was produced by Jingmei Biotech Co., Ltd. (Beijing, P.R. China). Commercial kits used for biochemical determinations were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, P.R. China).

Caulophine (Fig. 1) was provided by Professor Langchong He of Xi'an Jiaotong University and dissolved in DMSO followed by dilution with distilled water. The final concentration of DMSO was ≤0.1%. Nifedipine, isoproterenol (ISO), and adriamycin were produced by Shaanxi Bohua (Xi'an, P. R. China), Shanghai Huofeng (Shanghai, P.R. China), and Zhejiang Haizheng (Taizhou, P.R. China) Pharmaceutical companies, respectively.

In vitro study

Primary cardiomyocyte culture and treatments: Cardiomyocytes from the neonatal rat were prepared using a previously published method (7). In brief, a 2 – 4-day-old Sprague–Dawley (SD) rat was killed, the heart was isolated and minced, and then the minced heart was treated with 0.125% trypsin solution. The cells were pelleted and then the cell pellet was resuspended in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. A selective adhesion procedure was performed after the incubation for 1.5 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in order to obtain cardiomyocytes at a high purity. More than 96% cells were cardiomyocytes as assessed by immunostaining for α-sarcomeric actin. Finally, the medium was changed to serum-free medium 12 h prior to the experiments.

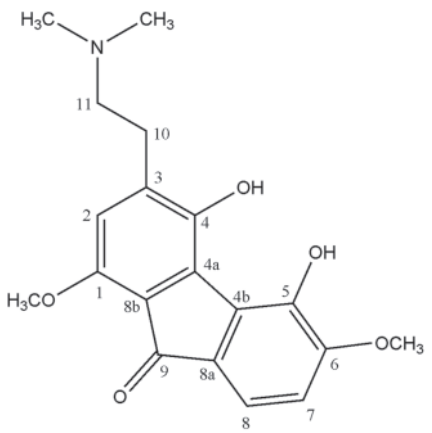

Fig. 1. Structure of caulophine.

In the experiment to determine time–effect and concentration–effect relationships of H_2O_2, cardiomyocytes were exposed to H_2O_2 at the final concentration of 0.1 – 0.6 mM for 4 – 24 h. Then, the cell viability was monitored. To determine the protective effect of caulophine on H_2O_2- or adriamycin-induced cardiomyocyte injury, cardiomyocytes were divided into the control group, H_2O_2-injured group, adriamycin-injured group, DMSO control group, and caulophine-pretreated groups. The cells in the H_2O_2- or adriamycin-injured group were incubated with DMEM for 24 h before exposure to 0.5 mM H_2O_2 for 4 h or 1.84 μM adriamycin for 24 h. The cells in the DMSO control group were incubated with 0.1% DMSO for 24 h before exposure to 0.5 mM H_2O_2 for 4 h or 1.84 μM adriamycin for 24 h. The cells in the caulophine-pretreated groups were pretreated with caulophine for 24 h before exposure to the same concentration of H_2O_2 or adriamycin. Finally, the cell viability and the biochemical parameters were monitored.

Assay of the cell viability with the MTT method: After exposure of cardiomyocytes to H_2O_2 or adriamycin, 0.5 mg/ml MTT was added and incubation carried out for an additional 4 h. Then, 100 μl of DMSO was added to dissolve the formazan particles. Finally, absorbance at 490 nm was measured with a microplate reader.

Measurement of the biochemical parameters with spectrophotometry: After exposure of cardiomyocytes to H_2O_2 or adriamycin, the cell supernatant was collected for the spectrophotometrical measurement of creatine kinase (CK) at 520 nm and lactate dehydrogenase (LDH) at 450 nm according to the procedures provided by the assay kits. The cell pellet was lysed in 0.5 ml lysis buffer and homogenized by three freeze/thaw cycles. Subsequently, the homogenate was centrifuged at 3000 × *g* for 20 min at 4°C. The supernatant was used for the spectrophotometrical assay of superoxide dismutase (SOD) activity at 550 nm and malondialdehyde (MDA) level at 532 nm according to the instructions provided by the assay kits.

Detection of apoptotic cells with flow cytometry: Apoptosis was assayed by the double staining with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) and analyzed with flow cytometry. In brief, the cells were treated with caulophine or DMSO and 0.5 mM H_2O_2 as mentioned above. Then, the medium was discarded. After the cells were removed from plates by using 0.25% trypsin, they were washed twice with cold phosphate-buffered saline (PBS, pH 7.4) and resuspended in binding buffer. Finally, the cells were incubated for 15 min with 5 μl FITC-conjugated annexin V molecules and 10 μl PI (20 μg/ml) and measured by a

flow cytometer (BioRad, Berkeley, CA, USA) (8).

In vivo study

Animal and pretreatment: Male SD rats weighing 200 – 250 g were provided by the Experimental Animal Center of Xi'an Jiaotong University School of Medicine (Xi'an, P.R. China) and housed in a room with temperature of 21°C – 25°C, relative humidity of 50% – 60%, and a 12-h light / 12-h dark cycle. Animals had free access to pellet food and tap water. The research was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care Committee of Xi'an Jiaotong University.

Rats were randomly divided into the following 6 groups with 8 – 12 rats in each group: sham-operated group, model control, three caulophine-treated groups, and positive control. Rats in the caulophine-treated groups were pretreated with intragastric administration of caulophine (12.5, 25, and 50 $mg \cdot kg^{-1} \cdot day^{-1}$; 1.25, 2.5 and 5 mg/ml, 10 ml/kg) for 5 days, and rats in the positive control group were intragastrically administered nifedipine (10.7 $mg \cdot kg^{-1} \cdot day^{-1}$; 1.07 mg/ml, 10 ml/kg) for 5 days. Rats in the sham-operated and model control groups received vehicle in an identical fashion to the drug-treated groups instead.

Myocardial infarction model in rats: Rat myocardial infarction was produced by ligating the coronary artery (LCA) as described previously (9). Briefly, rats in the model control and drug-treated groups were anesthetized with chloral hydrate (300 mg/kg). Then, the left chest cavity was opened, and the left anterior descending coronary artery was ligated with 40/50 thread. Rats in the sham-operated group experienced the same procedures except LCA.

After the ischemia for 24 h, rats were anaesthetized with urethane. Then, blood was collected from the abdominal aorta for examining serum CK, LDH, SOD, MDA, and free fatty acid (FFA) at 440 nm with the spectrophotometrical method as described above, and the heart was removed for assessing the myocardial infarction size.

Myocardial ischemia model in rats: Myocardial ischemia was induced by ISO according to the method described previously (10). On the 4th and 5th day of pretreating animals with the drugs, rats in the model control and drug-treated groups were subcutaneously injected with 20 mg/kg ISO once daily for 2 days, whereas rats in the sham-operated group received injection of 0.9% saline instead. At 30 min after the second injection, rats were anesthetized and the electrocardiogram (ECG) monitored. After 2 h, blood was collected for examining serum biochemical parameters, and the heart was removed for ultrastructural and pathological examinations.

Assessment of myocardial infarction size: The cardiac ventricle of rats was sliced into five horizontal sections. Then, the slices were weighed and incubated with 0.25% NBT in 0.2 M PBS for 15 min at 37°C to distinguish the normal and infarcted ventricular tissues (11). Finally, the infarcted ventricle was weighed, and the infarction ratio was calculated according to the following formula: infarction ratio (%) = (weight of infarcted ventricle / weight of whole ventricle) × 100.

Morphological observation of ischemic myocardium: After the sections of the myocardial tissue were stained with hematoxylin and eosin using the standard protocol, they were observed under light microscopy and degree of myocardial ischemia was graded according to the standard submitted by Rona (12). In the ultrastructural examination, the cardiac apex was processed with routine procedures and the slices were observed under transmission electronic microscope (Hitachi Co., Ltd., Tokyo).

Statistical analyses

Data were expressed as the mean ± S.E.M. Statistical significance of differences between groups was performed by One Way Analysis of Variance (ANOVA). The result from the histopathological examination was treated with the Ridit test. *P*-value of <0.05 was statistically considered significant.

Results

Effect of caulophine on H_2O_2-induced injury of cardiomyocytes

Following incubation of cardiomyocytes with the different concentrations of H_2O_2 for the indicated time, the cell viability was reduced in concentration- and time-dependent manners (Fig. 2). However, pretreatment of the cells with caulophine concentration-dependently increased the viability of H_2O_2-injured cells (Fig. 3, $P < 0.05$ or $P < 0.01$). As shown in Table 1, after exposure of cardiomyocytes to 0.5 mM H_2O_2 for 4 h, CK, LDH activities in the cellular supernatant, and MDA level in the cells were increased, while intracellular SOD activity was decreased. However, pretreatment of the cells with caulophine lowered CK, LDH activities and MDA level, and increased SOD activity in a concentration-dependent manner ($P < 0.05$ or $P < 0.01$). The results from Fig. 3 and Table 1 also showed that pretreatment of the cells with DMSO at the concentration contained in the caulophine solution had no effect on the cell viability and the biochemical parameters measured in H_2O_2-injured cells. The results suggest that caulophine protects cardiomyocytes from H_2O_2-induced injury.

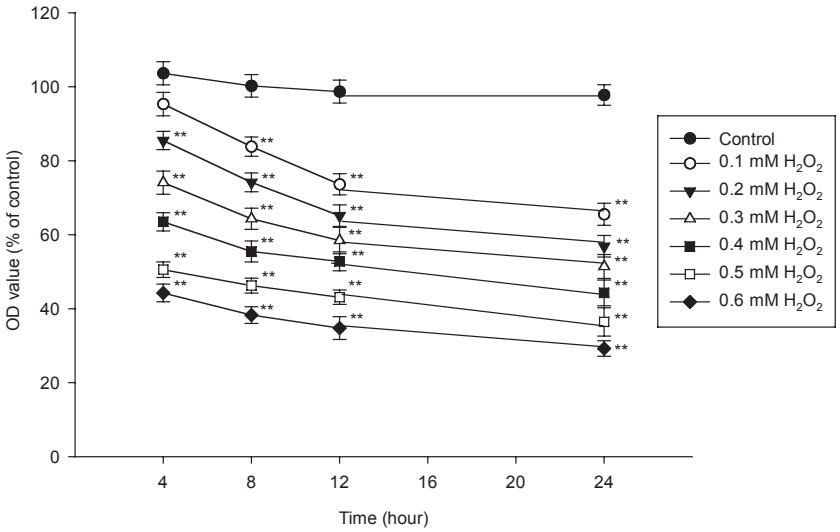

Fig. 2. Time- and concentration-dependent relationship of H_2O_2-induced cardiomyocyte injury. Cardiomyocytes were incubated with the different concentrations of H_2O_2 for the indicated time. Then, the cell viability was assayed with the MTT method. Data were expressed as the mean ± S.E.M. of 3 independent experiments. $^{**}P < 0.01$ vs. control.

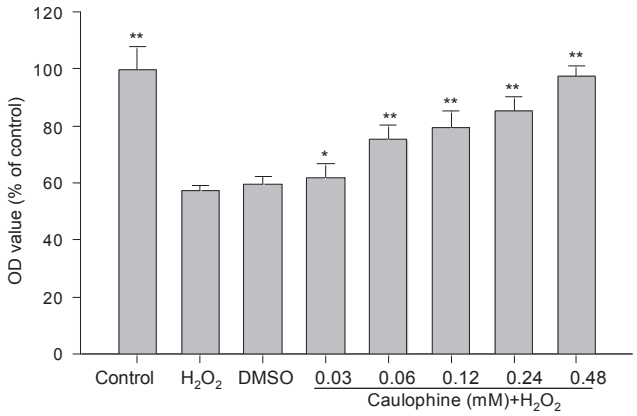

Fig. 3. Effect of caulophine on the viability of H_2O_2-injured cardiomyocytes. Cardiomyocytes were pretreated with the different concentrations of caulophine or 0.1% DMSO for 24 h before exposure to 0.5 mM H_2O_2 for 4 h. Then, the cell viability was assayed with the MTT method. Data were expressed as the mean ± S.E.M. of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. H_2O_2 group.

Table 1. Effects of caulophine on the biochemical parameters in H_2O_2-injured cardiomyocytes

Groups	CK (U/l)	LDH (U/l)	MDA (μM)	SOD (U/ml)
Control	688.8 ± 41.0	160.0 ± 14.6	1.06 ± 0.10	30.5 ± 1.1
H_2O_2	1396.0 ± 108.6**	283.3 ± 17.5**	2.32 ± 0.18**	16.8 ± 1.5**
DMSO	1287.6 ± 94.8	278.5 ± 13.6	2.19 ± 0.32	17.9 ± 1.2
Caulophine-pretreated groups (mM)				
0.03	1104.2 ± 80.9$^{\#}$	263.3 ± 16.7	1.89 ± 0.24	19.2 ± 1.8
0.06	939.3 ± 70.5$^{\#\#}$	236.7 ± 14.1$^{\#}$	1.66 ± 0.17$^{\#\#}$	21.2 ± 1.5$^{\#}$
0.12	785.2 ± 54.0$^{\#\#}$	206.7 ± 12.3$^{\#\#}$	1.42 ± 0.14$^{\#\#}$	23.4 ± 1.3$^{\#\#}$
0.24	740.7 ± 52.6$^{\#\#}$	196.7 ± 12.0$^{\#\#}$	1.24 ± 0.10$^{\#\#}$	25.8 ± 1.1$^{\#\#}$
0.48	718.3 ± 62.3$^{\#\#}$	176.7 ± 17.5$^{\#\#}$	1.14 ± 0.14$^{\#\#}$	28.6 ± 1.3$^{\#\#}$

Cardiomyocytes were pretreated with the different concentrations of caulophine or 0.1% DMSO for 24 h before exposure to 0.5 mM H_2O_2 for 4 h. Then, CK and LDH activities in the cellular supernatant, and MDA level and SOD activity in the cells were determined with spectrophotometry. The results were expressed as the mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. H_2O_2 group.

Effect of caulophine on adriamycin-induced injury of cardiomyocytes

Figure 4 and Table 2 showed that pretreatment of cardiomyocytes with caulophine increased the viability of adriamycin-injured cells as well as reversed adriamycin-induced increase of CK, LDH activity, and MDA level and decrease of SOD activity concentration-dependently ($P < 0.01$ or $P < 0.05$). Similarly, DMSO pretreatment had no effect on the cell viability and the biochemical parameters in adriamycin-injured cells as well. These observations implicate that caulophine also protects cardiomyocytes against adriamycin-induced injury.

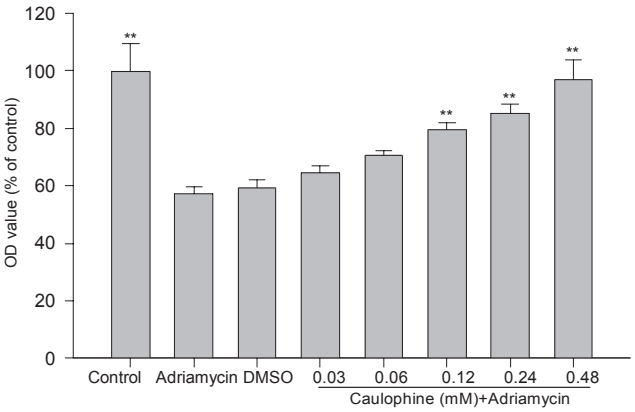

Fig. 4. Effect of caulophine on the viability of adriamycin-injured cardiomyocytes. Cardiomyocytes were pretreated with the different concentrations of caulophine or 0.1% DMSO for 24 h before exposure to 1.84 μM adriamycin for 24 h. Then, the cell viability was assayed with the MTT method. Data were expressed as the mean ± S.E.M. of 3 independent experiments. $^{**}P < 0.01$ vs. adriamycin group.

Effect of caulophine on H_2O_2-induced apoptosis of cardiomyocytes

As seen from Fig. 5, apoptotic rate of cardiomyocytes was increased to 75.8 ± 6.9% from 6.6 ± 0.9% in control group after exposure to 0.5 mM H_2O_2 for 4 h ($P < 0.01$). However, preincubation of the cells with caulophine at 0.06 and 0.12 mM reduced the cellular apoptosis to 51.9 ± 4.5% and 32.7 ± 3.2%, respectively ($P < 0.01$),

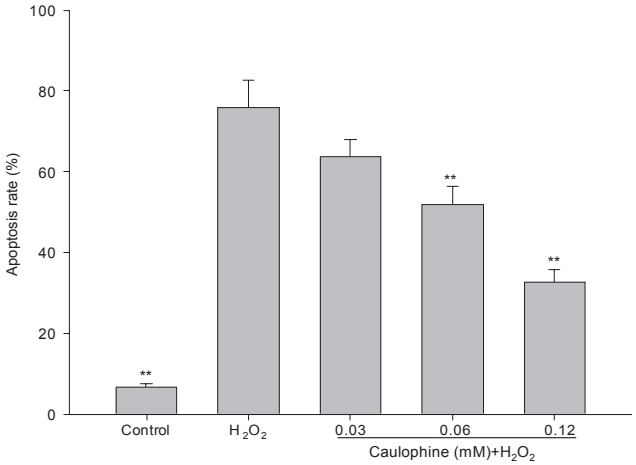

Fig. 5. Effect of caulophine on H_2O_2-induced apoptosis of cardiomyocytes. Cardiomyocytes were pretreated with the different concentrations of caulophine or 0.1% DMSO for 24 h before exposure to 0.5 mM H_2O_2 for 4 h. Then, cell apoptosis was assayed by annexin V-FITC and PI staining and analyzed by flow cytometry. Data were expressed as the mean ± S.E.M. of 3 independent experiments. $^{**}P < 0.01$ vs. H_2O_2 group.

Table 2. Effects of caulophine on the biochemical parameters in adriamycin-injured cardiomyocytes

Groups	CK (U/l)	LDH (U/l)	MDA (μM)	SOD (U/ml)
Control	750.8 ± 44.7	175.5 ± 8.4	1.12 ± 0.08	31.2 ± 1.2
Adriamycin	1507.7 ± 117.2**	295.6 ± 7.2**	2.10 ± 0.10**	18.7 ± 1.1**
DMSO	1386.3 ± 98.6	289.5 ± 9.6	2.03 ± 0.06	19.2 ± 0.5
Caulophine-pretreated groups (mM)				
0.03	1203.6 ± 86.4$^{\#}$	270.2 ± 13.7	1.91 ± 0.07	19.6 ± 0.9
0.06	1005.1 ± 75.4$^{\#\#}$	250.2 ± 10.6$^{\#\#}$	1.760 ± 0.08$^{\#\#}$	22.2 ± 1.2$^{\#}$
0.12	848.0 ± 58.3$^{\#\#}$	231.3 ± 13.0$^{\#\#}$	1.55 ± 0.09$^{\#\#}$	24.2 ± 1.1$^{\#\#}$
0.24	792.5 ± 56.3$^{\#\#}$	207.2 ± 11.8$^{\#\#}$	1.34 ± 0.08$^{\#\#}$	26.1 ± 1.2$^{\#\#}$
0.48	782.9 ± 67.9$^{\#\#}$	185.7 ± 9.7$^{\#\#}$	1.16 ± 0.08$^{\#\#}$	29.7 ± 1.5$^{\#\#}$

Cardiomyocytes were pretreated with the different concentrations of caulophine or 0.1% DMSO for 24 h before exposure to 1.84 μM adriamycin for 24 h. Then, CK and LDH activities in the cellular supernatant, and MDA level and SOD activity in the cells were determined with spectrophotometry. The results were expressed as the mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. adriamycin group.

while pretreatment of the cells with DMSO, the solvent for caulophine, did not produce any anti-apoptotic effect. These observations indicate that caulophine inhibits H_2O_2-induced apoptosis of cardiomyocytes.

Effect of caulophine on LCA-induced myocardial infarction in rats

As shown in Fig. 6, myocardial infarction was not seen in sham-operated rats. In model rats, the myocardial infarction ratio boosted to 30.02 ± 1.66% 24 h after LCA ($P < 0.01$). Compared with model rats, pretreatment of rats with caulophine (25 and 50 mg/kg) decreased the infarction ratio to 24.68 ± 2.07% and 21.84 ± 2.95%, respectively ($P < 0.01$), which is similar with the primary result we reported previously (6).

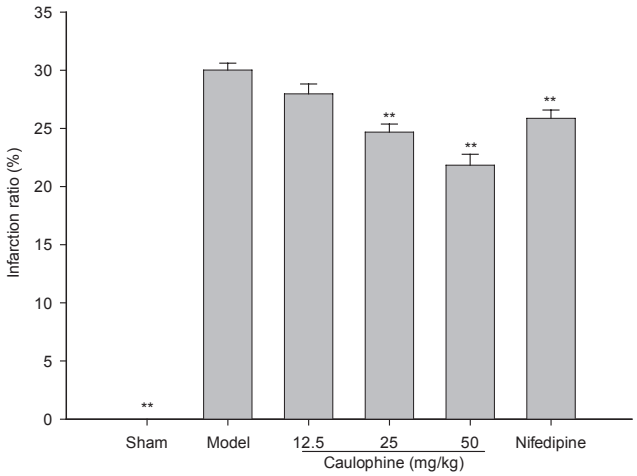

Fig. 6. Effect of caulophine on myocardial infarction size in rats. Rats in caulophine-treated groups and positive control group were pretreated with intragastric administration of caulophine or nifedipine (10.7 mg/kg) for 5 days. Rats in sham-operated and model groups received vehicle in an identical fashion to the drug-treated groups instead. Then, the myocardial infarction was produced by LCA. Rats in the sham-operated group experienced the same procedures except LCA. The myocardial infarction ratio was estimated with NBT staining after the infarction for 24 h. Data were expressed as the mean ± S.E.M. (n = 8 – 10). $^{**}P < 0.01$ vs. model group.

As seen from Table 3, CK, LDH, MDA, and FFA in the serum of model rats were much higher than those in the serum of sham-operated rats ($P < 0.05$ or $P < 0.01$), while SOD activity was significantly decreased ($P < 0.01$). However, pretreatment of rats with caulophine decreased serum CK, LDH, MDA, FFA, and increased SOD ($P < 0.05$ or $P < 0.01$).

Effect of caulophine on ISO-induced myocardial ischemia in rats

In rats subjected to ISO-induced myocardial ischemia, serum CK, LDH, MDA, and FFA were increased and SOD was decreased ($P < 0.01$). Histological observation showed that as compared with sham-operated rats (Fig. 7A), there existed different sizes of focal myocardial necrosis with edema and infiltration of the inflammatory cells in the local interstitial tissue in model rats, which were mainly distributed in the endocardium and even in the myocardium (Fig. 7B). The ultrastructural examination indicated that the myocardial fibers of model rats were disorderly arranged, and even dissolved. The necrotic focus was in the myocardium, and clumping chromatin was present under the nuclear membrane (Fig. 8: B1). Mitochondria were markedly swollen, even exhibiting vacuolar degeneration, and the inner compartments were electron-translucent and expanded (Fig. 8: B2) in comparison with the normal myocardium from sham-operated rats (Fig. 8: A1 and A2). However, pretreatment of rats with caulophine decreased serum CK,

Table 3. Effects of caulophine on the biochemical parameters in serum of rats with myocardial infarction

Group	Dose (mg/kg)	n	LDH (U/l)	CK (U/l)	FFA (μM)	MDA (μM)	SOD (U/ml)
Sham		8	822.8 ± 43.39	654.9 ± 52.06	657.8 ± 47.11	4.94 ± 0.55	260.4 ± 12.52
Model		8	1268.1 ± 73.32**	1006.9 ± 42.88**	1275.03 ± 92.01**	6.98 ± 0.54*	216.8 ± 9.20*
Caulophine	12.5	8	1080.1 ± 111.66	941.8 ± 74.05	1010.91 ± 87.77$^{\#}$	5.48 ± 0.48	239.8 ± 6.10
	25	9	948.1 ± 89.92$^{\#\#}$	849.2 ± 68.65	731.8 ± 63.73$^{\#\#}$	5.08 ± 0.21$^{\#}$	243.3 ± 7.62$^{\#}$
	50	10	920.6 ± 74.62$^{\#\#}$	768.5 ± 71.17$^{\#\#}$	694.9 ± 59.23$^{\#\#}$	4.76 ± 0.65$^{\#}$	249.7 ± 8.01$^{\#}$
Nifedipine	10.7	10	930.9 ± 79.96$^{\#\#}$	743.5 ± 40.29$^{\#\#}$	724.9 ± 54.39$^{\#\#}$	4.73 ± 0.55$^{\#}$	244.1 ± 7.24$^{\#}$

Rats in caulophine-treated groups and the positive control group were pretreated with intragastric administration of caulophine or nifedipine for 5 days prior to the myocardial infarction, respectively. Rats in the sham-operated and model groups received vehicle in an identical fashion to the drug-treated groups instead. Myocardial infarction was produced by LCA. Rats in the sham-operated group experienced the same procedures except LCA. LDH, CK, and SOD activities and MDA and FFA levels in serum were spectrophotometrically determined after the infarction for 24 h. Data were expressed as the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. model group.

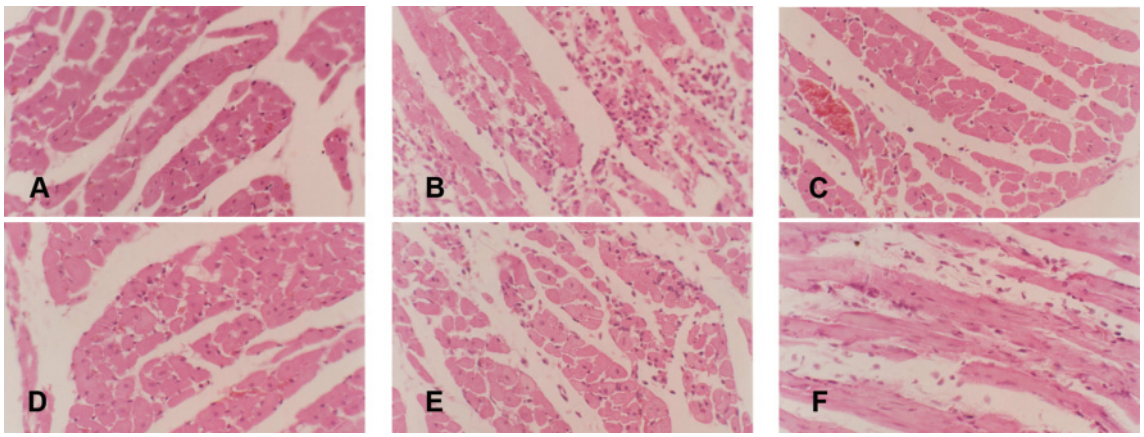

Fig. 7. Histological examination of ISO-induced ischemic myocardium in rats. Groups and drug pretreatment of rats were the same as Fig. 6. Myocardial ischemia was induced by ISO. Sections of the myocardial tissue were stained with hematoxylin-eosin and observed under light microscopy. A) Sham-operated group: normal myocardium. B) Model group: large scope of myocardial necrosis with edema and infiltration of the inflammatory cells in the local interstitial tissue. C) Nifedipine-treated group: roughly normal myocardial tissue except the mild infiltration of the inflammatory cells. D) 50 mg/kg caulophine–treated group: roughly normal myocardial tissue except the mild infiltration of the inflammatory cells. E) 25 mg/kg caulophine–treated group: small scope of myocardial necrosis with mild edema and infiltration of the inflammatory cells. F) 12.5 mg/kg caulophine–treated group: similar myocardial histopathological changes to model group (Magnification 200×).

LDH, MDA, and FFA; increased SOD (Table 4, $P < 0.05$ or $P < 0.01$); relieved the abnormal histopathological (Fig. 7: D, E, and F) and ultrastructural (Fig. 8: D1, D2, E, and F) changes of the ischemic myocardium; and reduced pathological grades of myocardial ischemia (Table 5).

Discussion

Caulophine is a new component isolated from the radix of *Caulophyllum robustum* Maxim by using cell membrane chromatography as a screening method and identified as 3-(2-(dimethylamino) ethyl)-4,5-dihydroxy-1,6-dimethoxy-9*H*-fluoren-9-one (Fig. 1) on the basis of physicochemical properties and spectroscopic analyses (6). As caulophine shows a high affinity for the myocardial cell membrane of the rat in cell membrane chromatography, this suggests that it possibly possesses a bioactivity on the heart. Thus, the present study examined the effect of caulophine on cardiomyocyte injury and the primary mechanism of action in four models, which involve different pathogenesis of cardiomyocyte injury. In the in vitro models, H_2O_2 causes cardiomyocyte injury mainly through lipid peroxidation, and adriamycin may produce a significant cardiotoxicity through the oxidative lesion and calcium overload (13 – 16). In the in vivo experiments, LCA is the most classical and reliable model for myocardial ischemic injury, while a large dose of ISO can increase myocardial oxygen consumption via activating β_1-adrenoceptor in the heart to lead to myocardial lesion (17).

Oxidative stress has been implicated in the pathogenesis of many cardiovascular diseases, including myocardial ischemia and/or reperfusion through inducing ROS production (18). In physiological conditions, ROS can be scavenged by some enzymes such as SOD, glutathione peroxidase, and catalase. However, ROS cause membrane phospholipid and protein oxidation and DNA injury if ROS production exceeds the capacity of antioxidant defenses under the pathological status (19).

As one of the ROS, H_2O_2 has been widely used as an in vitro inducer of oxidative injury (20). The present data showed that H_2O_2 at 0.1 – 0.6 mM decreased the viability of cardiomyocytes in concentration- and time-dependent manners.

The results from the in vitro experiment showed that caulophine increased the viability of H_2O_2- and adriamycin-injured cardiomyocytes and decreased release of CK and LDH from the injured cells. As caulophine was prepared in 0.1% DMSO and DMSO possibly affected the results of caulophine, we also observed its effects on the cell viability and biochemical parameters in H_2O_2- and adriamycin-injured cardiomyocytes. The results showed that DMSO alone did not produce any protective effect on H_2O_2- and adriamycin-induced cardiomyocyte injury. These observations indicate that caulophine indeed protects cardiomyocytes from H_2O_2- and adriamycin-induced injury in vitro. In the in vivo evaluation, we found that caulophine reduced the myocardial infarct size and serum CK and LDH activities in the rats sub-

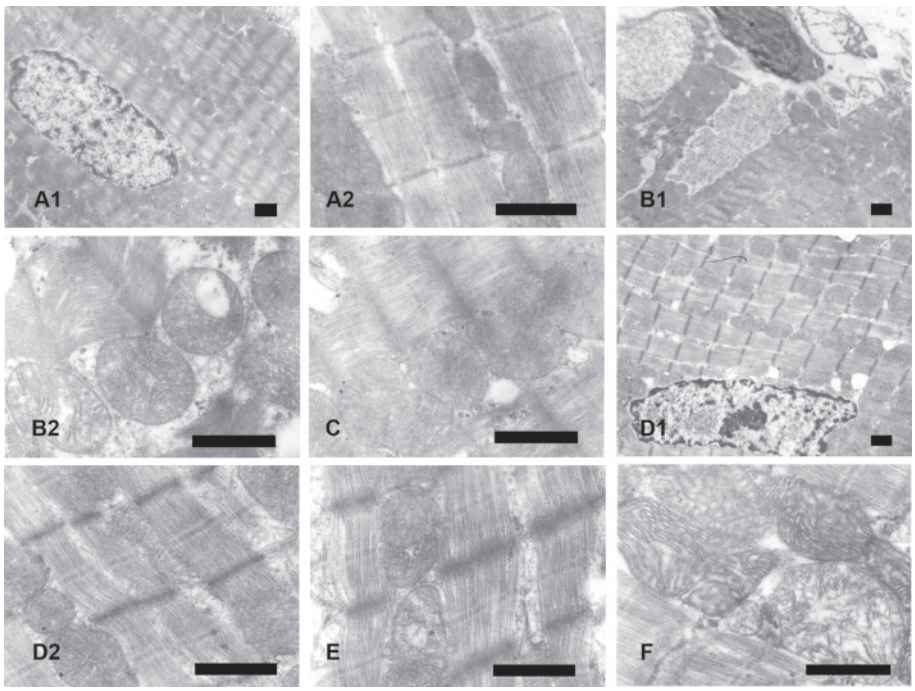

Fig. 8. Ultrastructural observation of ISO-induced ischemic myocardium in rats. Groups and drug pretreatment of rats were the same as Fig. 6. Myocardial ischemia was induced by ISO. Slices of the cardiac apex were stained with routine procedures and observed under a transmission electronic microscope. A) Sham-operated group: the myocyte nuclei were elliptic in shape, and the chromatin was distributed evenly. Many mitochondria were present around the nucleus (A1). The mitochondria, myocardial fiber, myocomma, and cross striation were distinct (A2). B) Model group: there were three scopes of focal necroses (B1). The myocardial fiber was disordered, even dissolved. The mitochondria were enlarged, even exhibiting vacuolar degeneration (B2). C) Nifedipine-treated group: the ultrastructural changes of myocytes were extensively attenuated. Mitochondria and myocardial fiber showed slight edema. D) 50 mg/kg caulophine–treated group: myocyte nuclei (D1), mitochondria, myocardial fiber, and cross striation (D2) were basically normal. E) 25 mg/kg caulophine–treated group: mitochondria, myocardial fiber, and cross striation were slightly enlarged. F) 12.5 mg/kg caulophine-treated group: mitochondria were seriously enlarged, even showing flocculent degeneration, and the myocardial fibers were clearly enlarged. Long bar scale = 20,000× magnification, short bar scale = 5,000× magnification.

Table 4. Effects of caulophine on the biochemical parameters in serum of rats with ISO-induced myocardial ischemia

Group	Dose (mg/kg)	n	LDH (U/l)	CK (U/l)	FFA (μM)	MDA (μM)	SOD (U/ml)
Sham		8	917.3 ± 19.82	585.0 ± 28.32	812.7 ± 173.4	4.49 ± 0.62	207.9 ± 3.21
Model		8	1122.6 ± 58.58**	755.5 ± 42.83**	1711.3 ± 468.2**	6.66 ± 0.48**	152.2 ± 14.79**
Caulophine	12.5	8	1075.5 ± 76.57	693.5 ± 44.27	1410.4 ± 305.6	5.03 ± 0.62$^{\#}$	181.1 ± 10.82$^{\#}$
	25	9	932.8 ± 51.55$^{\#}$	597.8 ± 49.95$^{\#}$	1292.1 ± 171.0$^{\#\#}$	4.57 ± 0.52$^{\#\#}$	189.0 ± 8.69$^{\#\#}$
	50	10	850.0 ± 48.22$^{\#\#}$	566.1 ± 40.77$^{\#}$	1047.2 ± 248.7$^{\#\#}$	4.51 ± 0.39$^{\#\#}$	192.5 ± 5.77$^{\#\#}$
Nifedipine	10.7	9	956.2 ± 32.41$^{\#}$	603.9 ± 31.55$^{\#}$	1139.7 ± 237.4$^{\#\#}$	4.81 ± 0.44$^{\#}$	192.0 ± 7.02$^{\#\#}$

Rats in caulophine-treated groups and the positive control group were pretreated with intragastric administration of caulophine or nifedipine for 5 days prior to the myocardial ischemia, respectively. Rats in the sham-operated and model groups received vehicle in an identical fashion to the drug-treated groups instead. Myocardial ischemia was induced by ISO. LDH, CK, and SOD activities and MDA and FFA levels in serum were spectrophotometrically determined. Data were expressed as the mean ± S.E.M. $^{**}P < 0.01$ vs. sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. model group.

jected to LCA, suggesting that caulophine has a protective effect on ischemic myocardium in rats. To further confirm the conclusion, we established the second model of myocardial ischemia with ISO. We found that caulo-

Table 5. The pathological grades of ISO-induced myocardial ischemia in rats

Groups	Dose (mg/kg)	n	Grade 0	Grade I	Grade II	Grade III	Grade IV
Sham		8	8	0	0	0	0
Model		8	0	1	3	2	2
Caulophine	12.5	8	0	3	3	2	0
	25	9	0	6	2	1	0
	50	10	1	7	1	1	0
Nifedipine	10.7	9	0	6	3	0	0

Rats in caulophine-treated groups and the positive control group were pretreated with intragastric administration of caulophine or nifedipine for 5 days prior to the myocardial ischemia, respectively. Rats in the sham-operated and model groups received vehicle in an identical fashion to the drug-treated groups instead. Myocardial ischemia was induced by ISO. The degree of ischemia was graded according to the standard submitted by Rona. Data were treated with the Ridit test method, and the pathological grades between the middle and large doses of caulophine-treated groups, nifedipine-treated group, and model group were $P < 0.05$.

phine also lowered serum CK and LDH activities, relieved the necrotic degree of ischemic myocardium, and improved the abnormal ultrastructural changes of ischemic myocardium in the rat with ISO-induced myocardial ischemia.

As active components of biological membranes, phospholipids play an important role in signal transduction and cellular metabolism. FFA is one of the degraded products of membrane phospholipids during lipid peroxidation. The accumulated FFA in cells contributes to myocardial ischemic injury by affecting Na^+/K^+-ATPase activity (21) and mitochondrial calcium handling (22). Our results revealed that serum FFA level was markedly elevated in rats subjected to LCA- and ISO-induced myocardial ischemia, which is consistent with the previous results (23). However, caulophine decreased FFA level in serum of rats with myocardial ischemia, suggesting that caulophine may correct the disorder of lipid metabolism to diminish FFA-induced damage to the myocardium during myocardial ischemia.

As one of the peroxidized products of polyunsaturated fatty acid (24), MDA concentration in the cells or serum reflects the degree of lipid peroxide. As an antioxidant enzyme, SOD activity represents the ability to clear oxygen free radicals (25). The present results indicated that caulophine both decreased MDA level and increased SOD activity in H_2O_2- and adriamycin-injured cardiomyocytes and in serum of rats with myocardial ischemia, implicating that caulophine is able to relieve injury of cardiomyocytes via antioxidation.

In addition to producing oxidative injury, H_2O_2 is able to cause cardiomyocyte apoptosis via Fas or other signaling pathways (26 – 28). We found that pretreatment of cardiomyocytes with caulophine significantly inhibited H_2O_2-induced apoptosis of cardiomyocytes, which was not related to DMSO contained in the caulophine solution. As H_2O_2 causes both cell death and apoptosis at high and low concentrations, further investigations are necessary to clarify the anti-apoptotic effect and related signal pathways.

In summary, we present the first evidence that caulophine has ability to protect myocardium from oxidative and ischemic damage in vitro and in vivo, which is associated with its antioxidative action. Although the precise mechanisms underlying the protection remain to be clarified, this finding suggests that caulophine may be a promising agent for treatment of coronary heart disease.

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