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Cryptotanshinone, a lipophilic compound of *Salvia miltiorrriza* root, inhibits TNF-α-induced expression of adhesion molecules in HUVEC and attenuates rat myocardial ischemia/reperfusion injury *in vivo*

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ABSTRACT

The aim of the present study was to evaluate the protective effect of cryptotanshinone (CTS), one of active ingredients of *Salvia miltiorrhiza* root, on myocardial ischemia-reperfusion injury in rat due to inhibition of some inflammatory events that occur by NF-kB-activation during ischemia and reperfusion. Myocardial ischemia and reperfusion injury was induced by occluding the left anterior descending coronary artery for 30 min followed by either 2 h (biochemical analysis) or 24 h (myocardial function and infarct size measurement) reperfusion. CTS injected (*i.v.*) 10 min before ischemia and reperfusion insult. CTS significantly reduced the infarct size and improved ischemia and reperfusion-induced myocardial contractile dysfunction. Furthermore, CTS inhibited NF-kB translocation, expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), neutrophil infiltration and MPO activity in ischemic myocardial tissues. CTS also significantly reduced plasma levels of TNF-α, IL-1β due to ischemia and reperfusion. Interestingly, H_2O_2-stimulated NF-kB-luciferase activity and TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) expressions in human umbilical vein endothelial cells (HUVEC) were significantly inhibited by CTS. Taken together, it is concluded that CTS may attenuate ischemia and reperfusion-induced microcirculatory disturbances by inhibition of proinflammatory cytokine production, reduction of neutrophil infiltration and possibly inhibition of adhesion molecules through inhibition of NF-*k*B-activation during ischemia and reperfusion.

1. Introduction

Ischemia and reperfusion occurs in a wide range of situations including trauma, vascular reflow after contraction, percutaneous transluminal coronary angioplasty, thrombolysis treatment, organ transplantation, and hypovolemic shock with resuscitation. Ischemia and reperfusion exerts multiple insults in microcirculation, frequently accompanied by endothelial cell injury, enhanced adhesion of leukocytes, macromolecular efflux, production of oxygen free radicals and mast cell degranulation (Han et al., 2001). In addition, myocardial damage due to reperfusion of ischemic tissue is caused primarily by proinflammatory cytokine, tumor necrosis factor-alpha (TNF-α) (Shames et al., 2002). TNF-α is indeed produced by the cardiac myocytes following ischemia. Infiltrating neutrophils also play a crucial role for the production of TNF-α in ischemia and reperfusion-injury. Furthermore, the rapid release of TNF-α, a cytokine found in reperfused myocardium (Gurevitch et al., 1996), contributed to the transcriptional activation of intracellular adhesion molecule-1 (ICAM-1). In addition, it has been suggested that early cleavage of TNF-α triggers a cascade of NF-κB activation and ICAM-1 induction in response to postischemic reperfusion. In fact, NF-κB activation can occur after ischemia and reperfusion of the heart, as shown in a study by Chandrasekar and Freeman (1997). Other factors besides enhanced ICAM-1 expression on the endothelial surface may have contributed to enhanced polymorph nuclear leukocytes adhesion after prolonged reperfusion, especially induction of P-selectin (Armstead et al., 1997) and E-selectin (Weyrich et al., 1995). Previously we reported that anthocyanins protected myocardial injury from ischemia and reperfusion in rats by inhibition of TNF-α-induced vascular cell adhesion molecule-1 (VCAM-1), and ICAM-1 (Kim et al., 2006). Recently, Han et al. (2008) reviewed the ameliorating effects of several active compounds including

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cryptotanshinone (CTS) derived from *Salvia miltiorrhiza* root extract on microcirculatory distance and target organ injury by ischemia and reperfusion. Although many cardioprotective effect of *Salvia miltiorrhiza* root extract has been reported *in vivo* (Fung et al., 1993; Wu et al., 1993; Kuang et al., 1996; Ji et al., 2000), the effect of CTS on myocardial ischemia and reperfusion animal model *in vivo* and especially on the expression of adhesion molecules *in vitro* has not been investigated so far. Therefore, in the present study, we determined whether CTS could reduce myocardial infarction after ischemia and reperfusion injury in anesthetized rat.

2. Materials and methods

2.1. Materials

Monoclonal antibodies to p-ERK, p-p38, NF-*k*B p65, ICAM-1 and VCAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies to JNK and p-JNK were from Cell Signaling (Beverly, MA, USA). Total RNA extraction kit was obtained from Tri Pure Reagent (Roche Molecular Biochemicals, Mannheim, Germany). Other chemicals were purchased from Sigma Chemical Co. (St., Louis, MO, USA) unless otherwise indicated.

2.2. Isolation and identification of cryptotanshinone

The procedure of isolation and identification of CTS was reported previously by us (Kim et al., 2007). Briefly, the dried roots of *S. miltiorrhiza* (17.45 kg) were percolated with 70% ethanol three times and the ethanolic extracts obtained were concentrated in vacuum. The residue (2.4 kg) was then suspended in water and partitioned successively with hexane, CH_2Cl_2, ethylacetate and butanol, to give hexane (48.8 g), CH_2Cl_2 (27.9 g), ethylacetate (212.2 g) and butanol (149.1 kg) soluble fractions, respectively. A portion of the hexane and CH_2Cl_2 fractions (76.7 g) were subjected to silica gel column chromatography and eluted using a stepwise gradient of hexane: CH_2Cl_2 to yield eleven fractions (HC1–HC11). Fraction HC8.3 was re-crystallized with methanol to yield pure cryptotanshinone. The structure of cryptotanshinone was verified by comparing NMR data with those reported in the literature (Kang et al., 1997; Ryu et al., 1977) and its chemical purities was 96.1%.

2.3. Surgical preparation of animals

Male Sprague-Dawley rats weighing 200–250 g were anesthetized with ketamine 100 mg/kg (i.m.) and xylazine 10 mg/kg (i.m.). The animals were ventilated with room air using a rodent respirator. The chest was opened by middle thoracotomy. After pericardiotomy, a 4–0 black silk ligature was placed under the left aortic descending coronary artery, and the ends of the tie were threaded through a small vinyl tube to form a snare for reversible left aortic descending coronary artery occlusion. After 30 min of ischemia, the myocardium was reperfused by loosening the snare for 2 h or 24 h. Cardiac function was analyzed using 2 F Millar catheter (Millar instruments, Houston, TX, USA). All animals were maintained in accordance with the guide of the Gyeongsang National University Institutional Animal Care and Use Committee.

2.4. Experimental protocol

The rats were assigned one of four groups. 1) Sham ($n=32$): the ligature was placed under left anterior descending coronary artery without occlusion; 2) Ischemia and reperfusion (control) group ($n=40$), rats pretreated with vehicle (0.1% DMSO 0.25 ml), injected through the femoral vein 10 min before ischemia; 3) Ischemia and reperfusion + CTS 125 ($n=34$); 4) Ischemia and reperfusion + CTS 250 ($n=42$). CTS (125 or 250 μg/kg) were administered intravenously 10 min before ischemia. Blood samples (0.3 ml in each group) were collected in tubes containing heparin at baseline and 2 h of reperfusion for measurement of cytokine concentration. The entire sample was immediately centrifuged. Plasma was collected and frozen under −70 °C until analyzed.

2.5. Hemodynamic measurements

Rats were anesthetized with ketamine (75 mg/kg) and xylazine (7.5 mg/kg). The right common carotid artery was exposed and cannulated with a 2 F Millar Catheter (Millar instruments, Inc. USA), into the ascending aorta to measure systolic and diastolic blood pressure, mean arterial pressure and heart rate. The pressure transducer was then advanced into the left ventricle to measure systolic end-diastolic and the first derivatives (positive and negative) of pressure over time ($\pm dp/dt$).

2.6. Determination of area at risk and infarct size

Infarct size was determined according to previous report (Kim et al., 2006). In brief, after measurement heart function, the left aortic descending coronary artery was occluded again and 2 ml of 1% Evans blue dye was injected into the femoral vein to distinguish between perfused and non-perfused sections of the heart. The area at risk was separated from the remaining of the left ventricle and then cut into small pieces and incubated with a TTC for 20 min at 37 °C to visualize the infarct area. The left ventricles were separated according to staining from the remainder of the heart and weighed to determine the infarct size as a percentage of the weight of the area at risk. Area at risk is expressed as a percentage of the left ventricles.

2.7. Isolation of nuclear and cytoplasmic protein

After 2 h of reperfusion, the heart samples (area at risk) were suspended in a buffer that contained 10 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% Triton X-100 and lysed by homogenization. Nuclei were recovered by microcentrifugation at 7500 rpm for 5 min. The supernatant that contained cytoplasmic and membrane protein was collected and stored at −70 °C for Western blot analysis. Nuclear proteins were extracted at 4 °C by gently resuspending the nuclei pellet in buffer that contained 20 mM Tris (pH 7.5), 20% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, and 0.1% Triton X-100, followed by 1 h incubation at 4 °C with occasional vortexing. After microcentrifugation at 13,000 rpm for 15 min at 4 °C, the supernatant that contained nuclear protein was collected. Protein concentration of each sample was measured with modified Bradford assay.

2.8. Western blot analysis

Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with monoclonal antibodies to p-ERK, p-p38, NF-*k*B p65, ICAM-1 and VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), JNK and p-JNK (Cell Signaling, Beverly, MA, USA), polyclonal antibodies to ERK, p38 (Santa Cruz Biotechnology) at 4 °C overnight. Proteins were detected with HRP conjugated secondary antibody (1:5000 dilutions in TBS-T containing 5% skim milk powder, 1 h, room temperature) and were visualized by ECL method.

2.9. RNA isolation and RT-PCR

TNF-α, IL-1β and IL-6 mRNA expressions were measured by RT-PCR. Total RNA was extract with TriPure Reagent (Roche Molecular Biochemicals, Mannheim, Germany) and quantified by absorption at 260 nm. RT was implemented using Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's protocol. GAPDH was used as the normalization control. The primer

sequences of rat TNF-α, IL-1β, IL-6 and GAPDH have also been described previously (Matsubara et al., 2004).

2.10. Determination of plasma TNF-α and IL-1β levels

Concentrations of immunoreactive TNF-α and IL-1β were determined with an ELISA kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's protocol. In brief, the extracted plasma was reacted with the assay reagents in TNF-α and IL-1β kits, respectively, and analyzed spectrophotometrically (Infinite® F200, Tecan Group Ltd, Switzerland) at 450 nm absorbance. Levels of TNF-α and IL-6 were calculated from the kit standards and expressed as pg/ml plasma.

2.11. Estimation of myocardial neutrophil infiltration and MPO activity in cardiac tissue

To determine the extent of polymorphonuclear infiltration, the hearts were rinsed free of blood after myocardial ischemia and reperfusion protocol, and then fixed in 10% formalin. Samples were washed, dehydrated with graded alcohols, and embedded in paraffin. The mid-ventricular cardiac sections were stained with monoclonal anti-polymorphonuclear antibody (Accurate Chemical and Scientific Corp., NY). Four hearts from each group were examined. MPO activity was determined in the ischemic cardiac tissue as described previously (Mullane et al., 1985).

2.12. Cell culture

HUVEC were obtained by collagenase digestion of umbilical veins as previously described (Gimbrone et al., 1974). The isolated HUVEC were cultured in M199 medium (Life Technologies, Grand Island, NY), containing 20% fetal bovine serum, 2 mM L-glutamine, endothelial cell growth supplement (30 μg/ml; Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml), and streptomycin (100 μg/ml). Endothelial cells were identified by cobblestone appearance and specific staining for Von Willebrand factor (DAKO Co., Carponteria, CA). The protocol related with HUVEC was approved from ethical committee of Gyeongsang National University Hospital.

2.13. Transfection

NF-*k*B-luciferase constructs (consensus NF-*k*B binding sequence was cloned into the pGL3 basic luciferase expression vector) were kindly provided by Dr. G. Koretzky (University of Pennsylvania, PA). Transient transfections were performed using Lipofectin (Gibco-BRL, Grand Island, NY) according to the manufacturer's protocol. Briefly, 5×10^5 cells were plated in 60 mm dish plate the day before transfection and grown to about 70% confluence. Cells were transfected with empty vector (pGL3 and/or pcDNA3), 1 μg of NF-*k*B-luciferase + 0.5 μg of pRL-TK-luciferase. Transfection was allowed to proceed for 12 h. The transfected cells were washed with 4 ml of PBS and then stimulated with 100 μM H_2O_2. The cells were continually cultured in serum-free DMEM until they were harvested. Luciferase activity was normalized using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample.

Table 1
Effect of CTS on functional parameters after 30 min ischemia and 24 h reperfusion of rat heart.

Groups	Left ventricular end diastolic pressure (mmHg)	±dp/dt (mmHg/s)	Mean arterial pressure (mmHg)
Sham ($n=8$)	6 ± 1	3896 ± 25/−3074 ± 27	77 ± 2
I/R ($n=10$)	11 ± 1[a]	3254 ± 17[a]/−2613 ± 14[a]	78 ± 1
Ischemia and reperfusion + CTS 125 μg/kg ($n=10$)	9 ± 1	3384 ± 18/−2942 ± 9[b]	83 ± 1
Ischemia and reperfusion + CTS 250 μg/kg ($n=12$)	8 ± 1[b]	3571 ± 19[b]/−2975 ± 18[b]	82 ± 1

Results are expressed as mean ± S.E.M.
[a]$P<0.05$ vs sham, [b]$P<0.05$ vs I/R control.

Table 2
Hemodynamic data under sham-operative condition.

	Baseline	30 min	60 min	90 min	120 min
Heart rates (bpm)					
Vehicle	249 ± 10	312 ± 8	327 ± 13	284 ± 8	296 ± 14
CTS 250	252 ± 11	305 ± 11	332 ± 10	274 ± 7	308 ± 10
Mean arterial pressure (mmHg)					
Vehicle	85 ± 1	110 ± 3	111 ± 3	114 ± 2	112 ± 3
CTS 250	88 ± 1	99 ± 2	120 ± 2	121 ± 3	110 ± 3

Results are expressed as mean ± S.E.M. ($n=6$/group).
No significant differences in each parameter were detected during this sham-operated, time-control, study. The baseline was measured at 30 min after preparation to allow hemodynamics to stabilize.

2.14. Luciferase assay

After experimental treatments, the cells were washed twice with cold PBS, lysed in a passive lysis buffer provided in the dual luciferase kit (Promega, Madison, WI), and assayed for luciferase activity using TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) according to the manufacturer's protocol. All transfections were done in triplicate. Data were presented as a ratio between Firefly and Renilla luciferase activity.

2.15. Statistical analysis

All data are expressed as mean ± standard error mean (S.E.M) unless otherwise indicated. Data were analyzed by factorial ANOVA and Scheffé test. Statistical significance was defined as $P<0.05$.

3. Results

3.1. Hemodynamic parameters

Twenty-four hours after reperfusion, both left ventricular end diastolic pressure and ±dp/dt significantly increased and decreased in ischemia and reperfusion control group compared to sham, respectively. Moreover, ±dp/dt in left ventricles increased in the CTS 250 group than in ischemia and reperfusion control group (3571 ± 19 vs. 3254 ± 17 mmHg/S, $P<0.05$; −2975 ± 18 vs. −2613 ± 14 mmHg/S, $P<0.05$; respectively; Table 1) and elevated left ventricular end diastolic pressure was significantly attenuated in IR + CTS 250 group (11 ± 1 vs 8 ± 1 mmHg, $P<0.05$; Table 1). In all groups left ventricular systolic pressure, systolic blood pressure, diastolic blood pressure and heart rates were not significantly different (data not shown). The effect of CTS was tested on normal hearts under the sham-operated condition (Table 2). No significant difference was observed in the values of heart rates and mean arterial pressure among the vehicle and CTS 250 μg/kg group.

3.2. Reduction of ischemia and reperfusion-induced myocardial infarction

As shown in Fig. 1A, the mean values for area at risk/left ventricles were similar in all treatment groups. However, infarct area/ area at

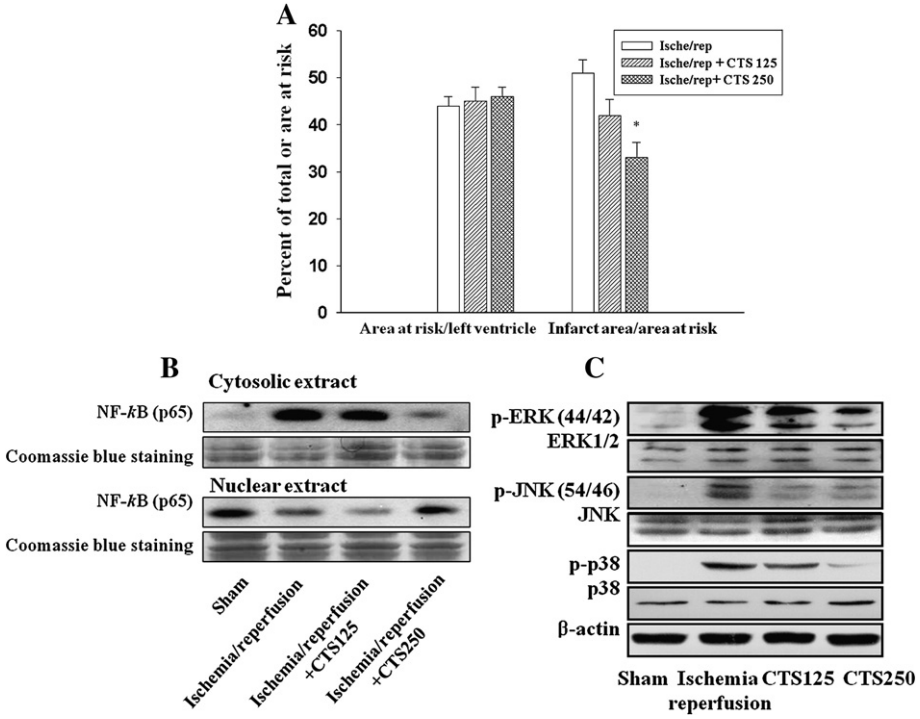

Fig. 1. Comparison of myocardial infarct size in ischemia and reperfusion control, ischemia and reperfusion + CTS 125 and ischemia and reperfusion + CTS 250 groups. Values are mean ± S.E.M. ($n = 10–12$ in each group). $^{*}P<0.05$ compared to ischemia and reperfusion control. (A). Effect of CTS on ischemia and reperfusion-induced translocation of NF-*k*B and protein phosphorylation of MAPK: (B) Representative illustration of NF-*k*B expression in cytosolic and nuclear fractions in rat ischemic myocardium by Western blotting. To examine the purity of the nuclear extracts and cytoplasmic extracts, gels were reprobed with antibodies against lactate dehydrogenase (LDH; cytoplasmic marker) and against PARP (nuclear marker). (C) MAPK (ERK1/2, JNK and p38) activation was determined in sham and underwent ischemia and 2 h after reperfusion. Heart protein lysates from ischemic area were obtained. Blot shown is representative of four experiments with similar results.

risk values were reduced significantly by the administration of CTS in a dose dependent manner relative to the control groups (vehicle, 51 ± 2%; 125 μg/kg of CTS, 42 ± 3%; 250 μg/kg of CTS, 33 ± 3%, $P<0.05$).

3.3. CTS modulates inflammatory signaling pathways

The changes in expression of NF-*k*B in cytosolic and nuclear extractions are shown in Fig. 1B. Densitometrically, expression of NF-*k*B in the cytosolic fraction was significantly attenuated in ischemia and reperfusion control group compared to sham group. In contrast, the expression of NF-*k*B in the nuclear fraction was enhanced in ischemia and reperfusion group. However, this change was inhibited by pretreatment with CTS (250 μg/kg). To determine whether CTS influences MAPK activation, after ischemia and reperfusion, phosphorylation of JNK, p38, and ERK was evaluated in heart tissue from animals. As shown in Fig. 1C, phosphorylation of three kinds of MAPK was increased in the ischemia and reperfusion control. In contrast, phosphorylation of these MAPK was decreased but did not affect total cellular levels of MAPK in CTS-treated ones. Thus, these results suggest that CTS acts upstream of MAPK activation.

3.4. CTS decreases production of inflammatory mediators

Inflammatory cytokines, such as TNF-α, IL-1β and IL-6 were shown to play key roles in the pathophysiology of ischemia and reperfusion injury (Valen et al., 2001). Compared with sham-treated animals, ischemia and reperfusion in vehicle-treated animals resulted in increased mRNA levels of TNF-α, IL-1β, and IL-6 mRNA after 2 h of reperfusion (Fig. 2A). However, animals that were treated with CTS exhibited minimal increases in myocardial TNF-α, IL-1β and IL-6 mRNA levels. As shown in Fig. 2B, ischemia and reperfusion induced a significant rise of plasma level of inflammatory cytokines during or after reperfusion. However, CTS 250 group significantly inhibited the

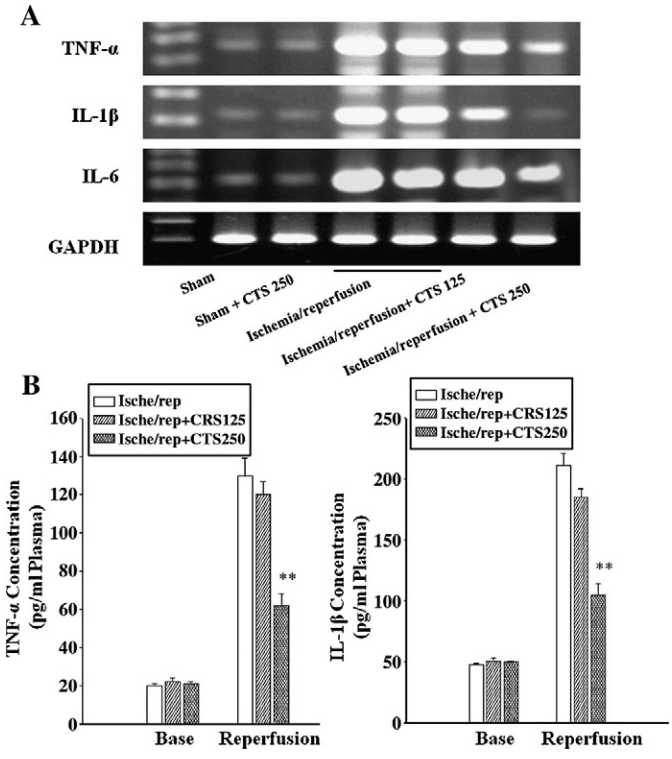

Fig. 2. Cardiac production of proinflammatory cytokines: (A) Heart TNF-α, IL-1β and IL-6 mRNA expression was measured after 2 h reperfusion in rats. Results were obtained using RT-PCR. Assay shown is representative of three experiments with similar results. (B) Plasma levels of TNF-α and IL-1β in baseline and 2 h after reperfusion time points in each group. Values are mean ± S.E.M. ($n = 6$ in each group). $^{**}P<0.01$ compared to ischemia and reperfusion control. Baseline, after anesthesia; R2, 2 h after reperfusion.

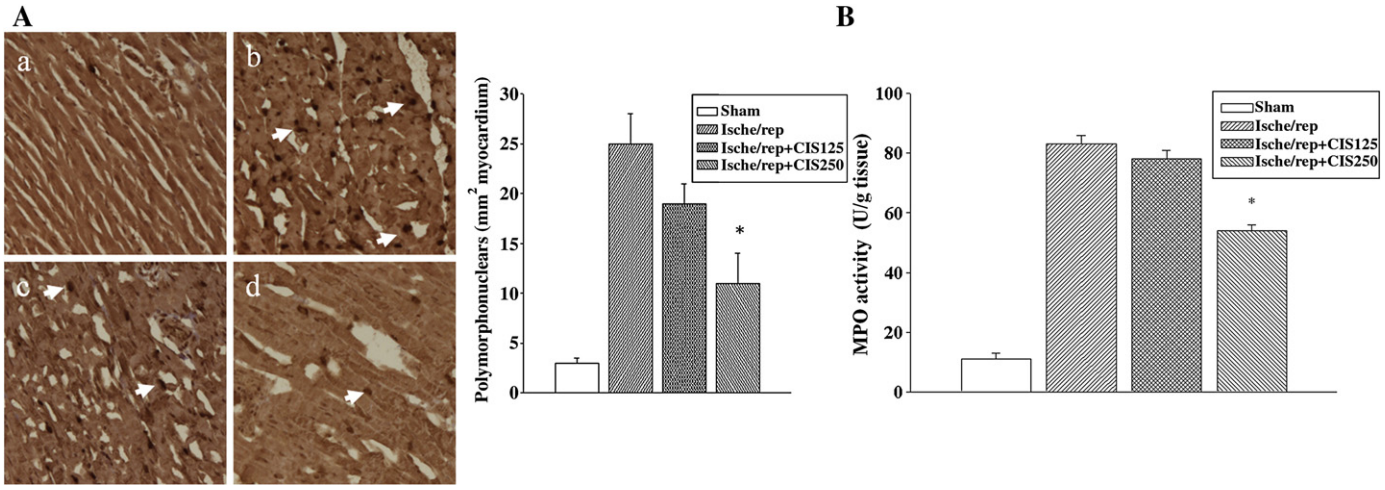

Fig. 3. Effect of CTS on myocardial polymorphonuclear accumulation: (A) Immunohistochemical staining with monoclonal anti-polymorphonuclear antibody. a) Sham, nonischemic tissue; b) Ischemia and reperfusion control, ischemia/reperfused myocardium; c) Ischemia and reperfusion + CTS 125 D) Ischemia and reperfusion + CTS 250. CTS 250 significantly inhibited polymorhonuclear accumulation after ischemia and reperfusion vs. control group. Values are mean ± S.E.M. ($n = 6$ in each group). $^{*}P<0.05$ compared to ischemia and reperfusion control. (B) MPO activity in the area at risk of cardiac tissue samples obtained from sham, ischemia and reperfusion control and CTS treated rats. Values are mean ± S.E.M. ($n = 6$ in each group). $^{*}P<0.05$ compared to ischemia and reperfusion control.

elevation in plasma TNF-α and IL-1β concentration compared to ischemia and reperfusion group after 2 h of reperfusion (62 ± 8 vs. 130 ± 12 pg/ml plasma, $P<0.01$; 105 ± 12 vs. 211 ± 15 pg/ml plasma, $P<0.01$). No statistical difference in changes of TNF-α and IL-1β level during the course of experiment was observed in baseline.

3.5. Polymorphonuclear accumulation in ischemic myocardium

Polymorphonuclear accumulation in the ischemic myocardium in the four groups was detected by immunohistochemistry with monoclonal anti-rat polymorphonuclear CD18 antibody (Fig. 3A).

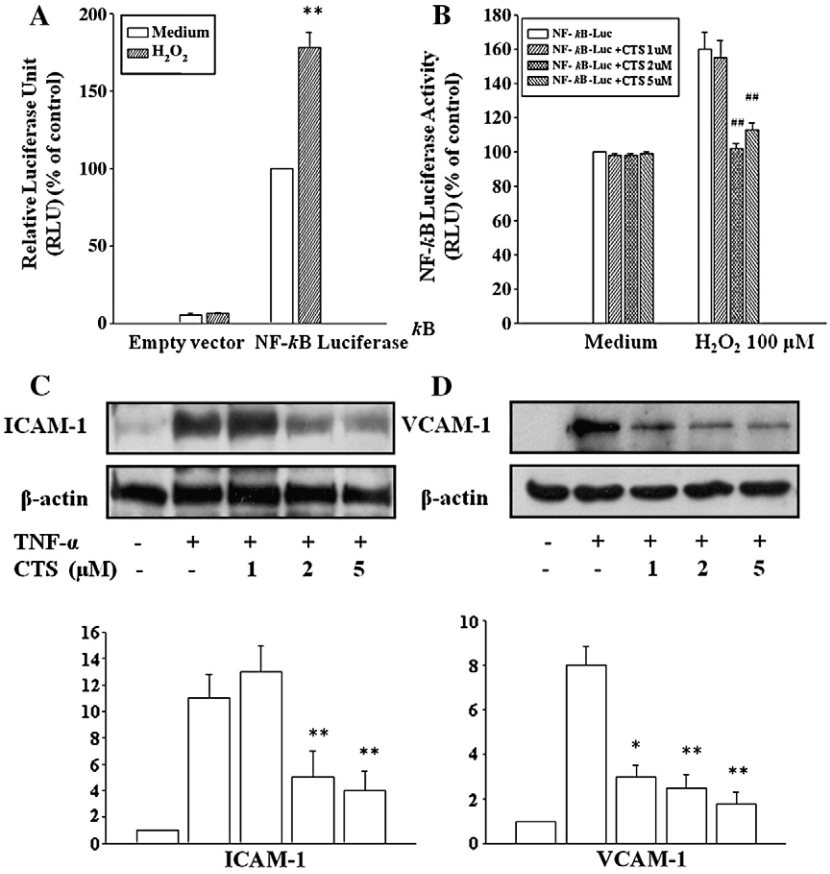

Fig. 4. Inhibition of H_2O_2-induced NF-*k*B-luciferase activity by CTS in HUVECs: (A) Cells were transfected with empty vector or 1 μg of NF-*k*B-luciferase + 0.5 μg of pRL-TK-luciferase. (B) Cells were allowed to recover for 24 h and then treated with 100 μM of H_2O_2 with/without CTS (1 or 2 or 5 μM), and harvested 2 h post-treatment. Luciferase activities are presented as the fold activation relative to that of the untreated cells. Values are mean ± S.E.M of four independent experiments. $^{**}P<0.01$ compared to control; $^{\#\#}P<0.01$ compared to NF-*k*B-Luc. Inhibition of TNF-α-induced ICAM-1 and VCAM-1 expression by CTS in HUVECs. Cells were pretreated with CTS (1, 2, or 5 μM) for 10 min and then treated with TNF-α (10 ng/ml) for 6 h. After treatment, ICAM-1 (C) and VCAM-1 (D) protein level was determined by Western blot analysis. Assay shown is representative of four experiments with similar results. $^{*}P<0.05$, $^{**}P<0.01$ compared to TNF-α.

Among all groups, few CD18 positive cells (cells/mm^2 myocardium) were found in non-ischemic tissue sections in control or sham groups. In contrast, the number of CD18 positive cells in ischemic/reperfused zone in control group was significantly increased compared with that in non-ischemic myocardium (25 ± 6 VS 3 ± 1, $P<0.05$). CTS 250 group reduced the number of accumulated polymorphonuclear in ischemia and reperfusion zone relative to control (11 ± 5 VS 25 ± 6, $P<0.05$) (Fig. 3A). MPO activity in the area at risk after ischemia and reperfusion was significantly increased. However, MPO activity in the area at risk of myocardium was significantly less in the CTS 250 group compared to ischemia and reperfusion control group (vehicle, 83 ± 5 U/g tissue; 250 μg/kg of CTS, 54 ± 4 U/g tissues, $P<0.05$; Fig. 3B).

3.6. Inhibition of H_2O_2-induced NF-kB activity

H_2O_2 increased NF-*k*B-luciferase activity by approximately $178\pm20\%$ ($P<0.01$, Fig. 4A) which was higher than that of the untreated control. In addition, CTS efficiently inhibited NF-*k*B-luciferase activity by H_2O_2 (1 μM of CTS, $160\pm21\%$; 2 μM of CTS, $102\pm3\%$, $P<0.01$; 5 μM of CTS, $113\pm8\%$, $P<0.01$; Fig. 4B).

3.7. Inhibition of TNF-α-induced ICAM-1 and VCAM-1 expression

Different concentrations (2 and 5 μM) of CTS were pretreated for 10 min before activating HUVECs with TNF-α (10 ng/ml) for 6 h. TNF-α increased the expression of ICAM-1, which was significantly decreased by CTS (Fig. 4C). Likewise, as the concentration of CTS increases, VCAM-1 expression by TNF-α was significantly diminished (Fig. 4D).

4. Discussion

In the present study, we demonstrated that CTS effectively improved cardiac function and reduced myocardial infarct size in rat myocardial ischemia and reperfusion model *in vivo*. It has been reported that ischemia and reperfusion leads to several injurious responses in microcirculation, such as an enhanced oxygen free radical production from endothelial cells (Granger, 1988), increased expression levels of CD11b/CD18 in leukocytes and ICAM-1 in endothelial cells (Harmon et al., 2004; Lan et al., 2004). Indeed, endothelial cell injury, leukocyte adhesion, platelet aggregation, release of oxygen radicals from the endothelium or leukocytes and mast cell degranulation after reperfusion are considered to be closely related and interplay in the process of microvasculatory injury induced by ischemia and reperfusion. Thus, ameliorating ischemia and reperfusion-induced microcirculatory disturbance may be one of the targets for drug therapy. We clearly demonstrated that CTS improved cardiac function of ischemia and reperfusion heart. It should be noted that the variations of the loading conditions of the heart may influence cardiac function such as $\pm dP/dt$ and may be considered as a confounding factor. However, this is not the case, since CTS did not influence the heart rate and mean arterial pressure when measured in normal heart.

We confirmed that reperfusion after ischemia caused increase of plasma level of pro-inflammatory mediators, and enhancement of infiltration of neutrophils to infarct area which resulted in cardiac contractile dysfunction and ischemic damages in ischemia and reperfusion model in vivo (Chandrasekar and Freeman, 1997; Shimizu et al., 1998). We believe the protective effects of CTS could be contributed in large part to suppression of the inflammatory response. Indeed, much evidence was given that NF-*k*B is activated by ischemia and reperfusion in myocardium (Chandrasekar and Freeman, 1997; Shimizu et al., 1998) and activation of NF-*k*B is a very early regulatory event during reperfusion and precedes the occurrence of oxidative damage. Therefore, we performed western blot analysis after 2 h not 24 h reperfusion. We demonstrated that CTS inhibited translocation of NF-*k*B in ischemia and reperfusion rat heart in vivo and luciferase activity in vitro. Although we did not test effects of CTS on rat myocardial coronary endothelial cells in ischemia and reperfusion model, from TNF-α-activated HUVEC experiment, it can be highly speculated that CTS can inhibit NF-*k*B activation in myocardial endothelial cells as well. Since TNF-α is highly produced by the cardiac myocytes following ischemia. Infiltrating neutrophils also play a crucial role for the production of TNF-α in myocardial ischemia and reperfusion-injury. The rapid release of TNF-α, a cytokine found in reperfused myocardium (Gurevitch et al., 1996), contributed to the transcriptional activation of ICAM-1. Furthermore, it is well known that TNF-α triggers a cascade of NF-κB activation and ICAM-1 induction in response to postischemic reperfusion. In addition, oxygen free radicals, such as H_2O_2, generated in many cells during ischemia and reperfusion can activate NF-kB. In fact, we confirmed that NF-κB activation has occurred after ischemia and reperfusion of the heart, as shown in a study by Chandrasekar and Freeman (1997). We also demonstrated that H_2O_2 increased NF-kB luciferase activity in HUVECs which CTS inhibited, implying that inhibition of NF-kB activation by CTS from H_2O_2 during ischemia and reperfusion can be expected *in vivo*. Other factors besides enhanced ICAM-1 expression on the endothelial surface may have contributed to enhanced polymorphonuclear leukocytes adhesion after prolonged reperfusion, especially induction of P-selectin (Armstead et al., 1997). Our data demonstrated a significant reduction of CTS in MPO activity, infiltration of neutrophils into the ischemic myocardium, and plasma levels of TNF-α and IL-1β in *in vivo* and also reduction of expression of adhesion molecules in endothelial cells *in vitro*, suggesting a potential mechanism by which CTS ameliorates ischemia and reperfusion-induced microcirculatory disturbance. Thus, CTS may have beneficial effect for the protection of endothelial cells when administered during myocardial ischemia and reperfusion-injury.

In summary and conclusion, we demonstrated that the *in vivo* administration of CTS reduced myocardial infarct size and improved cardiac function after ischemia and reperfusion injury. CTS inhibited the translocation of NF-*k*B and also suppressed the expression and production of inflammatory cytokines such as TNF-α, IL-1β, and decreased the MPO activity. Importantly, CTS inhibited the expression of adhesion molecules such as ICAM-1 and VCAM-1 in endothelial cells activated with TNF-α. We, thus, concluded that the inhibition of NF-*k*B activation, cytokine production, infiltration of neutrophil, and adhesion molecules might be one of the important mechanisms of CTS that protects myocardial injury from ischemia and reperfusion-induced microcirculatory disturbance. These findings suggest that CTS, one of lipophilic active components of *Salvia miltiorrriza* root, can be used as therapeutic agent in myocardial ischemia and reperfusion injury.

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