Total Ginsenosides Inhibit the Right Ventricular Hypertrophy Induced by Monocrotaline in Rats

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Ginsenosides have been reported to release nitric oxide (NO) and decrease intracellular free Ca^{2+} in cardiovascular system, which play important roles in antihypertrophic effect. This study investigated the potential inhibitory effect of total ginsenosides (TG) on right ventricular hypertrophy induced by monocrotaline (MCT, 60 mg/kg/d) and examined the possible antihypertrophic mechanism in male Sprague Dawley rats. MCT-intoxicated animals were treated with TG (20, 40, 60 mg/kg/d) for 18 d. TG treatment ameliorated MCT-induced elevations in right ventricular peak systolic pressure, right ventricular hypertrophy and the expression of atrial natriuretic peptide; N^G-nitro-L-arginine-methyl ester (L-NAME), an NO synthase (NOS) inhibitor, had no influence on these inhibitory effects of TG 40 mg/kg/d, and TG at this dose had no any effect on the eNOS mRNA expression, suggesting the limited rule of NO in TG's effects. To further examine the mechanisms of the protection, the expression of calcineurin and its catalytic subunit CnA, as well as extracellular signal-regulated kinase-1 (ERK-1) and mitogen-activated protein kinase (MAPK) Phosphatase-1 (MKP-1) was examined. TG treatment significantly suppressed MCT-induced elevations of these signaling pathways in a dose-dependent manner. In summary, TG is effective in protecting against MCT-induced right ventricle hypertrophy, possibly through lowering pulmonary hypertension. Multiple molecular mechanisms appeared to be involved in this protection, such as the suppression of MCT-activated calcineurin and ERK signaling pathways.

Key words total ginsenoside; right ventricular hypertrophy; calcineurin; extracellular signal-regulated kinase-1; nitro oxide; monocrotaline

Ginseng, the root of *Panax ginseng* C. A. MEYER, is a well-known and popular herbal medicine used in China for centuries and is now a world wide used natural medicine. Ginsenosides, the pharmacologically active components found in Ginseng, are also found in the leaf and stem of *Panax ginseng* C. A. MEYER,[1] and more than 30 different ginsenosides have been isolated from Ginseng. Ginsenosides exert various pharmacological effects on the central nervous, cardiovascular, endocrine, and immune systems.[2—4] Notably, it is shown that Ginsenosides could reduce both Ca^{2+} influx and catecholamine secretion in bovine adrenal chromaffin cells,[5,6] inhibit voltage-dependent Ca^{2+} channels in sensory neurons as well as in chromaffin in cells,[7] and decrease the level of intracellular free calcium concentration ($[Ca^{2+}]_i$) in dog's myocardium suffered from ischemia-reperfusion injury[8]; Ginsenosides could also stimulate endogenous product of nitric oxide (NO) in rat kidney[9] and in cardiovascular tissues,[10] evoke endothelium-dependent vascular relaxation in rat aorta.[11] Ca^{2+} elevation has been known to be a critical signaling in the development of cardiac hypertrophy induced by various hypertrophic stimuli,[12] and NO, produced in virtually every cell type in the heart, shows potent antihypertrophic effects.[13] Both effects of Ca^{2+}-decrease and NO-release of ginsenosides indicate that ginsenosides may have a potential inhibitory effect on cardiac hypertrophy.

The mechanism(s) at molecular level of pathological cardiac hypertrophy remains unclear. It has been reported that Ca^{2+} elevation activates the calcineurin (CaN) pathway, and in turn enhances hypertrophy or apoptosis of cardiomyocytes.[14] In addition, extracellular signal-regulated kinase-1/2 (ERK-1/2, the important members of mitogen-activated protein kinase (MAPK) family) likely occupies a central regulatory position in the signaling hierarchy of a cardiac myocyte given its unique ability to respond to virtually every characterized hypertrophic agonist and stress stimuli.[15]

In the present study, we investigated whether total ginsenosides (TG) extracted from the leaf and stem of *Panax ginseng* C. A. MEYER have inhibitory effect on right ventricle hypertrophy (RVH) induced by injection of monocrotaline (MCT) which lead to pulmonary hypertension by causing injury of the lung vasculature, and examine the possible influences of TG on NO-release, CaN and ERK signaling pathways.

MATERIALS AND METHODS

Materials TG (purity >93%), extracted from the leaf and stem of *Panax ginseng* C. A. MEYER, was presented by professor Rui Zhao (Beijing Naturally Occurring Drugs Research Institute, China), and was composed of Rb_1 (5.26%), Rg_1 (5.20%), Re (21.60%), Rd (13.65%), and other ginsenosides. MCT was purchased from Sigma Chemical Co. (U.S.A.). The antibodies against CnA, MKP-1, actin were obtained from Stressgen, Santa Cruz Company. Horseradish peroxidase conjugated goat anti-rabbit IgG were from Dako Cytomation Company. The reverse transcription kit and the primers of ANF, CaN, ERK-1 were purchased from TaKaRa Biological Engineering Company.

Animals and RVH Model Male Sprague Dawley rats ($n=64$, 200 ± 20 g), obtained from Animal Center of the Third Military Medical University (Chongqing, China), were housed in a standard environment with a 12-h light/12-h dark cycle, where they were free access to food and water. One week after being fed adaptively, the rats were injected (i.p.) with either MCT (60 mg/kg/d, which was dissolved in 0.5 N HCl and adjusted to pH 7.4 with 0.5 N NaOH, the final concentration was adjusted to 2% with PBS) or equal volume of

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Table 1. Primer Pairs Used in RT-PCR

Gene	GenBank Acc.	Forward primer (5′—3′)	Reverse primer (5′—3′)
ANF	NM 012612	TGACAGGATTGGAGCCCAGAG	TCGAGCAGATTTGGCTGTTATCTTC
eNOS	NM 021838	GGACTGAAGGCTGGCATCTGG	CATGTTACTGTGCGTCCACTCTGC
CaN	NM 017041	CTGAGATGCTGGTAAACGTCCTGA	TGCTCGGATCTTGTTCCTGATG
ERK1	NM 053842	GTTCCCAAACGCTGACTCCAA	GTAAGTCGTCCAGCTCCATGTCAA
β-Actin	NM 031144	GGCCAACCGTGAAAAGATGA	CAGCCTGGATGGCTACGTACA

saline. Then, they were randomly divided into eight groups: (1) vehicle-treated rats (Control group): the rats were given i.p. with vehicle once a day for 18 d; (2) MCT-treated rats (seven groups): after MCT-treatment, the rats were given i.p. vehicle (MCT group) or TG 20, 40, 60 mg/kg/d or L-arginine (L-arg) 200 mg/kg/d, for analyzing whether TG's effect was related to NO-release, another two groups were given with N^G-nitro-L-arginine-methyl ester (L-NAME) 20 mg/kg/d (*p.o.*) combined with TG 40 mg/kg/d by i.p. or L-arg 200 mg/kg/d by i.p., respectively. The rats in every group were administered for 18 d, and $n=8$ for all groups. All animal study procedures are followed by the WHO guideline for humane use of experimental animal.

Measurements of Right Ventricular Peak Systolic Pressure and Assessment of RV Hypertrophy In day 19 after MCT-injection, the rat body weights (BW) in each group were weighed, and the right ventricular peak systolic pressure (RVPSP) of the rats were monitored by polygraph system through the cannulation of polyvinyl tube into right ventricle, under the anesthesia with sodium pentobarbital solution (40 mg/kg/d i.p.). the heart was removed and weighed quickly. The heart was separated into the right ventricle (RV) and the left ventricle with septum (LV) and weighed separately. Finally, RVW/BW (RV weight/BW) and RVH index (RVHI =RV/LV) were calculated. After weighing, the RV tissue were quickly frozen and kept at −80 °C for extracting total RNA or protein.

Observation of Cardiac Tissue Ultrastructure The RV wall (1 mm^3) was fixed by immersion in 2.5% cold glutaraldehyde solution (pH 7.4), followed by rinsing and postfixing with 1% osmium tetroxide in 0.1 mol/l PBS for 2 h at room temperature, then the tissues were dehydrated through a graded series of ethanol to propylene oxide and embedded in epoxy resin. 600-A sections in thick were made and then observed under the transmission electron microscope.

Preparation of Lung Tissue for Morphometric Analysis Fixation was performed by immersion of the lung tissues in a 4% paraformaldehyde solution. For paraffin embedding, all lobes from entire lungs were dissected in to tissue blocks, sectioned at 5 μm. H&E and elastica stainings were performed according to common histopathological procedures. For examing whether TG can inhibit the pulmonary artery hypertrophy, the vessel diameter and wall thickness (WT) of at least 15 pulmonary arteries (50—100 μm in diameter) in each rat in MCT, control and TG 40 mg/kg/d groups were observed by a blinded observer under ×40 magnification using a computerized morphometric system (QWin; Leica). The WT of each artery was expressed as a percent of external diameter (% wall thickness) according to the formula: (2×wall thickness /external diameter)×100.

Cardiac Protein Extract and Western Blotting RV tissues were rapidly excised and rinsed in cold phosphate-buffered saline then homogenized on ice in 1 ml of protein extract. Homogenates were sonicated on ice for three bursts of 5 s each and centrifuged for 15 min at 14000 ***g***/min at 4 °C. Lysates were kept frozen until used or added with SDS-PAGE loading buffer (125 mM Tris, 4% SDS, 20% glycerol, 100 mM dithiothreitol, 0.2% bromphenol blue, pH 6.8) to reach a final concentration of 25%. Lysates were then heated at 95 °C for 5 min and run in 10% SDS-PAGE, and transferred on polyvinylidene difluoride nylon membranes. The blots were probed with mouse anti-CnA (1 : 1000), anti-MKP-1 (1 : 400), or anti-actin antibodies (1 : 600) followed by horseradish peroxidase-conjugated goat anti-rabbits IgG (1 : 1000) antibodies. Immunodetection was carried out using gel image analysis system.

RNA Isolation and Real Time PCR Total RNA was extracted from right ventricle tissues using TRIzol™ (MRC COMPANY). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed with RT-PCR kit according to the manufacturer's instructions, and carried out in iCycler iQ Real-Time PCR Detection System (BIO-RAD, U.S.A.), with SYBR® Green PCR Master Mix (ABI Company, U.K.). The nucleotide sequence of the primers were synthesized by TaKaRa Biological Engineering Company and listed in Table 1. The reactions conditions were: (1) 95 °C 8 min 1 Cycle; (2) 95 °C 15 s 60 °C 1 min 40 Cycles .The results (Ct values) of target gene were normalized with β-actin of the same sample, and expressed as were relative to controls.

Statistical Analysis Data were expressed as mean±standard deviation (S.D.). Statistical evaluations where appropriate were carried out with analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Student's *t*-test using the SPSS 11.0 for Windows statistical program. Data were considered statistically significant if p value were lower than 0.05.

RESULTS

Effects of TG on RVPSP, RVW/BW and RVHI in MCT-Treated Rats Compared with that of vehicle-treated group, the RVW/BW and RVHI significantly increased by 44%, 30% in MCT group respectively ($p<0.01$); MCT-treatment could also evoke an increase in RVPSP ($p<0.01$), indicating indirectly that pulmonary hypertension had been established. However, preventive administrations of TG or L-arg significantly prevented the increases of RVPSP, RVW/BW and RVHI ($p<0.01$). No significant difference could be seen in the inhibitory effect of TG on RVW/BW and RVHI at the doses of 20, 40 and 60 mg/kg/d (Figs. 1B, C) while its decreasing effect on RVPSP was dose-dependent (Fig. 1A). Administration of L-NAME (i.g.), a nitric oxide synthase (NOS) inhibitor, could abolish the effects of L-arg ($p<0.05$), but had no influence on the effects of TG

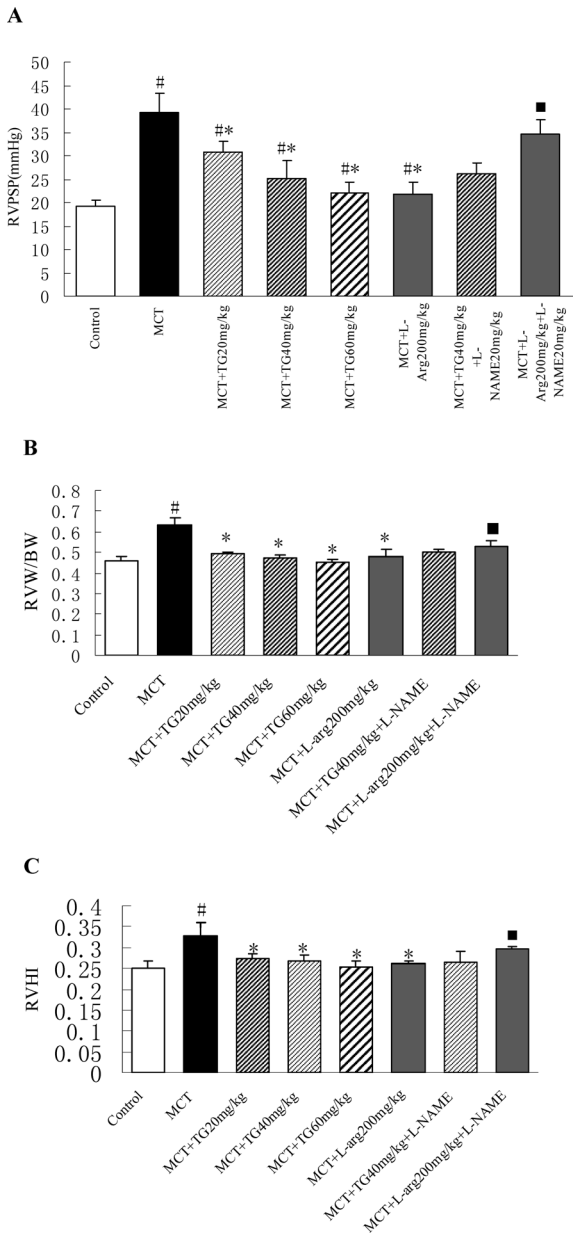

Fig. 1. Effects of Preventive Administration with TG on RVPSP (A), RVW/BW (B) and RVHI (C) in Rat Pretreated by MCT (Mean±S.D.)

RVPSP: right ventricular peak systolic pressure; RVW/BW: right ventricular weight/body weight; RVHI: right ventricular hypertrophy index. Group: male Sprague Dawley rats were given i.p. a single monocrotaline (MCT, 60 mg/kg/d) (MCT group) or equal volume of saline (control group) and then fed with vehicle for 18 d. In other MCT-treated groups, total ginsenosides at doses of 20, 40, 60 mg/kg/d, L-arg 200 mg/kg/d i.p. and L-NAME 20 mg/kg/d (*p.o.*) combined with TG 40 mg/kg/d by i.p. or L-arg 200 mg/kg/d by i.p. were given from day 1 to day 18, respectively. MCT group *vs.* control group $^{\#}p<0.01$; *vs.* MCT group $^{*}p<0.05$; MCT+L-arg 200 mg/kg/d+L-NAME group *vs.* MCT+L-arg 200 mg/kg/d group $^{\blacksquare}p<0.05$.

40 mg/kg/d (Figs. 1A, B, C).

Myocardial Morphology Figure 2A showed the normal morphology of the RV tissues in vehicle-treated rats; MCT-induced myocardial injuries in RV were observed, such as disruption of the intercalated disk, irregular pattern of cross-striation, and aggregation of swollen and misshapen mitochondria (Fig. 2B). Treatment with TG 40 mg/kg/d significantly improved the ultrastructure damages to RV, even though the swollen mitochondria still existed (Fig. 2C).

Assessment of Lung Morphology The normal lung tissue was shown in Fig. 3A, MCT-treatment induced a marked medial hypertrophy in pulmonary arteries, widening on alveolar septa, muscularization in the distal arterioles, and marked periarteritis in vascular artery (Fig. 3B). Administration of TG 40 mg/kg/d significantly ameliorated MCT-induced pulmonary vascular remodeling, reduced muscularization of the pulmonary arteries and arterioles, and relieved the periarteritis (Fig. 3C). For quantitatively analyzing, the protective effect of TG on MCT-induced pulmonary vascular remodeling, the % wall thickness of pulmonary arteries (50—100 μm in diameter) was calculated. MCT-injection caused an increase in % wall thickness to near 3 times, administration of TG 40 mg/kg/d siginificantly decreased the elevated % wall thickness ($p<0.05$), (Fig. 3D).

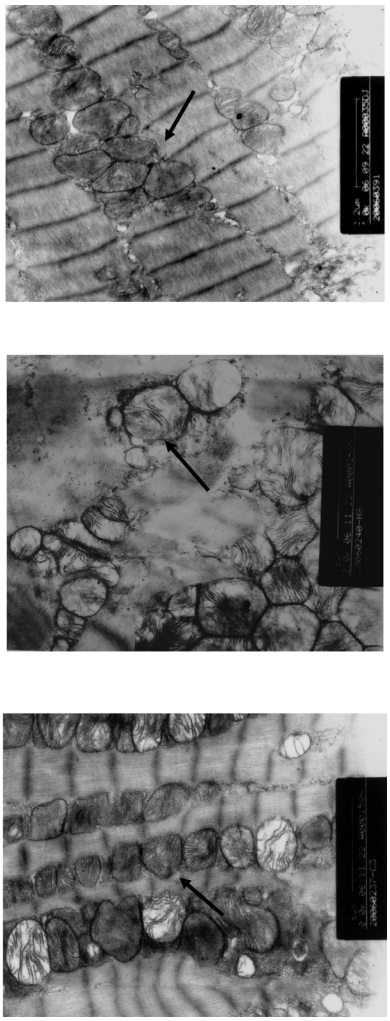

Fig. 2. Effects of TG 40 mg/kg/d Preventive Administration on the Ultrastructural Changes in RV Myocardial Cell of the Rats Pretreated by MCT

(A) Control group; (B) MCT group, the monocrotaline-treated rats showed some myocardial injuries such as disruption of the intercalated disk, irregular pattern of cross-striation, and aggregation of swollen and misshapen mitochondria (arrow); (C) MCT+TG 40 mg/kg/d group, TG 40 mg/kg/d administration significantly ameliorated the ultrastructure damages to the right ventricular striated patterns and intercalated disk. Scale bar is 2.2 μm.

Effects of TG on the Expressions of ANF, eNOS, CaN and ERK-1 mRNA in Hypertrophic RV Tissue Expressions of ANF (Fig. 4A), eNOS (Fig. 4B), CaN (Fig. 4C) and ERK-1 (Fig. 4D) mRNA from the cardiac RV were shown in Fig. 4. It was obvious that the basic expressions of ANF,

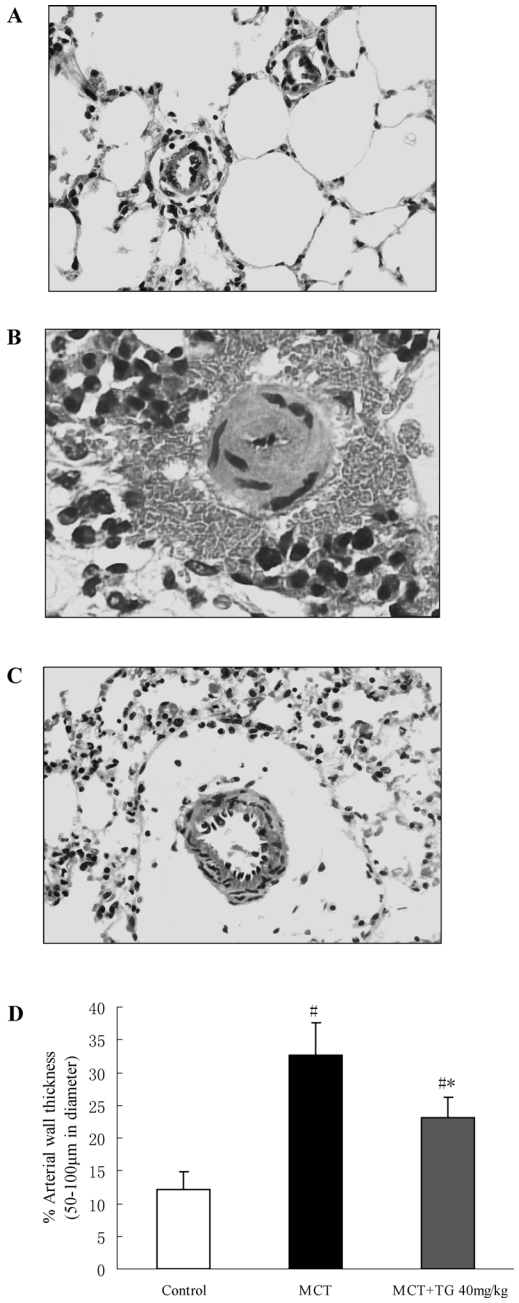

Fig. 3. Effect of Total Ginsenosides 40 mg/kg/d Administration on the Histomorphological Changes of the Small Muscular Arteries (H.E. Staining, 40×, A, B, C) and % Wall Thickness (D) in the Rats Pretreated by Monocrotaline

(A) Control group, the normal lung tissue of the vehicle-treated rat; (B) MCT group, the lung tissue of the MCT-treated rat: exhibiting the medial hypertrophy and muscularization of the arteriole, and a marked periarteritis; (C) MCT+TG 40 mg/kg/d group, the lung tissue of the rat administered with TG 40 mg/kg/d for 18 d after MCT-treatment: exhibiting the relief of the medial hypertrophy and muscularization of arteriole, and the relieving periarteritis. (D) % wall thickness (2×wall thickness/external diameter)×100.

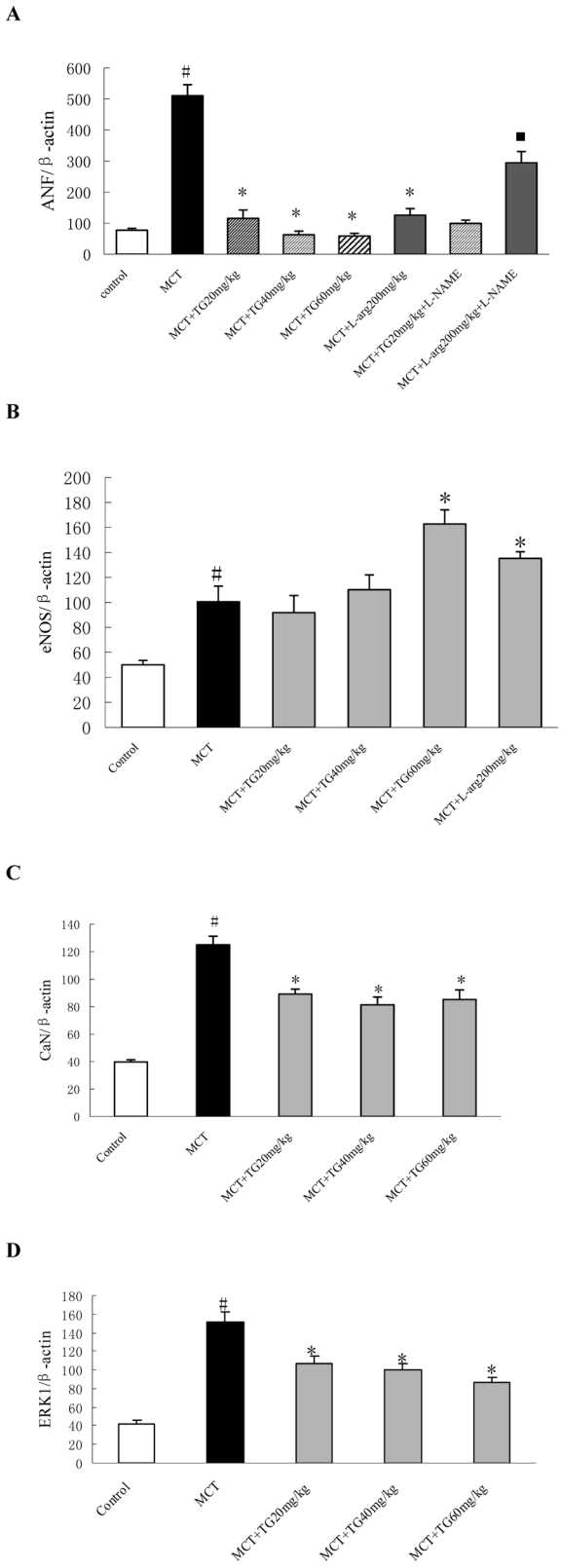

Fig. 4. Effects of Total Ginsenosides Administration on the Expressions of ANF (A), eNOS (B), CaN (C) and ERK1 (D) mRNA in Right Ventricle of Rats Pretreated by Monotrotaline (Mean±S.D.)

Group: male Sprague Dawley rats were given i.p. a single monocrotaline (MCT, 60 mg/kg/d) (MCT group) or with vehicle (control group) and then fed for 18 d. In other MCT-treated groups, total ginsenosides at doses of 20, 40, 60 mg/kg/d and L-arg 200 mg/kg/d by i.p., and L-NAME 20 mg/kg/d (*p.o.*) combined with TG 40 mg/kg/d by i.p. or L-arg 200 mg/kg/d by i.p. were given from day 1 to day 18, respectively. MCT group *vs.* control group $^{\#}p<0.01$; *vs.* MCT group $*p<0.01$; MCT+L-arg 200 mg/kg/d+L-NAME group *vs.* MCT+L-arg 200 mg/kg/d group $\blacksquare p<0.01$.

eNOS, CaN and ERK-1 mRNA were very low in normal control group. However, their expressions increased by 5.6, 1.0, 2.1 and 2.6 times, respectively, in MCT-treated rats (MCT group) ($p<0.01$). Administration of TG 20, 40, 60 mg/kg/d and L-arg 200 mg/kg/d significantly blunted the elevation of ANF mRNA expression, and administration of L-NAME (i.g.) could abolish the effect of L-arg on this parameter ($p<0.05$), but not influence on the effect of TG

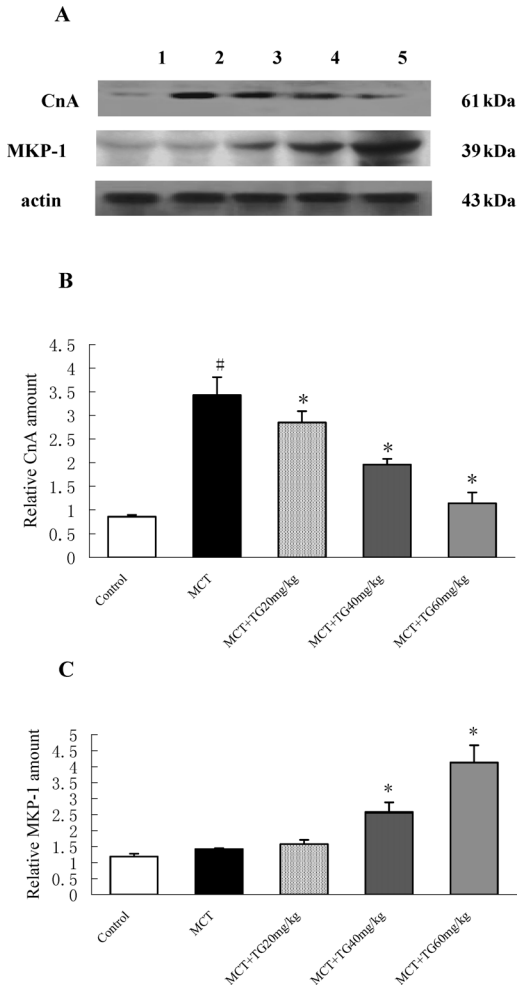

Fig. 5. Effects of Total Ginsenosides Administrations on the Protein Expressions of CnA and MKP-1 in Right Ventricle of the Rats Pretreated by MCT

Group: male Sprague Dawley rats were given i.p. a single monocrotaline (MCT, 60 mg/kg/d) (MCT group) or with vehicle (control group) and then fed for 18 d. In other MCT-treated groups, total ginsenosides at doses of 20, 40, 60 mg/kg/d by i.p. were given from day 1 to day 18, respectively. MCT group *vs.* control group $^{\#}p<0.01$; *vs.* MCT group $^{*}p<0.01$. (A) Lane 1: control group; Lane 2: MCT-treated group; Lane 3: MCT+TG 20 mg/kg/d; Lane 4: MCT+TG 40 mg/kg/d; Lane 5: MCT+TG 60 mg/kg/d; (B, C) the relative amount of CnA and MKP-1 were quantified by NIH Image program and normalized against the amount of actin.

40 mg/kg/d ($p<0.05$). Notably, administrations of TG 20 and 40 mg/kg/d had no any effect on eNOS mRNA Expression, but when the dose of TG was increased to 60 mg/kg/d, it caused an elevation in eNOS mRNA Expression. Similar to the effect of TG on ANF mRNA expression, the elevations of CaN and ERK-1mRNA expressions induced MCT were also inhibited by the administrations of TG 20, 40, 60 mg/kg/d.

Effects of TG on CnA and MKP-1 Protein Levels in RV The protein expression levels of CnA were markedly increased in MCT-treated rats ($p<0.05$), but there was a tendency for the increase in protein expression level of MKP-1 ($p>0.05$), compared with that of normal control group. TG administrations could significantly decrease the protein expression level of CnA ($p<0.01$), and further increase the protein expression levels of MKP-1 in a dose-dependent manner ($p<0.01$), compared with that in MCT-treated group (Fig. 5).

DISCUSSION

It has been well known that MCT causes injury of the vasculature and leads to pulmonary hypertension and RVH through its metabolite MCT-pyrrole.[16,17] In the present study, we used MCT-induced RVH in rat to examine the possible inhibitory effect of TG on cardiac hypertrophy. In MCT-treated animals, the increase in RVPSP and the findings in the electron microscopic observation strongly indicated that there existed the formation of pulmonary hypertension. The elevations of RVW/BW, RVHI and ANF mRNA expression (a marker of hypertrophy), as well as the RV tissue morphological changes, further suggested that the establishment of RVH model in our experiment was successful.

Intraperitoneal injection as an administering method was used widely by many investigators for studies on the pharmacological effects of ginsenosides in rats.[9,18] In this study, we designed to administer TG (20, 40, 60 mg/kg/d, i.p.) to MCT-treated rats for 18 d, and found that TG could significantly reduce the elevated RVW/BW RVHI, ANF mRNA expression induced by MCT, suggesting that TG could ameliorate the RVH in this model. However, it was unclear whether TG attenuate or delay the genesis of MCT-induced RVH. Our previous study demonstrated that ginsenoside Rb1, an active ingredient (5.26%) in TG, could reduce the MCT-induced RVH, when it was administered (i.p.) after 3 weeks of MCT-injection (RVH had been generated) at the dose of 40 mg/kg/d for 21 d,[19] suggesting that Rb1 could attenuate the genesis of RVH. Whether the effect of TG is the same as Rb1 remains to be studied. To our attention, TG 20 mg/kg/d almost completely suppressed the increases in RVW/BW and RVHI, suggesting that the optimal dosage for the antihypertrophic effect of TG in this model was at 20 mg/kg/d, whereas the suppressing effect of TG on the elevated RVPSP was dose-dependent at doses of 20, 40 and 60 mg/kg/d, and the inhibition of TG 40 mg/kg/d on % wall thickness was not complete in our experiment. The results indicated that other mechanisms rather than the suppressing effect on pulmonary hypertension might involve in the antihypertrophic effect of TG. Furthermore, we also found that TG had an antihypertrophic effect on cardiomyocyte *in vitro* which was not related to pulmonary hypertension (data not shown). Thus we consider that the lowering effect on pulmonary hypertension is not the only mechanism for the antihypertrophy of myocardium.

TG has been reported to stimulate endogenous NO release in cardiovascular system[10] and other tissue,[9] and NO was well-known to be a potent inhibitor of cardiac hypertrophy.[13] However, we observed that L-NAME, an NOS inhibitor, could abolish the inhibitory effects of L-arg on RVH, but had no influence on the antihypertrophic effects of TG 40 mg/kg/d, which was consistent with that TG at this dose or 20 mg/kg/d had no any effect on eNOS mRNA expression, in spite of it could increase the eNOS expression at the dose of 60 mg/kg/d, compared with that in MCT group. The results suggested that the antihypertrophic effect of TG might come from other mechanism rather than NO release when the dose was 40 or lower than 40 mg/kg/d, but when the dose of TG was increased to 60 mg/kg/d, the NO release might be partially responsible for its antihypertrophic effect.

In the past several years, a number of studies have impli-

cated that the Calcineurin signal transduction pathway activated by the increased $[Ca^{2+}]_i$ may play an important role in the cardiomyocyte hypertrophy process,[12,20—22] and TG has been reported to reduce the Ca^{2+} influx,[5] inhibit the voltage-dependent channels[7,23] and decrease the $[Ca^{2+}]_i$[8] in some tissues. In the present paper, the fact that the elevated expressions of CaN mRNA and CnA (the catalytic subunit of CaN) protein induced by MCT were significantly blunted by TG suggested that an inhibition on the CaN signaling pathway might be involved in the antihypertrophic mechanisms of TG. Moreover, many studies have indicated that ERK1/2, the members of the mitogen-activated protein kinase (MAPK) family, are crucial regulators in cardiac hypertrophy[15,24,25]; while the mitogen-activated protein kinase phosphatase-1 (MKP-1) can dephosphorylate and then inactivate ERK, functioning as a negative feedback mechanism in the control of MAPK activity.[26—28] It was interesting to note that TG not only markedly reduced the elevated ERK-1 mRNA expression induced by MCT, but also increased the MKP-1 protein expression in a dose-dependent manner. The results strongly demonstrated that the molecular mechanism for the antihypertrophic effect of TG might be also involved in the inhibition on the ERK signaling pathway. Nevertheless, the MCT-induced RVH was suppressed almost completely by TG at the dose of 20 mg/lg, but only a part of expressions of CaN mRNA, ERK-1 mRNA and CnA protein was inhibited by TG at this dose. The reason for this discrepancy was unclear, it was possible that the activities of signaling pathways were also inhibited by TG through some other mechanisms, excepting the inhibition on the transcription or translation of the members of signaling pathways.

In conclusion, our study demonstrates that TG can alleviate cardiac hypertrophy induced by MCT in rats, which may in part be, mediated by lowering pulmonary hypertension; the molecular mechanism for the antihypertrophic effect of TG may be involved in the inhibitions on the CaN and ERK signaling pathways.

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