Original Paper

Ginsenoside Rb1 Attenuates Agonist-Induced Contractile Response via Inhibition of Store-Operated Calcium Entry in Pulmonary Arteries of Normal and Pulmonary Hypertensive Rats

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Key Words

Ginsenoside Rb1 • Store-operated calcium entry • Pulmonary hypertension • Monocrotaline • Chronic hypoxia

Abstract

Background: Pulmonary hypertension (PH) is characterized by sustained vasoconstriction, enhanced vasoreactivity and vascular remodeling, which leads to right heart failure and death. Despite several treatments are available, many forms of PH are still incurable. Ginsenoside Rb1, a principle active ingredient of *Panax ginseng*, exhibits multiple pharmacological effects on cardiovascular system, and suppresses monocrotaline (MCT)-induced right heart hypertrophy. However, its effect on the pulmonary vascular functions related to PH is unknown. **Methods:** We examined the vasorelaxing effects of ginsenoside Rb1 on endothelin-1 (ET-1) induced contraction of pulmonary arteries (PAs) and store-operated Ca^{2+} entry (SOCE) in pulmonary arterial smooth muscle cells (PASMCs) from chronic hypoxia (CH) and MCT-induced PH. **Results:** Ginsenoside Rb1 elicited concentration-dependent relaxation of ET-1-induced PA contraction. The vasorelaxing effect was unaffected by nifedipine, but abolished by the SOCE blocker Gd^{3+} . Ginsenoside Rb1 suppressed cyclopiazonic acid (CPA)-induced PA contraction, and CPA-activated cation entry and Ca^{2+} transient in PASMCs. ET-1 and CPA-induced contraction, and CPA-activated cation entry and Ca^{2+} transients were enhanced in PA and PASMCs of CH and MCT-treated rats; the enhanced responses were abolished by ginsenoside Rb1. **Conclusion:** Ginsenoside Rb1 attenuates ET-1-induced contractile response via inhibition of SOCE, and it can effectively antagonize the enhanced pulmonary vasoreactivity in PH.

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Introduction

Pulmonary hypertension (PH) is a pathophysiological condition associated with a broad spectrum of diseases of different pathological features and etiological mechanisms [1]. It is characterized by progressive pulmonary vascular remodeling, sustained vasoconstriction and enhanced vasoreactivity, resulting in elevated pulmonary vascular resistance and eventually leading to right heart failure and even death [2]. The pathogenesis of PH is complex involving many different mechanisms and signaling pathways. Accumulating evidence suggest that there are major intrinsic changes in ionic balance and Ca^{2+} homeostasis in pulmonary arterial smooth muscle cells (PASMCs) in many forms of PH, and the aberrant alterations in Ca^{2+} signaling in PASMCs play pivotal roles in the augmented pulmonary vasoconstriction and vascular remodeling in PH [3]. However, despite several effective treatments have become available in recent years, many forms of PH are still incurable and the mortality rates are very high [4, 5]. Hence, research on novel therapies that can improve the modality of treatment is urgently needed.

Ginseng, the root of *Panax ginseng*, is a well-known oriental traditional medicine. It has been used widely as a tonic for various disorders in China, Korea and Japan for over two thousand years and is now a popular herbal remedy worldwide. Ginsenosides, the bioactive ingredients of ginseng, have been proven to provide therapeutic effects affecting the cardiovascular, endocrine, immune, and central nervous systems [6-9]. Ginsenosides are generally classified into two categories, namely the panaxadiols and the panaxatriols. Ginsenoside Rb1 is one of most abundant panaxadiols that possesses many beneficial effects on the cardiovascular system. Previous studies have demonstrated that ginsenoside Rb1 promotes endothelial nitric oxide production [10], suppresses intimal hyperplasia after vascular injury [11-13], protects against endothelial dysfunction [14] and inhibits inflammatory responses in vascular smooth muscle cells [15]. It also suppresses apoptosis and oxidative injury of cardiomyocytes [16-20]; and protect against ischemic-reperfusion injury [21, 22], dilated cardiomyopathy [23] and cardiac hypertrophy [24]. A previous study showed that ginsenoside Rb1 can suppress and reverse the development of right heart hypertrophy in monocrotaline (MCT)-treated rats, and the finding was explained by an inhibitory effect of ginsenoside Rb1 on the calcineurin-NFAT signaling pathway in cardiomyocytes [24]. However, the primary cause of MCT-induced right heart hypertrophy is pulmonary arterial hypertension (PAH) triggered by the toxic effect of the metabolite monocrotaline pyrrole on the pulmonary endothelial cells [25]. Hence, this raised the interesting possibility that ginsenoside Rb1 may exert its cardiac protective effect on MCT-treated rats through the suppression of PAH.

Alteration in pulmonary vasoreactivity is one of the salient features of PH [26]. Previous studies have shown that vasoreactivity to agonists such as ET-1, 5-HT and Ang II are enhanced in PAs of different PH rat and mouse models [27-33]. We have recently shown that the enhanced vasoreactivity to ET-1 in PAs of MCT-treated rats is related to the upregulation of store-operated Ca^{2+} entry (SOCE). In the present study, we sought to investigate the possible therapeutic actions of ginsenoside Rb1 on PH by examining its effects on the contractile responses and vasoreactivity to ET-1 in PAs of chronic hypoxia (CH) and MCT-induced PH rats, and by interrogating the underlying mechanisms.

Materials and Methods

Animals and PH models

Experiments were performed on male Sprague-Dawley rats (200–250 g) supplied by the animal center of the Fujian Medical University (Fuzhou, China). All procedures were approved by the Animal Care and Use Committee of Fujian Medical University. CH-induced PH was produced by established method [34, 35]. Male Sprague-Dawley rats were placed in a hypoxic chamber and exposed to either normoxia or normobaric hypoxia 10% O_2 for 3 weeks. MCT-induced PH was generated by a single intraperitoneal injection of MCT (50

mg/kg) [36]. Twenty-one days after MCT injection or CH exposure, rats were anesthetized with urethane (1 g/kg). Right ventricle systolic pressure (RVSP) was measured by accessing the right ventricle through the jugular vein, using a polyethylene catheter connected to a pressure transducer (YPJ01; Chengyi, China). Pressure signals were displayed continuously on an RM6240 polygraph (Chengyi, China). At the end of hemodynamic measurement, the rat was sacrificed with an overdose of urethane. The heart was removed, and right ventricular mass index (RVMI) was calculated as the ratio of wet weight of the right ventricle to the left ventricular wall plus septum $[RV/(LV+S)]$.

Isometric contraction of pulmonary artery ring

Rats were injected with heparin and anesthetized with urethane (1 g/kg). They were exsanguinated, and the lungs were removed and transferred to a petri dish filled with cold (4°C) oxygenated modified Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.18 KH_2PO_4 , 25 $NaHCO_3$, 10 glucose, and 2 $CaCl_2$. Under a dissecting microscope, the third- and fourth-generation pulmonary arteries (PAs) (~300 to 800 μm) were isolated and cleaned free of connective tissue. Then they were cut into 4-mm-length rings. The endothelium was removed by gently rubbing the lumen with a small wooden stick, and the arterial rings were suspended between two stainless steel stirrups in organ chambers filled with modified Krebs solution for isometric tension recording. The solution was gassed with 95% O_2 /5% CO_2 to maintain a pH of 7.4 and a temperature at 37°C. Isometric contraction was measured using a force transducer (JZJ01H; Chengyi, China) connected to an RM6240 polygraph. Resting tension was adjusted to 0.8–1.0 g. Arteries were exposed to 60 mM KCl to establish maximum contraction and to phenylephrine (3×10^{-7} M) followed by acetylcholine (10^{-6} M) to verify complete disruption of endothelium. Because Gd^{3+} is known to be insoluble in phosphate and bicarbonate-containing buffer [15], a bath solution containing (in mM) 118.3 NaCl, 4.7 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 11.1 glucose, and pH adjusted to 7.4 with NaOH, was used in the Gd^{3+} experiment.

Culture of pulmonary arterial smooth muscle cells

Pulmonary arterial smooth muscle cells (PASMCs) were enzymatically isolated and transiently cultured as previously described [34, 36]. Briefly, lungs were removed and transferred to a petri dish filled with cold HEPES-buffered salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). The third- and fourth-generation PAs (~300 to 800 μm) were isolated and cleaned free of connective tissue. The endothelium was removed by gently rubbing the luminal surface with a cotton swab. Arteries were then allowed to recover for 30 min in cold HBSS, followed by 20 min in reduced- Ca^{2+} (20 μM) HBSS at room temperature. The tissue was digested at 37°C for 20 min in 20 μM Ca^{2+} HBSS containing collagenase (type I, 1750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM), then removed and washed with Ca^{2+} -free HBSS to stop digestion. PASMCs were dispersed gently by trituration with a small-bore pipette in Ca^{2+} -free HBSS at room temperature. The cell suspension was placed on 25 mm glass cover slips and transiently (18–24 h) cultured in Ham's F-12 medium (with L-glutamine) supplemented with 0.5% fetal calf serum, 100 U/ml of streptomycin, and 0.1 mg/ml of penicillin.

Measurement of $[Ca^{2+}]_i$ and Mn^{2+} quenching of Fura-2

$[Ca^{2+}]_i$ were monitored using the membrane-permeable Ca^{2+} -sensitive fluorescent dye fluo-3 AM as previously described [34, 36]. PASMCs were loaded with 5–10 μM fluo-3 AM for 30–45 min at room temperature (~22 °C) in normal Tyrode solution containing (in mM) 137 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Cells were then washed thoroughly with Tyrode solution to remove extracellular fluo-3 AM and rested for 15–30 min to allow for complete deesterification of cytosolic dye. fluo-3 AM was excited at 488 nm, and emission light at >515 nm was detected using a Nikon TE2000U microscope equipped with epifluorescence attachments and a microfluorimeter (PTI, USA). Protocols were executed, and data were collected online with an analog-to-digital interface (FeliX32; PTI). $[Ca^{2+}]_i$ was calibrated using the equation $[Ca^{2+}]_i = K_d(F - F_{bg}) / (F_{max} - F)$, where F_{bg} is background fluorescence and F_{max} is the maximum fluorescence determined in situ in cells superfused with 10 μM 4-bromo-A-23187 and 10 mM Ca^{2+} , and K_d of fluo-3 AM is 1.1 μM . Cation entry through SOCC was quantified by quenching of fura-2 with Mn^{2+} . PASMCs were loaded with fura-2 AM as described above. Fura-2 was excited at the Ca^{2+} -insensitive isobestic point of 360 nm, and emission light was recorded at 510 nm (PTI, USA). PASMCs were then bathed in a Ca^{2+} free (with 0.1 mM EGTA) Tyrode solution containing 3 μM nifedipine. After a stable

baseline fluorescence was attained, 500 μM Mn^{2+} was applied through a concentration-clamp system with the multi-barrel pipette positioned $<50\text{ }\mu\text{m}$ from PSMCs. The rates of quenching of fura-2 fluorescence and $[\text{Ca}^{2+}]_i$ in PSMCs with or without drugs treatments were determined and compared.

Solutions and Drugs

Ginsenoside Rb1 (purity $\geq 98\%$) was purchased from Chengdu Gelei Xiya Chemical Technology Co. Ltd. (Chengdu, China). Fluo-3 AM and fura-2 AM were purchased from Life Technologies (U.S.A.). All the other drugs were purchased from Sigma Chemicals Co. (U.S.A.). Ginsenoside Rb1, nifedipine and CPA were dissolved in dimethyl sulfoxide (DMSO). Fluo-3 AM and fura-2 AM were dissolved in DMSO with 20% pluronic acid. The final amount of DMSO in the bath solution was less than 0.1%. MCT was dissolved in 1 M HCl, neutralized to pH 7.4 with 1 M NaOH, and diluted with saline. All the other reagents were dissolved in distilled water. All stock solutions were stored at $-20\text{ }^{\circ}\text{C}$.

Statistical Analysis

Data are expressed as mean $\pm$ S.E.M., and n indicates the number of animals, PA rings, or cell samples used. Curve fitting was performed using the SigmaPlot 11.0 software. Statistical significance was assessed using unpaired or paired Student's t-tests and ANOVA wherever appropriate. Differences were considered significant at $P < 0.05$.

Results

Vasorelaxing effects of ginsenoside Rb1 in PAs

The vascular effect of ginsenoside Rb1 was examined in endothelium denuded PA rings. Ginsenoside Rb1 at concentration of 0.1 to 300 μM had negligible effect on the resting tension of PA. In PA precontracted with 60 mM KCl, ginsenoside Rb1 caused slight concentration-dependent relaxation (Fig. 1A and D) with the maximal relaxation (E_{max}) of $-19.7 \pm 1.4\%$ and an IC_{50} of $5.4 \pm 0.2\text{ }\mu\text{M}$, suggesting that it has a very small inhibitory effect on the voltage-gated Ca^{2+} channels. The KCl-induced contraction was very stable in the absence of ginsenoside Rb1 throughout the course of experiment ($n=9$) (Fig. 1E). Ginsenoside Rb1 caused dramatic relaxation in PA precontracted with 10 nM endothelin-1 (ET-1) in the absence or presence of nifedipine (3 μM) (Fig. 1B and C). The E_{max} were -48.3 ± 1.8 and $-44.2 \pm 1.2\%$; and the IC_{50} s were 4.3 ± 0.2 and $3.1 \pm 0.2\text{ }\mu\text{M}$ in PA with and without nifedipine treatment, respectively. There was no significant difference between the nifedipine-treated and untreated PAs. The ET-1 induced contraction was relatively stable in the absence of ginsenoside Rb1, with only about 10% reduction throughout the course of experiment ($n=9$) (Fig. 1F). These results suggest that ginsenoside Rb1 relaxes ET-1 induced PA contraction mainly through a mechanism independent of voltage-gated Ca^{2+} entry.

Effect of ginsenoside Rb1 on ET-1-induced contraction in PAs of PH rats

Previous study showed that ET-1 induced PA constriction is enhanced in PH [26, 36, 37]. We compared the vasorelaxing effect of ginsenoside Rb1 on ET-1 induced contraction of PAs isolated from control, CH- and MCT-induced PH rats. In the presence of 3 μM nifedipine, ET-1 (10 nM) elicited significantly greater contraction in PAs of CH- and MCT-treated rats compared to control rats (control: $105.6 \pm 3.3\%$ 60 mM KCl-induced contraction, $n=12$; CH: $126.7 \pm 4.2\%$, $n=12$, $p < 0.05$; MCT: $129.2 \pm 5.3\%$, $n=13$, $p < 0.05$) (Fig. 2). Preincubation with 10 μM ginsenoside Rb1 for 8 minutes significantly reduced the ET-1 induced response in PAs of all three groups (control: $84.2 \pm 2.9\%$, $n=12$, $p < 0.01$; CH: $58.4 \pm 2.6\%$, $n=12$, $p < 0.01$; and MCT: $66.6 \pm 3.9\%$, $n=13$, $p < 0.01$) (Fig. 2D). The percent inhibition of ET-1 induced contraction by ginsenoside Rb1 was significantly higher in PAs of CH- and MCT-treated rats (control: $-20.3 \pm 2.7\%$, $n=12$; CH: $-53.9 \pm 2.1\%$, $n=12$, $p < 0.01$; and MCT: $-48.5 \pm 3.0\%$, $n=13$, $p < 0.01$) (Fig. 2E).

We have previously shown that ET-1 induced pulmonary vasoconstriction is in part mediated through store-operated Ca^{2+} entry (SOCE), which is upregulated in PAs of CH and

Fig. 1. Vascular effect of ginsenoside Rb1 on pulmonary artery (PA) rings in control rats. (A-C) Typical traces of ginsenoside Rb1-induced concentration-dependent relaxation in PA rings precontracted with 60 mM KCl, 10 nM ET-1 and 10 nM ET-1 pretreated with 3 μ M nifedipine (Nif). (D) The average percent relaxation elicited by ginsenoside Rb1 in PAs precontracted with KCl (n=12), ET-1 (n=13) and Nif+ET-1 (n=14). Vasorelaxation was expressed as percentage change in tension in the contractile response to KCl or ET-1. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared to the KCl group. (E) The average time-course of KCl-induced contraction generated from 9 different PAs. (F) The average time-course of ET-1 induced contraction generated from 9 different PAs.

Fig. 2. Effect of ginsenoside Rb1 pretreatment on ET-1-induced contraction in PAs of control, CH-exposed and MCT-treated rats. (A-C) Representative traces of ET-1-induced contraction in PAs of control, CH-exposed and MCT-treated rats with or without pretreated with ginsenoside Rb1. (D) The average values of ET-1-induced contraction in PAs of control (n=12 and 12), CH-exposed (n=12 and 12) and MCT-treated (n=13 and 13) rats with or without pretreatment with ginsenoside Rb1. Contractile force was expressed as percentage of 60 mM KCl-induced contraction. (E) The average percent inhibition of ET-1-induced PA contraction caused by Rb1 in control, CH-exposed and MCT-treated rats. ** indicates $P < 0.01$ compared to no pretreatment with ginsenoside Rb1. # indicates $P < 0.05$, and ## indicates $P < 0.01$ compared to the control group.

MCT induced PH rats [34, 36]. To examine the possible involvement of SOCE, ginsenoside Rb1 dependent inhibition of ET-1 induced response was examined in the presence or absence of the SOCE inhibitor gadolinium (Gd^{3+} , 0.4 μ M)(Fig. 3). Ginsenoside Rb1 (10 μ M)

Fig. 3. Relaxation effect of ginsenoside Rb1 and Gd^{3+} on ET-1-precontracted PA of control, CH-exposed and MCT-treated rats. Typical traces of ginsenoside Rb1-induced vasorelaxation in ET-1 (10 nM) precontracted PAs of (A, B) control, (C, D) CH-exposed and (E, F) MCT-treated rats in the absence or presence of 0.4 μ M Gd^{3+} . (G) The average values of ET-1 induced contraction before and after the addition of ginsenoside Rb1 (10 μ M) in PAs of control (n=12), CH-exposed (n=12) and MCT-treated (n=10) rats. Contractile force was expressed as percentage of 60 mM KCl-induced contraction. (H) The average values of ET-1 induced contraction with or without Gd^{3+} and Gd^{3+} +Rb1 in PAs of control (n=12), CH-exposed (n=15) and MCT-treated (n=12) rats. (I) Percent relaxation of ET-1-induced contractions caused by Rb1, Gd^{3+} and Gd^{3+} +Rb1 in PAs of control, CH-exposed and MCT-treated rats. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared to the control. ## indicates $P < 0.01$ compared to the ET-1 group without treatment.

when applied to ET-1 precontracted PAs caused relaxation from $100.5 \pm 4.6\%$ to $69.3 \pm 3.9\%$ (n=12, $p < 0.01$) in control PAs (Fig. 3A). Application of Gd^{3+} (0.4 μ M) also caused dramatic relaxation of ET-1 contracted PAs from $106.6 \pm 4.8\%$ to $54.9 \pm 6.0\%$ (n=12, $p < 0.01$), but further application of ginsenoside Rb1 failed to cause addition relaxation ($42.7 \pm 5.6\%$) (Fig. 3B). Similarly, application of Gd^{3+} relaxed ET-1 precontraction from $123.1 \pm 5.9\%$ to $26.4 \pm 2.4\%$ (n=15, $p < 0.01$) in PAs of CH rats; and from $130.6 \pm 7.1\%$ to $34.5 \pm 4.7\%$ (n=12, $p < 0.01$) in MCT-treated rats (Fig. 3C-F). Application of ginsenoside Rb1 in the presence of Gd^{3+} did not cause further relaxation (CH: $19.4 \pm 2.8\%$; MCT: $28.9 \pm 4.4\%$). Moreover, ginsenoside Rb1, Gd^{3+} and Gd^{3+} +ginsenoside Rb1 caused significant greater relaxation in CH-exposed and MCT-treated group compared to the control (Fig. 3I). These results suggest that ginsenoside Rb1 causes relaxation of ET-1 precontracted PA through inhibition of SOCE, and the inhibition is enhanced in PAs of PH rats.

Fig. 4. Effect of ginsenoside Rb1 pretreatment on CPA-induced contraction in PAs of control, CH-exposed and MCT-treated rats. (A-C) Representative traces of CPA-induced contraction in PAs of control, CH-exposed and MCT-treated rats with or without pretreated with ginsenoside Rb1. 10 μ M CPA was applied to PAs in Ca^{2+} free solution for 10 min, and 2 mM Ca^{2+} was then reintroduced to allow store-operated Ca^{2+} entry. (D) The average values of CPA-induced contraction in PAs of control (n=11 and 11), CH-exposed (n=10 and 11) and MCT-treated (n=12 and 13) rats in the absence or presence of ginsenoside Rb1. Contractile force was expressed as percentage of 60 mM KCl-induced contraction. (E) The average percent inhibition of CPA-induced contractions caused by Rb1 in PAs of control, CH-exposed and MCT-treated rats. ** indicates $P < 0.01$ compared to PAs without Rb1 pretreatment. # indicates $P < 0.05$, and ## indicates $P < 0.01$ compared to the control group.

Effect of ginsenoside Rb1 on CPA-induced PA rings contraction in control, CH-exposed and MCT-treated Rats

The effect of ginsenoside Rb1 on SOCE mediated PA contraction was further examined by depleting sarcoplasmic reticulum (SR) Ca^{2+} stores with cyclopiazonic acid (CPA). PA rings were exposed to Ca^{2+} -free nifedipine (3 μ M) containing Tyrode solution followed by pretreatment with 10 μ M CPA for 10 min. SOCE mediated contraction was initiated by readmission of Ca^{2+} to the external solution. Pretreatment with 10 μ M ginsenoside Rb1 significantly inhibited the contraction of PA rings induced by CPA (Fig. 4A and D) (control: $50.0 \pm 2.6\%$ of 60 mM KCl response, $n=11$; ginsenoside Rb1 treated: $32.1 \pm 2.1\%$, $n=11$, $p < 0.01$), indicating that ginsenoside Rb1 indeed inhibits SOCE.

CPA-induced contraction was examined in PAs of CH- and MCT-induced PH rats. The maximum contraction was $129.5 \pm 6.5\%$ of KCl-induced response ($n=10$, $p < 0.01$) in the CH group and $111.2 \pm 5.0\%$ ($n=12$, $p < 0.01$) in the MCT-treated groups. Ginsenoside Rb1 pretreatment caused significant inhibition of the CPA-induced contraction in PAs of CH- ($63.0 \pm 3.5\%$, $n=11$, $p < 0.01$) and MCT-treated ($77.0 \pm 3.8\%$, $n=13$, $p < 0.01$) groups (Fig. 4B, C, and D). The % inhibition of CPA-induced contraction by ginsenoside Rb1 was significantly greater ($P < 0.05$) in PAs of CH rats compared to the control PAs (Fig. 4E).

Application of ginsenoside (10 μ M) directly to PAs precontracted with CPA (Fig. 5) caused time-dependent relaxation from $47.2 \pm 3.0\%$ to $34.4 \pm 2.8\%$ 60 mM KCl induced contraction ($n=12$, $p < 0.01$) in control PA, from $103.7 \pm 8.0\%$ to $56.6 \pm 5.2\%$ ($n=8$, $p < 0.01$) in PAs of CH rats, and from $105.7 \pm 8.6\%$ to $67.0 \pm 7.0\%$ ($n=12$, $p < 0.01$) in PAs of MCT-treated rats (Fig. 5D). Relaxation induced by ginsenoside Rb1 was significant greater in PAs of CH-exposed and MCT-treated rats than those of control rats (control: $-27.8 \pm 2.0\%$, $n=12$; CH: $-45.5 \pm 2.3\%$,

Fig. 5. Effect of ginsenoside Rb1 on CPA-precontracted PAs of control, CH-exposed and MCT-treated rats. (A-C) Typical traces of ginsenoside Rb1-induced vasorelaxation of CPA (10 μ M)-induced contraction in PAs of control, CH-exposed and MCT-treated rats. (D) The average values of CPA-induced contraction before and after application of ginsenoside Rb1 in PAs of control (n=12), CH-exposed (n=8) and MCT-treated (n=12) rats. Contractile force was expressed as percentage of 60 mM KCl-induced contraction. (E) The average percent relaxation of CPA-induced contractions caused by ginsenoside Rb1 in PAs of control (n=12), CH-exposed (n=8) and MCT-treated (n=12) rats. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared to the control group. ## indicates $P < 0.01$ compared to CPA-induced contraction before application of Rb1.

n=8, $p < 0.01$; MCT: $-37.4 \pm 2.8\%$, n=12, $p < 0.05$) (Fig. 5E). These results are consistent with the notion that ginsenoside Rb1 is an inhibitor of SOCE, and its influence on SOCE in PAs is enhanced during PH.

Effect of ginsenoside Rb1 on CPA-induced cation entry and Ca^{2+} Transients in PSMCs of Control, and PH rats

To examine directly the effect of ginsenoside Rb1 on SOCE, CPA-induced cation entry was quantified using the Mn^{2+} quenching technique in PSMCs (Fig. 6). In control PSMC pretreated with 10 μ M CPA for 10 mins, application of 500 μ M Mn^{2+} caused significant reduction in fura-2 fluorescence. Pre-incubation of PSMCs with ginsenoside Rb1 for 10 min significantly reduced the maximal rate of Mn^{2+} quenching (control: $-1.24 \pm 0.14\%/s$, n=10; Ginsenoside Rb1: $-0.54 \pm 0.10\%/s$, n=10, $p < 0.01$). The rate of Mn^{2+} quenching was significantly enhanced in PSMCs of CH rats ($-2.40 \pm 0.34\%/s$, n=13, $p < 0.05$) and MCT-treated rats ($-2.47 \pm 0.33\%/s$, n=15, $p < 0.05$) (Fig. 6A-D). Pretreatment with ginsenoside Rb1 abolished the enhanced rate of Mn^{2+} entry in PSMCs of CH ($-0.77 \pm 0.06\%/s$, n=14, $p < 0.01$) and MCT-treated cells ($-0.57 \pm 0.10\%/s$, n=9, $p < 0.01$) (Fig. 6A-D).

The effect of ginsenoside Rb1 on SOCE was further examined by measuring CPA-induced Ca^{2+} transients (Fig. 7). Ca^{2+} transients was elicited in PSMCs treated with 10 μ M CPA for 10 min in Ca^{2+} free Tyrode solution (containing 0.1 mM EGTA and 3 μ M nifedipine) followed by readmission of Ca^{2+} to external solution. The resting $[Ca^{2+}]_i$ and the amplitude of the Ca^{2+} influx transient was significantly higher in PSMCs of CH and MCT-treated groups compared to the control (control: 133.0 ± 12.8 nM, n=14; CH: 356.2 ± 37.4 nM, n=12, $p < 0.01$; MCT: 291.2 ± 36.2 nM, n=12, $p < 0.01$) (Fig. 7D and F), whereas the CPA-induced Ca^{2+} release was similar in the three groups (Fig. 7E). Pretreatment with ginsenoside Rb1 (10 μ M for 10 min) reduced the elevated resting $[Ca^{2+}]_i$ in PSMCs of CH animals, but had no effect on the CPA-induced Ca^{2+} release. By contrast, ginsenoside Rb1 significantly reduced the CPA-induced Ca^{2+} influx transients in all three groups of PSMCs to a comparable levels (control: 80.9 ± 9.2 nM, n=9, $p < 0.01$; CH: 96.2 ± 15.5 nM, n=14, $p < 0.01$; MCT: 120.6 ± 13.8 nM, n=12, $p < 0.01$)

Fig. 6. Effect of ginsenoside Rb1 on CPA-induced cation entry measured by Mn^{2+} quenching in PSMCs of control, CH-exposed and MCT-treated rats. (A-C) The averaged time-course of Mn^{2+} quenching in PSMCs of control (n=10 and 10), CH-exposed (n=13 and 14) and MCT-treated (n=15 and 9) rats with or without pretreatment with ginsenoside Rb1. (D) The maximum rate of quenching in PSMCs of control, CH-exposed and MCT-treated rats with or without ginsenoside Rb1 pretreatment. ** indicates $P < 0.01$ comparing to the group without ginsenoside Rb1 treatment. # indicates $P < 0.05$ compared to control group.

Fig. 7. Effect of ginsenoside Rb1 on CPA-induced Ca^{2+} transients in PSMCs of control, CH-exposed and MCT-treated rats. (A-C) Representative traces of CPA-induced Ca^{2+} transients in PSMCs of control, CH-exposed and MCT-treated rats with or without pretreatment with ginsenoside Rb1. (D-F) The average resting $[Ca^{2+}]_i$, peak changes in Ca^{2+} transients elicited by CPA-induced Ca^{2+} release and Ca^{2+} entry in PSMCs of control (n=14 and 9), CH-exposed (n=12 and 14) and MCT-treated (n=12 and 12) rats in the presence or absence of ginsenoside Rb1. ** indicates $P < 0.01$ compared to PSMCs without ginsenoside Rb1 pretreatment. ## indicates $P < 0.01$ compared to control group.

(Fig. 7F). These results clearly indicate that ginsenoside Rb1 inhibits SOCE in PSMCs, and abolishes the enhanced SOCE in PSMCs of CH and MCT-treated rats.

Discussion

The present study examined the effects of ginsenoside Rb1 on the agonist-induced contraction of endothelium denuded PA rings, and the CPA-induced Ca^{2+} transients and cation entry in PSMCs of normal rats and two different PH models. We found that ginsenoside Rb1 caused dramatic relaxation of ET-1-induced PA contraction. The ginsenoside Rb1-induced relaxation was not related to the inhibition of voltage-gated Ca^{2+} channel, but was mainly due to the inhibition of SOCE. This is based on several lines of evidence. First, ginsenoside

Rb1 had a small effect on KCl-induced PA contraction, and its vasorelaxing effect on ET-1 induced contraction persisted in the presence of nifedipine. Second, the vasorelaxing effect of ginsenoside Rb1 on ET-1 mediated contraction was blocked by the SOCE inhibitor Gd^{3+} . Third, ginsenoside Rb1 suppressed CPA-induced contraction of PA, and attenuated CPA-activated non-selective cation entry and Ca^{2+} transient in PSMCs. Furthermore, CH and MCT-induced PH enhanced the ET-1 and CPA-activated PA contraction, and CPA-activated cation entry and Ca^{2+} transients in PSMC. The enhancements of these responses were suppressed or abolished by ginsenoside Rb1. These results demonstrate for the first time that ginsenoside Rb1 is a functional inhibitor of SOCE, and it antagonizes the enhanced pulmonary vascular reactivity in PH.

There is increasing evidence suggesting that ginsenoside Rb1 has therapeutic potential for the treatment of cardiovascular diseases [38]. Ginsenoside Rb1 has been shown to attenuate cellular hypertrophy, apoptosis and oxidative injury of cardiomyocytes [16-20]; and protect against ischemic-reperfusion injury [21, 22], dilated cardiomyopathy [23] and right heart hypertrophy [24]. It exerts these cardioprotective effects through different mechanisms, for example by reducing $[Ca^{2+}]_i$, enhancing the expression of eNOS and increasing nitric oxide production, attenuating reactive oxygen species and inhibiting the Ca^{2+} -calcineurin signal transduction pathway in cardiac myocytes [17, 20, 21, 24, 39]. Ginsenoside Rb1 is also known to suppress intimal hyperplasia of carotid arteries after injury [11-13], promote endothelial nitric oxide production and protect against homocysteine-induced endothelial dysfunction via PI3Kinase/Akt pathways [10, 14], inhibit tumor necrosis factor- α evoked inflammatory responses and proliferation of vascular smooth muscle cells [15], and reverse antiretroviral therapy drug-induced pulmonary vasomotor dysfunction [40]. However, there is little information available on the vasoactive effects of ginsenoside Rb1, except a previous report showing ginsenoside Rb1 stimulates endothelial-dependent relaxation of mouse coronary arteries through e-NOS activation and NO production [41]. The present study, hence, revealed a novel direct relaxant effect of ginsenoside Rb1 on vascular smooth muscle.

Previous studies had shown that ginsenoside Rb1 causes partial inhibition of several types of voltage-gated Ca^{2+} channel in chromaffin cells [42], neurons [43, 44] and other expression systems [45]. But the inhibitory effect on voltage-gated Ca^{2+} channels is small, ranging from 10-20% at the maximal concentrations of 10-100 μM [42-44]. This is comparable to the small vasorelaxing effect of ginsenoside Rb1 on the KCl-induced PA contraction. Since ET-1 induced PA contraction is only partially mediated by the L-type Ca^{2+} channels [37], the small inhibition on the voltage-gated Ca^{2+} channel could not account for the robust vasorelaxing effect of ginsenoside Rb1. Indeed, ginsenoside Rb1 caused similar relaxation in PA precontracted with ET-1 in the absence or presence of nifedipine, suggesting the vasorelaxing effect is unrelated to the inhibition of voltage-gated Ca^{2+} channel.

Our present results clearly suggest that ginsenoside Rb1 exerts its vasorelaxing effect on rat PAs through inhibition of SOCE. ET-1-induced contraction of PA is mediated in part through SOCE [36], which can be effectively suppressed by the SOCE blockers Gd^{3+} , La^{3+} , SKF-96365 and BTP-2. The complete abolition of the vasorelaxant effect of ginsenoside Rb1 in the presence of a low concentration of Gd^{3+} is congruent with SOCE inhibition. This is substantiated by three different methods of measuring CPA-induced contraction of PA, CPA-induced Ca^{2+} influx transients and Mn^{2+} quenching of Fura-2 fluorescence in PSMCs, which all showed consistent attenuation of SOCE by ginsenoside Rb1. SOCE has been well characterized in many different cell types including PSMCs. It is initiated by Ca^{2+} release from SR, leading to SR Ca^{2+} depletion, oligomerization and translocation of stromal interaction molecule (STIM) to the SR-plasma membrane junctions to couple with the store-operated Ca^{2+} channels (SOCCs) [46-48]. Previous studies from several laboratories including ours have identified the canonical transient receptor potential 1 (TRPC1) and Orai1 as the major components of SOCC in PSMCs [34, 49-53]; and other TRPC subtypes and non-selective cation channels may be also involved [54-56]. Inhibition of SOCE by ginsenoside Rb1 is not related to the suppression of SR Ca^{2+} release, because the CPA-induced Ca^{2+} release was unaltered in the presence of ginsenoside Rb1. It may inhibit SOCE by suppressing STIM activation and/or by

inhibiting the TRPC1/Orai channels of PSMCs. But, whether ginsenoside Rb1 acts directly through interactions with STIM1, TRPC1 and Orai1, or indirectly through other signaling pathways is unclear. Recent evidence has indicated that SOCE can be regulated by reactive oxygen species (ROS) through redox-dependent modification of STIM and Orai proteins [57]. Since ginsenoside Rb1 is known to reduce oxidative stress in different cells and tissues [17, 27, 58, 59], it is possible that it modulates SOCE through a ROS-related mechanism.

It is important to note that the efficacy of ginsenoside Rb1 for the inhibition of ET-1-induced response and SOCE was increased in PAs of PH rats. Ginsenoside Rb1 completely reversed the enhanced ET-1 induced contraction in PAs, and normalized the enhanced CPA-induced Ca^{2+} and cation entry in PSMCs of CH and MCT-treated rats. This is similar to our previous finding that ET-1-induced contraction in PA and Ca^{2+} response in PSMC of MCT-treated rats are more susceptible to the inhibition by SOCE blockers [36], and is consistent with the notion that the enhanced ET-1 induced response is mediated by upregulation of SOCE in PSMCs during PH. The finding of ginsenoside Rb1 as an effective inhibitor of ET-1 and SOCE raises the intriguing possibility that the ginsenoside may be considered as a potential therapeutic agent for the treatment of PH. ET-1 has been implicated as an important factor in the development of PH. Its circulating level is increased, its receptor density in PA is upregulated and vasoconstrictory effect is enhanced during PH; and antagonists of ET-receptors can effectively prevent and reverse PH [60-66]. Enhanced SOCE has been reported in many different models of experimental PH, including CHPH [34, 67], monocrotaline-induced PH [36] and cigarette smoke-induced PH [68], and in patients of idiopathic pulmonary arterial hypertension (IPAH)[69-71]. It contributes to the elevated $[\text{Ca}^{2+}]_i$ of PSMCs, increased pulmonary vascular tone, enhanced vasoreactivity and vascular remodeling, and plays a crucial pathogenic role in PH [28, 34, 54, 72]. Our observation that ginsenoside Rb1 reduces the elevated resting $[\text{Ca}^{2+}]_i$ in PSMCs of CH rat is consistent with inhibition of the enhanced SOCE in resting CH PSMCs. Our results also indicate that ginsenoside Rb1 inhibits the enhanced pulmonary vasoreactivity in two mechanistically different forms of PH. Hence, ginsenoside Rb1 by inhibiting ET-1 induced responses and SOCE may be effective for alleviating PH. Indeed, this is supported by the previous study showing ginsenoside Rb1 protected and reversed the MCT-induced right heart hypertrophy [24]. Future experiments will be needed for examining the effects of ginsenoside Rb1 on pulmonary vascular remodeling. This will further establish the therapeutic potentials of the ginsenoside in PH, and may provide an alternative treatment for the dreadful disease.

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