INTRODUCTION

Cardiovascular diseases are the leading cause of death in developed and developing countries (1), causing a great impact not only on human health, but also in social and economic areas. In recent decades, in an attempt to reduce this impact, researchers worldwide have been working extensively to improve the treatment of cardiovascular diseases including the discovery of new therapy strategies and drugs (2, 3). Despite enormous advances in the research, development, and use of natural products as therapeutic agents, detailed understanding on their action mechanisms is largely lacking (4, 5). On the other hand, evaluation of their pharmacological effects and clarification of the pathways of action for the natural compounds could be used as a logical research strategy for searching new drugs (6, 7).

Rubus species have been cultivated for centuries and used in many countries as natural remedies to treat several diseases, such as diabetes, many types of infection, colic, and burns (8). The dried, unripe fruit of Rubus chingii Hu (Rosaceae) has long been used as a food and a tonic in traditional Chinese medicine (9). Rubus chingii has been used to improve function of the kidney and treat excessive polyuria. However, the effects of Rubus chingii on the cardiovascular system and its pharmacological mechanisms of action have not been studied. The aim of the present study was to evaluate the cardiovascular effects of ethanol extract of Rubus chingii (ERC) in rats. The changes in systolic blood pressure and heart rate of rats and vascular tone of aortic rings in in vitro were measured using pressure transducer and force transducer, respectively, connected to a multichannel recording system. ERC decreased systolic blood pressure and heart rate in a concentration-dependent manner. ERC induced vasorelaxation in a concentration-dependent manner. The ERC-induced vasorelaxation was not observed in the absence of the endothelium. The vasorelaxant effect of ERC was significantly attenuated by inhibition of endothelial NO synthase (eNOS), soluble guanylyl cyclase (sGC), or Ca2+ entry from extracellular sources with L-NAME, ODQ, diltiazem, or extracellular Ca2+ depletion, respectively. Similarly, an inhibition of Akt with wortmannin attenuated the ERC-induced vasorelaxation. Modulators of the store-operated Ca2+ entry, thapsigargin, Gd3+, and 2-aminoethyl diphenylborinate markedly attenuated the ERC-induced vasorelaxation. Furthermore, 4-aminopyridine an inhibitor of voltage-dependent K+ (Kv) channel, had no significant effect on the ERC-induced vasorelaxation. However, tetraethylammonium and glibenclamide, had no significant effect on the ERC-induced vasorelaxation. Indomethacin, atropine, and propranolol had no effects on the ERC-induced vasorelaxation. The present study demonstrates that ERC induces vasorelaxation via endothelium-dependent two-step signaling: an activation of the Ca2+-eNOS-NO signaling in the endothelial cells and then subsequent stimulation of the NO-sGC-cGMP-Kv channel signaling in the vascular smooth muscle cells. The Akt-eNOS pathway is also suggested to be involved in this relaxation. Also, the findings suggest that the ERC-induced vasorelaxation is closely related to the hypotensive action of the agent.
Plant material and preparation of Rubus chingii extract

The dried fruits of Rubus chingii were purchased from Shanxi Sciphar Hi-Tech Industry (Xian, Shanxi, China). Professor Tongde Li, Department of Traditional Chinese Medicine, Taishan Medical University, Taian, Shandong, China, identified the plant. Herbarium voucher specimens of Rubus chingii (CH 65) were prepared and deposited in the herbarium of the Institute of Materia Medica, Taishan Medical University, Taian, Shandong, China. The powdered Rubus chingii (200 g) was soaked in 70% aqueous-ethanol for a week in amber colored bottle with occasional shaking. The soaked material was passed through a double layered muslin cloth to remove vegetative debris. Then, the fluid portion was filtered through Whatman No. 3 filter paper and the combined filtrate was concentrated using rotary evaporator (EYELA N-1000, Japan) at 40°C, which afforded 31.2 g of ethanol extract (ERC).

Animals

Male Sprague-Dawley rats (weighing 250–300 g) were used in all experiments and were housed at 23°C under a 12-hour light/12-hour dark cycle with free access to food and water. The protocols and procedures were approved by the Animal Care and Use Committee of the Institute of Atherosclerosis, Taishan Medical University, China and were in accordance with the guidelines on the care and use of animals required by the NIH (NIH publication No. 86-23, revised 1985).
atropine, propranolol, wortmannin, Gd 3+, 2-APB, thapsigargin, muscle, modulators, L-NAME, ODQ, indomethacin, diltiazem, define the mechanism by which ERC relaxes vascular smooth responses were recorded. In the next series of experiments, to ERC (0.1 to 100 µg/ml) added to the tissue bath and the aortic rings were exposed to the cumulative concentrations of vascular tension were tested. In the presence of PE (1 µM), contracted vessels.

Effects of Rubus chingii extract on blood pressure and heart rate in rats

Systolic blood pressure (SBP) and heart rate (HR) were recorded before and after intravenous administration of ERC in rats. After 30 s, the ERC at doses of 0.5 mg/kg induced a significant fall in SBP (from 112.8±2.4 to 81.2±4.9 mmHg) and HR (from 398±2.5 to 354±5.4 beats/min), and then both SBP and HR progressively returned back to the basal value in about 2–3 min (Figs. 1A and 1B). ERC decreased SBP and HR in a concentration-dependent manner (Figs. 1C and 1D).

Effects of Rubus chingii extract on vascular tone of aortic rings

To identify the effect of ERC on vascular tension, aortic rings were exposed to cumulative doses of ERC. PE-contracted aortic rings relaxed in response to ERC in a concentration-dependent manner (Fig. 2A). To determine the role of the endothelium in the vasorelaxation of ERC, experiments were conducted in the denuded aortic rings. In contrast to the effect in the presence of the endothelium, ERC had no significant effect in the absence of the endothelium (Fig. 2A). This finding indicates that ERC induces vasorelaxation via activation of the endothelium-dependent signaling pathway.

Effects of L-NAME and ODQ on Rubus chingii extract-induced vasorelaxation

To identify the mechanisms involved in the ERC-induced vasorelaxation, the role of the endothelial signaling pathways were dissected. Because the NO-cGMP signaling is important in the regulation of endothelium-dependent vasorelaxation, effects of an inhibition of endothelial NO synthase (eNOS) and soluble guanylyl cyclase (sGC) activity were examined. As shown in Fig. 2B, pretreatment of aortic rings with L-NAME, an inhibitor of eNOS, or ODQ, an inhibitor of sGC, significantly attenuated the ERC-induced vasorelaxation. These findings suggest that an activation of NO-sGC-cGMP signaling is involved in the ERC-induced vasorelaxation.

**RESULTS**

Vascular relaxation is expressed as percentage changes of PE contraction levels. Significant difference between groups was determined with one-way ANOVA and Student's t-test using Graph Pad Prism TM 3.0 software. Results were expressed as means ± S.E.M. Statistical significance was defined as P<0.05.

**Statistical analysis**

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**Fig. 2. Effects of ERC on vascular relaxation and its modulation by endothelium-denudation, L-NAME and ODQ in phenylephrine-precontracted aortic rings.** (A) Relaxing effects of ERC in the endothelium-intact (V, n=9) and endothelium-denuded (-Endo, n=6) aortic rings. (B) Effects of L-NAME (10 µM, n=6) and ODQ (10 µM, n=6) on the ERC-induced vasorelaxation in the endothelium-intact aortic rings. ERC control (V), data from (A). Each value shows mean ± S.E.M. *P<0.01 vs. vehicle group. Some of error bars are obscured by the size of symbol.
Fig. 3. Effects of Ca2⁺ depletion and inhibition of L-type Ca²⁺ channels on ERC-induced vasorelaxation in endothelium-intact aortic rings. (A) Effects of nominally Ca²⁺-free buffer on ERC-induced vasorelaxation. ERC control (V), data from Fig. 2A; Ca²⁺ depletion (nominally Ca²⁺-free)+ERC, n=5. (B) Effects of diltiazem (Dilt, 10 µM) on ERC-induced vasorelaxation. Vehicle (V), data from Fig. 2(A); Dilt+ERC, n=7. Each value shows mean ± S.E.M. *P<0.001 vs. vehicle. Some of error bars are obscured by symbol.

Fig. 4. Effects thapsigargin (TG, 1 µM), Gd³⁺ (10 µM) and 2-APB (75 µM) on ERC-induced vasorelaxation in phenylephrine-precontracted endothelium-intact aortic rings. Vehicle (V), data from Fig. 2A; TG+ERC, n=6; Gd³⁺+ERC, n=5; 2-APB+ERC, n=5. Each value shows mean ± S.E.M. *P<0.001 vs. vehicle.

Fig. 5. Effects of wortmannin (WT, 0.1 µM) on ERC-induced vasorelaxation in phenylephrine-precontracted endothelium-intact aortic rings. Vehicle (V), data from Fig. 2A; WT+ERC, n=7. Each value shows mean ± S.E.M. *P<0.01 vs. vehicle.

Fig. 6. Effects of tetraethylammonium (TEA, 1 mM), glibenclamide (Gli, 10 µM) and 4-aminopyridine (4-AP, 1 mM) on ERC-induced vasorelaxation in endothelium-intact aortic rings. Vehicle (V), data from Fig. 2A; TEA+ERC, n=6; Gli+ERC, n=6; 4-AP+ERC, n=6. Each value shows mean ± S.E.M. *P<0.001 vs. vehicle group.
Role of extracellular Ca\(^{2+}\) in Rubus chingii extract-induced vasorelaxation

To identify the role of Ca\(^{2+}\) entry in the ERC-induced vasorelaxation, effects of extracellular Ca\(^{2+}\) depletion and inhibition of L-type Ca\(^{2+}\) channels were tested. Nominally Ca\(^{2+}\)-free buffer significantly attenuated the ERC-induced vasorelaxation (Fig. 3A). Similarly, pretreatment of aortic rings with thapsigargin (1 µM), an inhibitor of L-type Ca\(^{2+}\) channel, attenuated the ERC-induced vasorelaxation (Fig. 3B). These findings indicate that Ca\(^{2+}\) entry from extracellular sources is involved in the ERC-induced vasorelaxation.

Role of intracellular Ca\(^{2+}\) in Rubus chingii extract-induced vasorelaxation

To further characterize the nature of the Ca\(^{2+}\) dependence of the ERC-induced vasorelaxation, another series of experiments were performed. To identify the role of intracellular Ca\(^{2+}\) release, effects of modulators of the intracellular Ca\(^{2+}\) homeostasis were tested. Pretreatment of aortic rings with wortmannin (0.1 µM), an inhibitor of Akt, significantly attenuated the ERC-induced vasorelaxation. These findings suggest that Ca\(^{2+}\) entry via inhibition of sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase, attenuated the ERC-induced vasorelaxation (Fig. 4). Similarly, Gd\(^{3+}\) (10 µM) and 2-APB (75 µM), which inhibits the Ca\(^{2+}\) entry through the store-operated Ca\(^{2+}\) entry (SOCE) from external sources, significantly attenuated the ERC-induced vasorelaxation. These findings suggest that ERC induces vasorelaxation via an increase in intracellular Ca\(^{2+}\) levels of the endothelium through an enhancement of intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) entry from external sources.

Role of Akt-eNOS signaling in Rubus chingii extract-induced vasorelaxation

Because ERC induces vasorelaxation via endothelium-dependent eNOS-NO-cGMP signaling pathway, the involvement of Akt signaling was tested. As shown in Fig. 5, pretreatment of aortic rings with wortmannin (0.1 µM), an inhibitor of Akt, significantly attenuated the ERC-induced vasorelaxation. Therefore, this finding suggests that ERC induces vasorelaxation via activation of Akt-eNOS-eNOS-cGMP signaling.

Role of K\(^{+}\) channels in Rubus chingii extract-induced vasorelaxation

To assess whether ERC-induced vasorelaxation was mediated by the activation of K\(^{+}\) channels, the effects of different K\(^{+}\) channel inhibitors were evaluated. As shown in Fig. 6, pretreatment of aortic rings with 4-AP (1 mM), an inhibitor of voltage-dependent K\(^{+}\) (K\(_{v}\)) channels, markedly inhibited the ERC-induced vasorelaxation, while both TEA (1 mM), a non-selective inhibitor of Ca\(^{2+}\)-activated K\(^{+}\) (K\(_{Ca}\)) channels, and glibenclamide (10 µM), an inhibitor of ATP-sensitive K\(^{+}\) (K\(_{ATP}\)) channels, had no significant effects on the relaxation. These findings indicate that activation of K\(_{v}\) channels is involved in the ERC-induced vasorelaxation.

Roles of cyclooxygenase and adrenergic and muscarinic receptors in Rubus chingii extract-induced vasorelaxation

To identify the role of the cyclooxygenase system in the ERC-induced vasorelaxation, the effect of an inhibition of cyclooxygenase with indomethacin was tested. Pretreatment of aortic rings with indomethacin showed no significant changes in the ERC-induced vasorelaxation, while both TEA (1 mM), a non-selective inhibitor of Ca\(^{2+}\)-activated K\(^{+}\) (K\(_{Ca}\)) channels, and glibenclamide (10 µM), an inhibitor of ATP-sensitive K\(^{+}\) (K\(_{ATP}\)) channels, had no significant effects on the relaxation. These findings indicate that the cyclooxygenase system and autonomic nervous system are not involved in the ERC-induced vasorelaxation.

DISCUSSION

The present study demonstrates that ERC produces a potent vasorelaxation of aortic ring, and hypotension and heart slowing in rats. ERC produced dose-dependent hypotension and bradycardia in normotensive rats, indicating that the ERC evokes cardiac effects. Therefore, the hypotensive effects observed after ERC infusion could be the result of the combined effects of

Fig. 7. Effects of inhibitors of cyclooxygenase and muscarinic and β-adrenergic receptors on ERC-induced vasorelaxation in endothelium-intact aortic rings. (A) Effects of indomethacin (Indo, 10 µM) on ERC-induced vasorelaxation. Vehicle (V), data from Fig. 2A; Indo+ERC, n=6. (B) Effects of atropine (Atro, 1 µM) and propranolol (Prop, 1 µM) on ERC-induced vasorelaxation. Vehicle (V), data from Fig. 2A; Atro+ERC, n=5; Prop+ERC, n=5. Each value shows mean ± S.E.M.
vasorelaxation and bradycardia. However, it is important to note that reduction in blood pressure due to vasorelaxation is usually followed by reflex tachycardia. However, under our experimental conditions, the bradycardia induced by ERC seems likely to be, at least in part, a direct effect of the ERC on the heart or on the regulatory system of blood pressure. Although we demonstrated that ERC can produce an acute and short-lasting hypotensive effect in normotensive rats, the beneficial effects of this *Rubus chingii* on hypertension and its clinical relevance still awaits further investigation.

The endothelium is involved in the regulation of vascular smooth muscle tone in conduit and resistance vessels by releasing endothelium-derived relaxing factors (EDRFs) (23), including NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) (24). In many instances, NO mediates its biological effects by activating soluble guanylyl cyclase and elevates intracellular cGMP synthesis from GTP (25). An increase in cytosolic levels of cGMP causes vasodilation by activation of cGMP-dependent protein kinases (26). In the present study, ERC relaxed pre-contracted endothelium-intact aortic rings in a concentration-dependent manner and this relaxation was abolished by endothelium denudation. This suggests that functional endothelium is involved in the action mechanism of ERC. The relaxation induced by ERC was inhibited by L-NAME, but not by indomethacin. This indicates that an activation of the NO system, but not prostaglandin pathway, is involved in the ERC-induced vasorelaxation. Also, the present study revealed that ODQ significantly attenuated the ERC-induced vasorelaxation. These results suggest that ERC-induced vasorelaxation involves endothelium-dependent eNOS-NO-sGC-cGMP signaling pathway. Although the eNOS-NO system in the vascular smooth muscle is also known to be involved in the vasorelaxation (27, 28), the present findings suggest that the ERC-induced vasorelaxation is mainly caused by endothelial NO-cGMP signaling.

It is known that many vasodilators relax vascular smooth muscle cells by activation of eNOS through an increase of intracellular Ca²⁺ concentration of the endothelial cells (29). It has been shown that Ca²⁺ plays an essential role in the NO synthesis and release in endothelial cells (29, 30). The cytosolic Ca²⁺ concentration is controlled by both extracellular Ca²⁺ influx and Ca²⁺ release from intracellular Ca²⁺ stores (31). The present findings show that both inhibitions of Ca²⁺ entry from the extracellular sources and intracellular Ca²⁺ release significantly attenuated the ERC-induced vasorelaxation. These findings indicate that ERC relaxes vascular smooth muscle cells via activation of extracellular Ca²⁺ entry and intracellular Ca²⁺ release. The vascular system is known to be equipped with variable Ca²⁺ entry pathways including L-type Ca²⁺ channels, store-operated Ca²⁺ entry (SOCE), and receptor-operated Ca²⁺ channels (ROCC) (31, 32). To further define the nature of the Ca²⁺ dependence of the ERC-induced vasorelaxation, effects of modulators of the intracellular Ca²⁺ homeostasis were tested. The modulators of the SOCE, thapsigargin, Gd³⁺, and 2-APB, significantly attenuated the ERC-induced vasorelaxation. These findings indicate that ERC induces vasorelaxation by an activation of SOCE in rat aorta. Depletion of Ca²⁺ stores with thapsigargin, an activator of SOCE, increases Ca²⁺ entry and intracellular Ca²⁺ levels in the endothelial cells (33, 34), which induces eNOS activation and NO production (32). Our results are in agreement with previous reports (22, 32) which show that the SOCE plays an important role in eNOS activation and vasodilation. In addition, eNOS is known to be activated via Akt-eNOS signaling (35). Phosphorylation of Akt at the endothelial membrane activates eNOS, leading to the production of NO (35, 36). In the present study, blockade of the Akt signaling with wortmannin significantly attenuated the ERC-induced vasorelaxation. This finding indicates that ERC induces vasorelaxation via activation of Akt-eNOS-cGMP signaling. Our results are consistent with the results of Fleming and Busse who reported that activation of eNOS is dependent on both Ca²⁺-dependent and Ca²⁺-independent pathways (37).

It is well known that membrane K⁺ channels are involved in the control of vascular tone, and are a potential target for anti-hypertensive drugs. Activation of K⁺ channels expected to hyperpolarize vascular smooth muscle cells and lead to
vasorelaxation (38, 39). To examine the role of different K⁺ channels in the ERC-induced vasorelaxation, we used different agents with K⁺ channel-inhibiting activity. Our results showed that ERC-induced vasorelaxation was significantly attenuated by 4-AP, but not by both TEA, and glibenclamide, suggesting that ERC preferentially activates Kᵥ channels in rat aorta. It is well known that membrane hyperpolarisation caused by an activation of K⁺ channels leading to the closure of L-type voltage-dependent Ca²⁺ channels is responsible for vessel relaxation. Both large conductance Ca²⁺ (BKᵥ) and Kᵥ channels could be activated under these conditions, thereby hyperpolarising the cell membrane (38). In the present study, an increase of intracellular Ca²⁺ by ERC at submembrane space through an increase of both release from stores and entry from external sources may accentuate Kᵥ channel activity and results in membrane hyperpolarization and lead to vasorelaxation.

Our study showed that propranolol and atropine had no effect on the ERC-induced vasorelaxation, indicating that the ERC-induced vasorelaxation was not related to activation of muscarinic and β-adrenergic receptors in vascular smooth muscle cells.

It has been shown that Rubus chingii contains chemical constituents of biological importance including triterpene acids, flavonoids, and phenolics (11-14, 16). The hypotensive and vasorelaxant effects of ERC attributed to a number of polyphenolic compounds such as flavonoids which are known for their cardiovascular effects such as hypolipidemic and hypoglycemic effects (20). Further work is necessary to isolate, identify, and characterize the active components of Rubus chingii and their actions on endothelial and smooth muscle cells.

In conclusion, our data derived from in vivo and in vitro approaches demonstrate that ERC induces hypotension associated with bradycardia and vascular relaxation. ERC induces vasorelaxation via endothelium-dependent signaling, which involves two-step signaling: first is an activation of the Ca²⁺-eNOS-NO signaling in the endothelial cells and then subsequent stimulation of the NO-sGC-cGMP-Kᵥ channel signaling in the vascular smooth muscle cells (Fig. 8). Our data also suggest that the Akt-eNOS pathway is involved in the ERC-induced vasorelaxation. The present study provides scientific evidence supporting the therapeutic uses of Rubus chingii in folk medicine.

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