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Introduction

Panax notoginseng (P.N.) is an herb that belongs to the Araliaceae family and is synonymous with Panax ginseng C.A. Meyer, Panax gracilistylus and Stephania sinica and pseudoginsen radix. It is one of the famous traditional medicinal herbs that have been used for hundreds of years in many East Asian countries [1]. P.N. consists of two major ingredients: crude ginseng saponin and crude ginseng non-saponin. To date, thirty different types of saponins [2] have been isolated from ginseng and identified chemically. They can be classified into three major groups according to their chemical structure: 20 (S)-protopanaxadiol, 20 (S)-protopanaxatriol and oleanolic acid saponins. Ginsenoside Rb1 (Rb1) and ginsenoside Rg1 (Rg1) are contained in Panax notoginseng (P.N.), P. ginseng (P.G.),

prior to application of a vasodilator agent [16]. Rb1 (10-9~10-5 g/mL) or Rg1 (10-9~10-5 g/mL) was used to examine the vasorelaxation of mouse coronary artery. To study the e ects of Rg1and Rb1 on NO signaling, pharmacological inhibitors including L-NAME (10-5M, Alexis,MA), the inhibitor of eNOS, and LY294002 (2*10-5M, Cayman Chemical, MI), the inhibitor

determined by real-time quantitative polymerase chain reaction (Q-PCR) analysis. Total RNA was isolated using an RNA miniprep kit according to the manufacturer's instructions (Stratagene, CA, USA). e speci c primer pair for CAT-1 resulting in a 328-bp PCR product was: forward primer: 5'-CTCTCCTACATCATCGGTAC-3', reverse primer: 5'-GGCATAGATAACTCGCG-3'. e primer sequences for GAPDH were: forward: 5'-CACGACCATGGAGAAGGCTG-3'and reverse: 5'-TCCACGATGCCGAAGTTGTC-3'. Ampli cation was followed by melting curve analysis to verify the accuracy of the amplicon. A negative control without cDNA was run with every PCR to assess the speci city of the reaction. Analysis of data was performed using Light Cycler so ware version 3.5.

Western blot analysis

e mouse hearts and PAECs were lysed in RIPA bu er (Boston Bioproducts, MA) with protease inhibitors, and proteins were resolved by SDS/PAGE gel and transferred to a PVDF membrane (Millipore, MA). A er being blocked for 1 h in TBS-T with 5% nonfat milk, the PVDF membrane was then probed with primary antibodies: polyclonal <</MCID 353 >>BDC T*--20(oo)-9(die5 <</h-A2(t)k6(h -9(die5S

and Rb1, were analyzed by HPLC (Supplementary gure 1). Mouse coronary arteries with intact endothelium were pre-contracted by U-46619 (a thromboxane mimic), and treated with a xed dosage of P.N. extract (10 7~10 4 g/mL), Rb1 (10 7~10 4 g/mL) or Rg1 (10 7~10 4 g/mL). Rb1 and Rg1 induced a signi cant vessel relaxation in a dose-dependent manner (Figure 1A). In order to further elicit the site of action of Rb1 and Rg1, either eNOS inhibitor (L-NAME) or PI3K inhibitor (LY294002) was injected for about 30 minutes before giving Rb1 and Rg1. It was observed that both L-NAME and LY294002 markedly diminished the vasodilator action of Rb1 (Figure 1B) as well as Rg1 (Figure 1C).

Rb1 and Rg1-induced vasodilatation is endothelial dependent

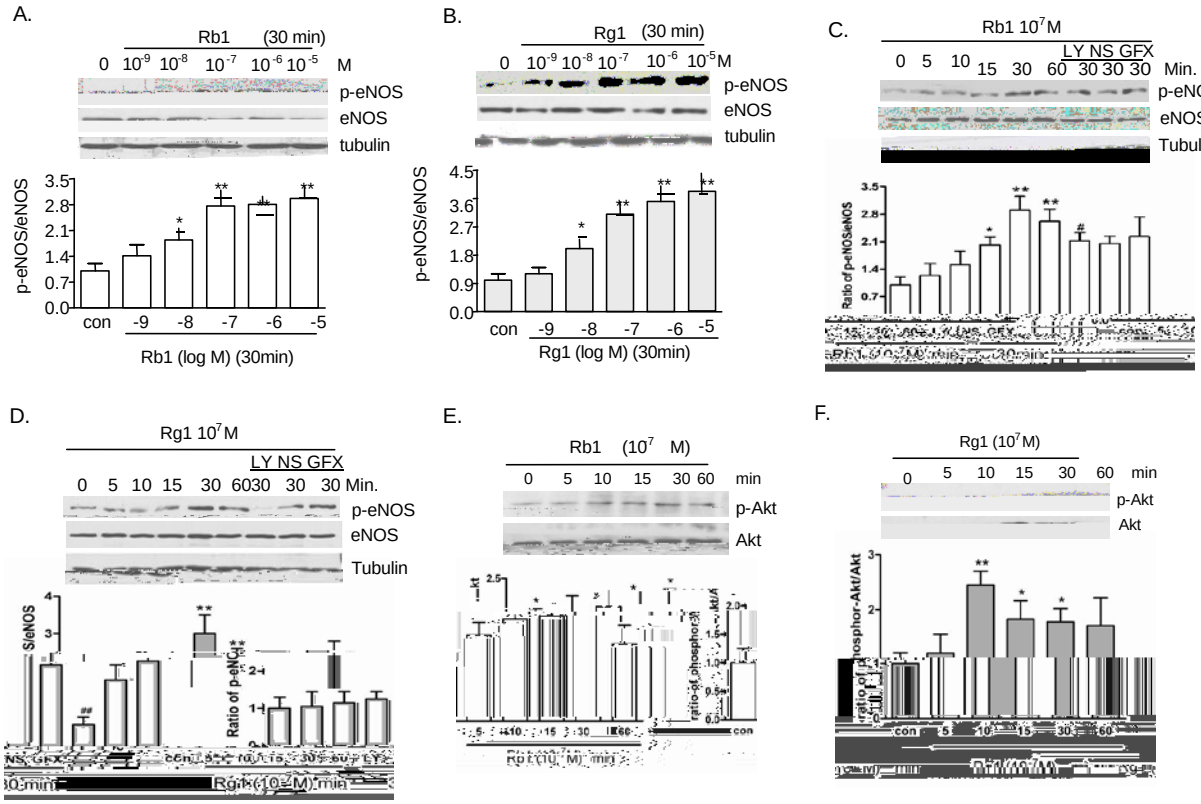
In order to prove the exact mechanism for relaxation of the coronary microvessels by Rb1 and Rg1, eNOS-/- mice whose endothelium cannot constitutively generate NO were used (Figure 1D). e phenomenon of vascular relaxation by Rb1 and Rg1 was also present in the coronary vessels of eNOS-/- mice (Figure 1F, 1G). Infusion of SNP, which operates through an endothelium-independent cGMP-mediated pathway, to the eNOS-/- coronaries, causes vasodilation (Figure 1E), while A23187, a calcium ionophore, whose activity required the presence of an intact endothelium, failed to do so (Figure 1E).

Rb1 and Rg1 activate phosphorylation of eNOS via the PI3K/Akt pathway

Both Rb1 and Rg1 at concentration of 10-7 g/ml stimulated the eNOS phosphorylation in cultured PAECs in a concentration-dependent manner beginning a er 10-15 minutes of incubation and peaking at 30 minutes (Figure 2A and 2B). When the endothelial cells were pretreated with an inhibitor of PI3K (LY294002), the Rg1 induced phosphorylated-eNOS (p-eNOS) was signi cantly diminished (Figure 2D). Interestingly, a similar e ect on Rb1- mediated phosphorylation was not seen (Figure 2C). Cox-2 inhibitor (NS398) and a PKC inhibitor (GFX) did not show any interference with the amount of p-eNOS (Figure 2C and 2D). In addition, both Rb1 and Rg1 also rapidly increased the phosphorylation of Akt (Ser473) (Figure 2E and 2F), suggesting the involvement of the PI3K/Akt pathway in Rg1-mediated phosphorylation of eNOS.

Rb1 and Rg1 stimulates production of NO

Whether Rb1 or Rg1-induced eNOS activation resulted in enhancing the NO production in endothelial cells were examined. e increases in the NO level in cultured medium were measured a er PAECs were incubated for 3 hours with or without Rb1 or Rg1, and the results were analyzed using the NO production assay. It was found that the NO production was una ected with lower concentrations of both Rb1 (10 9 g/ml) and Rg1 (10 8 g/ml), whereas exposure to slightly higher concentrations of Rb1 (10 8~10 5 g/ml) and Rg1 (10 7~10 5 g/ml) signi cantly increased NO production (P<0.01) (Figure 3A and 3B). In order to explore the upstream cascades involved in NO generation and to exclude the role of other secondary pathways other than PI3K-phospho-Akt-eNOS, PAECs in the absence or presence of Rb1 or Rg1 were treated in culture with supplemental L-arginine, L-arginine and L-NAME, D-arginine, or no supplement. L-Arginine, but not D-arginine, is the natural substrate for NOS in nature. Culture medium containing supplemental L-arginine demonstrated a signi cant increase in NO levels compared with all other controls. is stimulatory e ect of L-arginine was completely inhibited by L-NAME, and NO production was further enhanced in the presence of Rb1 or Rg1 (Figure 3C and 3D).



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Rb1 and Rg1 stimulates L-arginine transport and uptake

In order to examine if Rb1 and Rg1 are also involved in modulating with the latter. In addition, kinetic curve analysis of the data showed that both Rb1 and Rg1 do not a ect L-arginine transport by increasing Vmax values but by decreasing Km values of the transporter (Figure 4D). Eadie-Hofstee analyses of transport were linear (Figure 4E), suggesting the presence of a single transport site for L-arginine in cells using either Rb1 or Rg1.

Rb1 and Rg1 increased cationic amino acid transporter (CAT-1) mRNA expression

Quantitative-PCR was performed on the PAECs isolated from cultures at incremental time intervals and analyzed for the expression of CAT-1. e evidence shows that both Rb1 and Rg1 (10-7 g/ml) increased CAT-1 mRNA levels (Figure 5A and 5B).

P.N. extract lowered the blood pressure in spontaneously

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11. 1DWKDQ & ;LH 4: 1LWULF R[LGH V\QWKDVHV &HOO

12.

the Na+-independent system y+ carrier [30]. e activity of system y+ is encoded by four genes: CAT1, CAT2, CAT3, and CAT4. In endothelial cells, the vast majority of system y+-mediated Na+-independent L-arginine uptake is provided by the CAT1 transporter [31]. is work also con rms the role of L-arginine transport, its dependence on the y+ system, and the upstream regulation by CAT-1 mRNA in the generation of additional NO whenever needed by the cells (Figure 5A and 5B).

Conclusion

e major ndings include the following: (1) e vasodilator e ect of Rb1 and Rg1 is apparent, as evidenced by signi cant lowering of the caudal blood pressure; (2) e vasodilator e ect of P.N., Rb1 and Rg1 is endothelial-dependent, and uses a signal mechanism through the phosphorylation of eNOS via the PI3K/Akt pathway and the subsequent generation of NO; (3) Rb1 and Rg1 stimulates L-arginine transport and uptake to the endothelium cells, which is linked with increased cationic amino acid transporter (CAT-1) mRNA expression. All three points is graphically illustrated in (Figure 6). It is plausible that Rb1 and Rg1, the major components of P.N., could be responsible for the anti-hypertensive e ects, as are other members of the ginseng family as previously reported [32,33].

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2. 'X 4 -HU] * :DLEHO 5 :LQWHUKDOWHU 3 ,VRODWLRQ RI GDPPDUDQH VDSRQLQV IURP SDQD[QRWRJLQVHQJ E\ KLJK VSHHG FRXQWHU FXUUHQW FKURPDWRJUDSK\ - &KURPDWRJU $

3. /L / 6KHQJ < =KDQJ - *XR ' 'HWHUPLQDWLRQ RI IRXU DFWLYH VDSRQLQV RI SDQD[QRWRJLQVHQJ LQ UDW IHFHV E\ KLJK SHUIRUPDQFH OLTXLG FKURPDWRJUDSK\ - Chromatogr Sci 43: 421-425.

4. Li L, Zhang JL, Sheng YX, Guo DA, Wang Q, et al. (2005) Simultaneous TXDQWL¿FDWLRQ RI VL[PDMRU DFWLYH VDSRQLQV RI SDQD[QRWRJLQVHQJ E\ KLJK SHUIRUPDQFH OLTXLG FKURPDWRJUDSK\ 8Y PHWKRG - 3KDUP %LRPHG $QDO 51.

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