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Short communication

Ginsenoside Rb_1 inhibits cardiomyocyte apoptosis and rescues ischemic myocardium by targeting Caspase-3

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Myocardial ischemia (MI) is a pathophysiological condition in which the myocardium is unable to maintain normal cardiac function due to insufficient coronary artery blood and oxygen supply, as well as abnormal myocardial energy metabolism [1]. Ginsenoside Rb_1 (Rb_1), one of the most abundant natural ingredients in ginseng and *Panax notoginseng*, has been proven to protect the heart from MI/reperfusion injury (RI) [2]. However, the Rb_1 protective effect on the heart in post-MI state needs further investigation, and the underlying mechanism and targets remain unclear [3]. Therefore, this study aims to further confirm the therapeutic effects of Rb_1 on MI (not MI/RI) *in vitro* and *in vivo*, and explore its anti-MI targets by combining network pharmacology, proteomics, cellular thermal shift assay (CETSA), molecular dynamics (MD) simulation, protein recombination and activity testing; and UV and fluorescence spectroscopy analysis. The experimental materials, methods and discussion section are shown in Supplementary data. The results provide insights into the optimal utilization of Rb_1 in clinic, and feasible targets for screening anti-MI drugs.

Firstly, cardiomyocyte H9c2 injury models induced by H_2O_2 and *tert*-butyl hydroperoxide (tert-BHP), as well as MI mouse models induced by isoproterenol (ISO) and left anterior descending ligation (LADL), were established to verify the therapeutic effects of Rb_1 against MI *in vitro* and *in vivo*. The animal care and experimental procedures were approved by the Animal Ethics Committee of Fujian Medical University on March 8, 2024, with an approval number IUCAC FJMU 2024-Y-0516. As shown in Figs. 1A–C, S1 and S2, the results confirm Rb_1 significantly reduces oxidative stress, myocardial inflammation, and fibrosis, inhibits cardiomyocyte apoptosis, and rescues ischemic myocardium.

To predict the potential target protein of Rb_1 against MI, we conducted network pharmacology and molecular docking analysis (Figs. S3 and Table S1). The results suggested that Caspase-3 (CASP3), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), FK506-binding protein 1A (FKBP1A), etc., might be key targets for Rb_1 exerting its therapeutic effects. To further screen the targets for Rb_1 against MI, we conducted proteomic and phosphoproteomic studies on Rb_1 intervention in MI *in vitro* and *in vivo* models (Figs. S4, S5A and S5B, and Tables S2 and S3). The protection of Rb_1 against MI mainly involved endoplasmic reticulum, oxidoreductase activity and contractile fiber, etc. (Figs. S5C–F). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis found that dilated cardiomyopathy and hypertrophic cardiomyopathy pathways were enriched in both proteomic studies. All these pathways contain Lamin A/C (LMNA) (Fig. S6A), which is one of the substrates of CASP3 and up-regulated by the Rb_1 intervention in the MI cells and mouse hearts. Because LMNA is engaged in the nucleus composition, maintaining nuclear stability and being involved in mitosis and DNA replication, it is suggested

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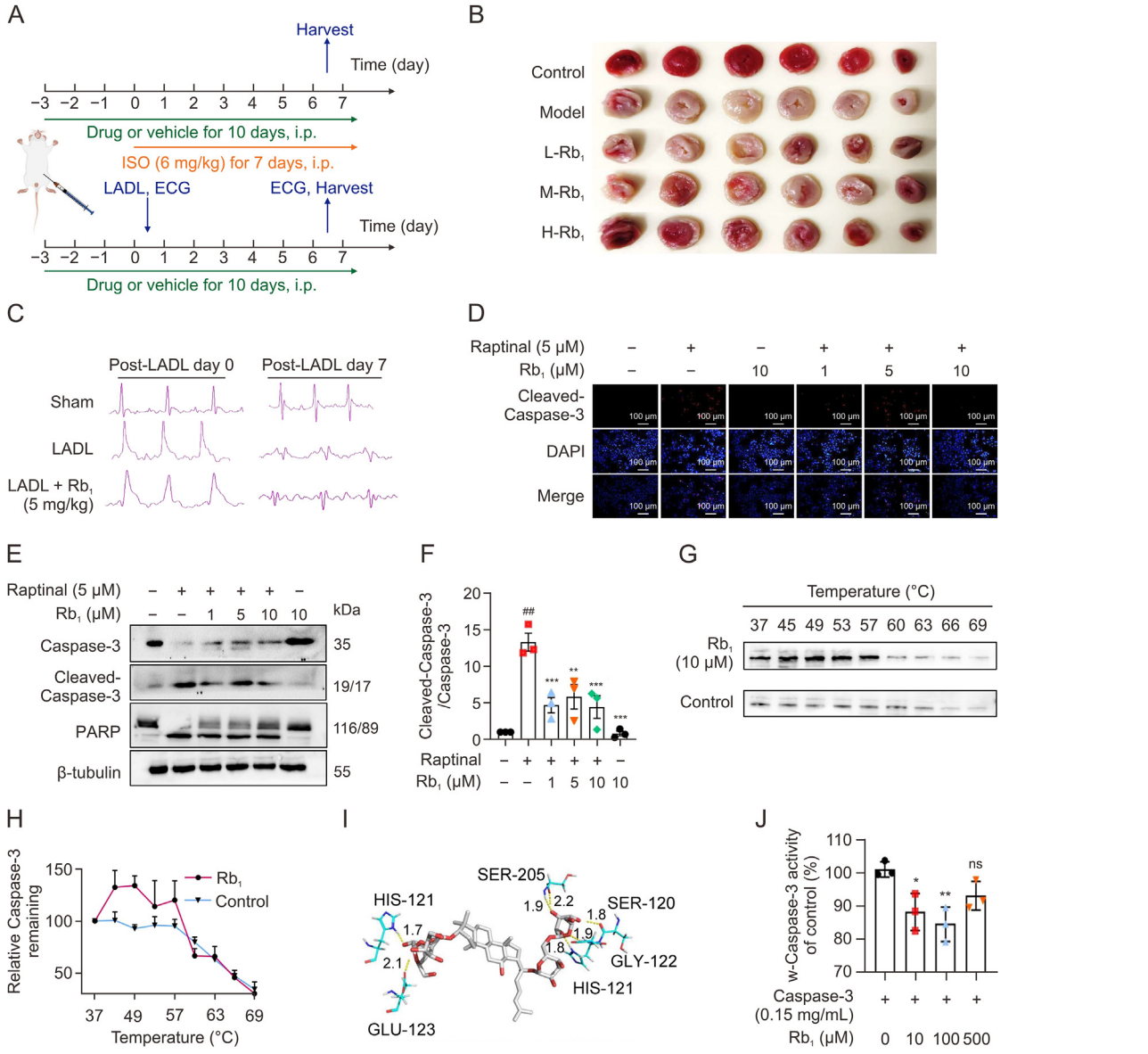

Fig. 1. Protection of ginsenoside Rb_1 (Rb_1) in *in vivo* myocardial ischemia (MI) model, and intervention of Rb_1 on the cleavage of Caspase-3 (CASP3) agonized by raptinal and the binding properties of CASP3 with Rb_1. (A) Schematic illustration of a mouse model of myocardial ischemia (MI) induced using isoproterenol (ISO) injection and left anterior descending ligation (LADL), respectively, and the timing and injection method of Rb_1. (B) Typical 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining of heart tissue sections for Rb_1 treatments (L-Rb_1, 5 mg/kg Rb_1; M-Rb_1, 10 mg/kg Rb_1; H-Rb_1, 20 mg/kg Rb_1; by single daily intraperitoneal injection for 10 days) and ISO modeling groups. It showed a significant reduction in myocardial infarct area in ISO-induced mice for the Rb_1 administration group. (C) Electrocardiography (ECG) characteristics of the groups before and after LADL ($n = 3$ each). Elevation of the ST segment on the electrocardiography represents successful MI modeling. On the 7th day post-operation, compared to the MI group, mice in the Rb_1 group showed a moderate recovery in QRS wave shape and reduced the ST segment elevation on the ECG, suggesting that Rb_1 can improve cardiac function and alleviate MI damage in LADL mice. (D) Immunofluorescence staining to evaluate the cleaved-CASP3 (C-CASP3) expression (red) in H9c2 cells via pre-treatment with different concentrations of Rb_1, followed by administration of 5 μM raptinal with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) as a nuclear stain agent (blue). (E) Western blot bands and (F) quantitative analysis for the expression ratio of C-CASP3 and CASP3 in H9c2 cells following Rb_1 intervention. Compared to control group, $^{\#\#}P < 0.01$. Compared to raptinal group, $^{**}P < 0.01$, $^{***}P < 0.001$. (G) Western blot bands and (H) quantitative analysis for cellular thermal shift assay (CETSA) to probe the heat stability of CASP3 from 37 to 69 °C upon incubation with Rb_1. (I) Visualization of the binding structure of Rb_1 to CASP3 by molecular docking, with Rb_1 as the gray structure and yellow hydrogen bonds to CASP3 protein amino acid residues. (J) The activity of wild-type CASP3 (w-CASP3) with different concentrations of Rb_1. Compared to Rb_1 0 μM group, $^{*}P < 0.05$, $^{**}P < 0.01$. ns: not significant. The data are presented as mean ± standard deviation (SD) ($n = 3$). HIS-121: histidine residue at position 121; GLU-123: glutamate residue at position 123; SER-120: serine residue at position 120; SER-205: serine residue at position 205; GLY-122: glycine residue at position 122; PARP: poly (adenosine diphosphate (ADP)-ribose) polymerase.

that Rb_1 protects the heart from ischemia injury via targeting CASP3, as predicted above. By combining the differential proteins from Disease Ontology Semantic and Enrichment (DOSE) analysis [4] with CASP3 (Figs. S6B and C), it is further found that CASP3 participates in multiple pathways, such as lipid metabolism and atherosclerosis, apoptosis, viral myocarditis, etc. (Fig. S7). All these findings intensely suggest that CASP3 is a regulatory target of Rb_1.

We further examined the effect of Rb_1 on the CASP3 expression *in vivo*. CASP3 did not show significant differences between model and treatment groups (Figs. S8A and B). ISO induced increased expression of cleaved-CASP3 (C-CASP3, active form of CASP3) in mouse hearts, while Rb_1 treatment reversed this trend (Fig. S8C). The expression of two substrates of CASP3, LMNA and poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP),

decreased in the ISO-induced MI model due to the cleavage of C-CASP3, but Rb_1 restored them to the control group level (Figs. S8D and E). In the LADL-induced MI model, consistent findings were also obtained, and Rb_1 did not affect the expression of the upstream regulatory factor cleaved-Caspase-9 of CASP3 (Figs. S8F–J). Afterwards, using the CASP3 agonist raptinal, we confirmed the targeted inhibitory effect of Rb_1 on CASP3 activity (Figs. 1D–F and S9A–D).

To further verify the direct interaction of Rb_1 with CASP3, CETSA was carried out. The thermal stability of CASP3 was significantly enhanced at 57 °C upon incubation with Rb_1, indicating in-situ binding of Rb_1 to CASP3 (Figs. 1G and H). Interestingly, when the testing temperature was above 60 °C, the stability of CASP3 incubated with Rb_1 decreased to the level of the control group, suggesting a moderate degree of binding of Rb_1 to CASP3. Next, molecular docking indicated that Rb_1 is linked to two His121 sites of CASP3 through the shortest hydrogen bonds (1.7 and 1.8 Å, respectively, Fig. 1I). MD simulations show that Rb_1 could alter the CASP3 conformation (Figs. S9E–H) and tighten the protein structure (Video shown in Supplementary data), indicating the CASP3-Rb_1 complex is more stable than CASP3 alone.

Afterwards, wild-type CASP3 (w-CASP3) and mutant CASP3 (*m*-CASP3, with two His121 mutated) were recombinantly synthesized *in vitro* to confirm the binding of Rb_1 at the special sites of CASP3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can observe proteins around 34 kDa and 17 kDa, which are likely full-length proteins of w-CASP3 and its subunits, respectively (Fig. S10A), while the *m*-CASP3 was observed near 42 kDa (Fig. S10B). In the gel chromatography, peak 1 is considered to be the w-CASP3, and peaks 2 and 3 are the two subunits of w-CASP3 (19 and 12 kDa, respectively) derived from the cleavage of w-CASP3. Excitingly, with the intervention of Rb_1 for 2 h, the height of peak 1 increased a little, while peaks 2 and 3 decreased a little (Fig. S10C), suggesting that Rb_1 could reverse the w-CASP3 cleavage. The purified w-CASP3 and *m*-CASP3 were also further characterized by the SDS-PAGE bands using mass spectrometry [5] and their activities were confirmed with a CASP3 activity assay kit (Figs. 1J and S10D). Inhibitory effect of Rb_1 at 10 and 100 μM on the w-CASP3 activity was obtained. This demonstrates that Rb_1 can inhibit CASP3 activity at appropriate concentrations (e.g. 10 μM). However, the enzyme activity of *m*-CASP3 was very weak, further confirming that two His121 are essential residues for CASP3 activity. In addition, the UV and fluorescence spectra for the Rb_1-CASP3 complex interaction were also examined (Figs. S10E–J). All these confirm that Rb_1 is capable of binding to CASP3 to generate a CASP3-Rb_1 complex, thereby inhibiting the C-CASP3 production.

Summarily, we for the first time confirm CASP3 as a key target for Rb_1 against MI. Rb_1 can bind to two His121 sites in CASP3 and block the cleavage of CASP3, thereby inhibiting cardiomyocyte apoptosis and rescuing cells under ischemic conditions (Fig. S11). The present findings provide insights into the optimal utilization of Rb_1 in clinical settings, and highlight CASP3 as a promising target for screening anti-MI drugs.

CRediT authorship contribution statement

Chenhui Zhong: Writing – original draft, Investigation, Formal analysis, Data curation. **Liyuan Ke:** Validation, Formal analysis, Data curation. **Fen Hu:** Supervision, Resources, Conceptualization. **Zuan Lin:** Investigation, Formal analysis, Data curation. **Shuming Ye:** Validation, Data curation. **Ziyao Zheng:** Investigation. **Shengnan Han:** Validation, Data curation. **Zan Lin:** Validation, Data curation. **Yuying Zhan:** Validation, Data curation. **Yan Hu:** Validation. **Peiying Shi:** Supervision, Resources, Conceptualization. **Lei Wen:** Writing – review & editing, Resources. **Hong Yao:** Writing – review & editing, Visualization, Software, Resources, Data curation.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.101142.

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