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Original Research Article

Rhynchophylline alleviates endothelial injury in spontaneously hypertensive rats by regulating PPARγ-mediated iron death

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Abstract

Purpose: *To determine the effects of rhynchophylline (Rhy) on the progression of cardiovascular diseases in rats.*
Methods: *Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR, 7 week old, weighing 180 – 200 g) were used in this study. The rats were divided into WKY, SHR, SHR+ Rhy (30 mg/kg), and SHR+ Rhy (50 mg/kg). WKY rats were used as the normal control group which had free access to distilled water. The rats were then orally administered Rhy (30 and 50 mg/kg daily by gavage). For inhibition of PPARr, T0070907 was given at a dose of 2 mg/kg for 24 h. The blood pressure of control, spontaneously hypertensive rats (SHR), and SHR rhynchophylline (Rhy) rats were measured. While PPARr levels in the hypertensive rats were assessed by Western blot. Cell apoptosis in response to SHR and Rhy were evaluated by TdT-mediated dUTP nick-end labeling (TUNEL) and Western blot assays, whereas oxidative stress and inflammatory response in SHR rats treated with Rhy were determined by enzyme linked immunosorbent assay (ELISA) and Immunoblot assays, respectively.*
Results: *Rhy decreased hypertension and up-regulated PPARγ in SHR rats ($p < 0.01$). In addition, Rhy improved SHR endothelial cell apoptosis by inducing PPARγ, and also produced reduction in proinflammatory factor secretion by inducing PPARγ. Furthermore, Rhy mitigated oxidative stress and iron death by inducing PPARγ ($p < 0.001$).*
Conclusion: *Rhynchophylline provides a promising method in alleviating endothelial injury in SHR rats by modulating PPARγ-mediated inflammation and oxidative stres. We therefore thought Rhy alleviated endothelial injury and could serve as a promising drug.*

Keywords: *Endothelial dysfunction, Hypertension, Rhynchophylline (Rhy), Uncaria, PPARγ, Oxidative stress*

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INTRODUCTION

Endothelial dysfunction is considered to be a predictor of cardiovascular disease and long-term clinical outcomes, such as heart disease, arteriosclerosis, stroke, kidney disease and hypertension [1]. Hypertension is a cardiovascular disease with a high mortality rate. However, its pathogenesis remains unclear. It is widely believed that hypertension is associated

with endothelial damage caused by inflammatory cytokines in vascular endothelial cells and oxidative stress [2].

Uncaria is a traditional Chinese herb that has been used to treat cardiovascular and central nervous system diseases [3, 4], and Rhynchophylline (Rhy) is a pharmacologically active substance isolated from Uncaria, and is widely used in the treatment of hypertension [5]. The antihypertensive effects of Rhy have been observed in many animal models and attributed to vasodilation, possibly through the inhibition of l-type Ca^{2+} channels and/or reduction of calcium sensitivity in smooth muscle cells. In addition, uncarine also ameliorates endothelial dysfunction in the renal artery cultured in spontaneously hypertensive rats through the SrC-PI3K/Akt-ENOS cascade. Previous studies have shown that Rhy can significantly upregulate PPARγ in human bone marrow mesenchymal stromal cells [3]. Therefore, it is speculated that Rhy may alleviate endothelial injury in hypertension by up-regulating PPARγ-mediated ferroptosis.

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor that regulates the transcription of several genes involved in fatty acids and energy metabolism [6]. It also has pleiotropic properties in the cardiovascular system, as well as anti-inflammatory and anti-atherosclerotic properties. PPARγ acts as a vascular protective metabolic regulator in smooth muscle and endothelial cells [7]. For example, the PPARγ agonist, pioglitazone, reverses pulmonary hypertension and prevents right heart failure through fatty acid oxidation [8]. Recent studies have shown that PPARγ is associated with iron death. Resveratrol alleviates ER stress and up-regulates PPARγ expression, thereby inhibiting acrolein induced iron death [9]. In addition, PPARγ can also upregulate lactoferrin (Ltf) through transcription, while PPARγ inhibition results in the deficiency of Ltf transcription and secretion, the accumulation of neuronal iron and the exacerbation of neuronal ferroptosis.

In this study, the possible effects of rhynchophylline (Rhy) on the progression of cardiovascular diseases were investigated to determine its potential for the management of cardiovascular diseases.

EXPERIMENTAL

Animal grouping and drug administration

Wistar Kyoto Rats (WKY) and spontaneously hypertensive rats (SHR, 7 weeks old, weighing 180 – 200 g) were obtained from Charles River (Beijing, China) and managed in accordance with the guidelines of the Ethics Committee of Huashan Hospital, Fudan University which gave approval to the study (approval no. 2022JS - 228). The study was performed conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [10]. The rats were divided into WKY, SHR, SHR+ Rhy (30 mg/kg), and SHR+ Rhy (50 mg/kg). WKY rats were used as the normal control group and given distilled water for free drinking. They were then orally administered Rhy 30, 50 mg/kg daily by gavage. For inhibition of PPARr, T0070907 was given at a dose of 2 mg/kg for 24 h.

Determination of systolic blood pressure (SBP) and diastolic blood pressure (DBP)

SBP and DBP of the rats were measured by non-invasive caudal arterial blood pressure meter (Techman, China) every week.

Quantitative real-time-polymerase chain reaction (qRT-PCR)

Cellular total RNA was extracted with TRIzol reagents (Thermo, Rockford, USA). Total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The cDNA was amplified using the primers listed in Table 1.

Western blot

Proteins were extracted with RIPA buffer (Beyotime, Shanghai, China).

Table 1: Primers used in this study

Gene	Forward primer	Reverse primer
DAPK1	CCAGACTGTCTTCCACC	TCCTCACACTCACGTTC
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
COX-2	CCGGGTACAATCGCACTTAT	GGCGCTCAGCCATACAG
iNOS	GCTCTACACCTCCAATGTGACC	CTGCCGAGATTTGAGCCTCATG
IL-6	AGACAGCCACTCACC	TTCTGCCAGTGCCTCTT
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

Then, the samples were collected and electrophoresed by 10 % SDS-PAGE, transferred onto PVDF membranes, then blocked with 5 % fat-free milk. Subsequently, the membranes were incubated with primary antibodies targeting PPARr (Mouse, 1:1000, Abcam), bax (Mouse, 1:1000, Abcam), bcl-2 (Mouse, 1:1000, Abcam), PARP (Mouse, 1:1000, Abcam), cleaved PARP (Mouse, 1:1000, Abcam), Nrf-2 (Mouse, 1:1000, Abcam), HO-1 (Mouse, 1:1000, Abcam), b-actin (Mouse, 1:10000, Abcam), GAPDH (Mouse, 1:10000, Abcam) for 1 h. Ultimately, the membranes were conjugated with anti-mouse IgG and anti-rabbit IgG (Abcam, Cambridge, UK) for 1 h. Specific proteins were visualized with enhanced chemiluminescence detection kit (ECL, Thermo, Rockford, USA).

TUNEL staining

The artery in each group were excised and snap frozen with OCT. Then the tissues were sliced and fixed with formaldehyde, rinsed with PBS and then stained with cell apoptosis detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The percentage of cell apoptosis was measured using a microscope (Olympus), and the apoptotic cells were counted manually.

Enzyme linked immunosorbent assay (ELISA) and qRT-PCR

The concentrations of tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), and Interleukin-6 (IL-6), in the serum were evaluated using ELISA kit in accordance with manufacturer's protocols. Samples were added into wells, and biotin-conjugated primary antibodies were plated into the wells before the addition of avidin conjugated horseradish peroxidase (HRP). Then, enzyme substrate was added for color development. The intensity of each well was measured using a microplate reader (R&D systems, Minneapolis, MN, USA). All experiments were carried out in accordance with the applicable guidelines.

Endothelia sample RNA was extracted with TRIzol reagents (Thermo, Rockford, USA). Total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The cDNA was amplified using the primers shown in Table 2.

Western blot

Proteins were extracted with RIPA buffer (Beyotime, Shanghai, China). Then, the samples were collected and electrophoresed using 10 % SDS-PAGE, transferred onto PVDF membranes, and then blocked with 5 % fat-free milk. Subsequently, membranes were incubated with primary antibodies targeting Nrf2 (Mouse, 1:1000, Abcam), NQO-1 (Mouse, 1:1000, Abcam), HO-1 (Mouse, 1:1000, Abcam), p-p65 (Mouse, 1:1000, Abcam), p65 (Mouse, 1:1000, Abcam), p-IκBα (Rabbit, 1:1000, Abcam), IκBα (Rabbit, 1:1000, Abcam), and β-actin (Mouse, 1:10000, Abcam) for 1 h. Ultimately, the membranes were conjugated with the anti-mouse IgG and anti-rabbit IgG (Abcam, Cambridge, UK) for 1 h. Specific proteins were visualized with enhanced chemiluminescence detection kit (ECL, Thermo, Rockford, USA).

Evaluation of superoxide dismutase (SOD), malondialdehyde (MDA), GSH and MPO

Aortic tissues were collected from the rats for the determination of H_2O_2, MDA, and GSH with the relevant commercial kits (Jiancheng Bioengineering Institute of Nanjing, China). The tissues were homogenized and centrifuged (1000 g) for 20 min and the supernatant was collected, to which the samples were added. The sample was gently shaken, mixed, and covered for reaction at 37 °C for 2 h. A microplate reader was then employed to determine the absorbance (A) of each well at the wavelength of 450 nm. The experiment was repeated three times.

Statistics

GraphPad 6.0 was used for the statistical analysis. Three replicates were performed for each experiment. One-way ANOVA and Student's t test were used for statistical comparisons. $P < 0.05$ was considered statistically significant.

Table 2: Primers for cytokines

Gene	Forward primer	Reverse primer
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
IL-1β	ACAAGGAGAAGAAAGTAATGAC	GCTGTAGAGTGGGCTTAT
IL-6	AGACAGCCACTCACC	TTCTGCCAGTGCCTCTT
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

RESULTS

Rhy reduced hypertension and up-regulated PPARγ in SHR rats

SHR rats exhibited higher level of SBP and DBP. And Rhy treatment effectively reduced the level of SBP and DBP in a dose dependent manner (Figure 1 A, $p < 0.01$). In addition, Rhy treatment induced a higher expression of PPARr in aortic tissues (Figure 1 B, $p < 0.01$). Collectively, Rhy reduces hypertension and up-regulates PPARγ in SHR rats.

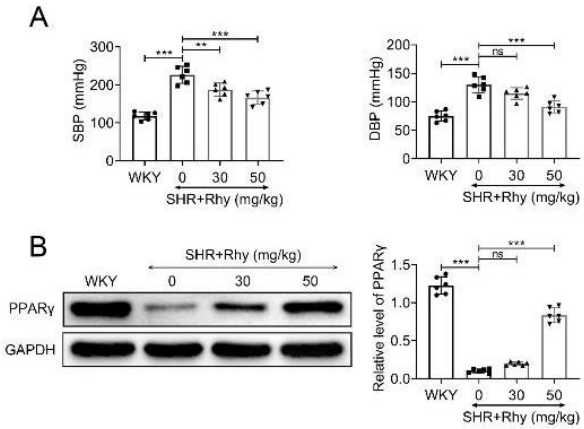

Figure 1: Rhy reduced hypertension and up-regulated PPARγ in SHR rats. **(**A): The SBP and DBP in WKY, SHR, SHR+ Rhy (30 mg/kg), SHR+ Rhy (50 mg/kg) rats; (B): The PPARr level in each group. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

Rhy inhibited endothelial cell apoptosis by enhancing PPARr

Apoptotic cells increased in SHR rats, but Rhy reduced cell apoptosis in the rats upon the treatment of a PPARr inhibitor. Also, T0070907 abolished the effect of Rhy in cell apoptosis (Figure 2 A, $p < 0.001$); furthermore, SHR rats showed elevated levels of Bax and cleaved-PARP, but reduced levels of Bcl-2 and PARP. Rhy therapy reversed these alterations, but were abolished by PPARr (Figure 2 B, $p < 0.001$). Overall, Rhy improved endothelial cell apoptosis by enhancing PPARr.

Rhy alleviated pro-inflammatory factor secretion by inducing PPARγ expression

To explore the inflammatory response in SHR and Rhy rats, the IL-6, IL-1β, and TNF-α levels were monitored in each rat group. Hypertension significantly increased the levels of IL-6, IL-1β, and TNF-α (Figure 3 A), but Rhy administration relieved the inflammation by reducing the levels of the cytokines (Figure 3 A, $p < 0.001$). The mRNA IL-6, IL-1β, and TNF-α levels after Rhy treatment were also reduced (Fig. 3 B, $p < 0.001$) and the inhibitive effect on pro-inflammatory factor by Rhy was abrogated by PPARr inhibitor ($p < 0.001$). Therefore, Rhy was responsible for the inhibited inflammation in SHR rats.

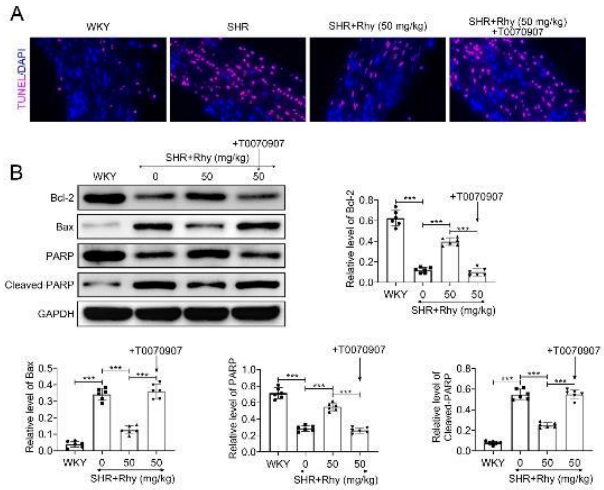

Figure 2: Rhy improved endothelial cell apoptosis by enhancing PPARr**. (**A): The cell apoptosis in each group; (B): The expression of Bcl-2, Bax, PARP, and cleaved-PARP in each group. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

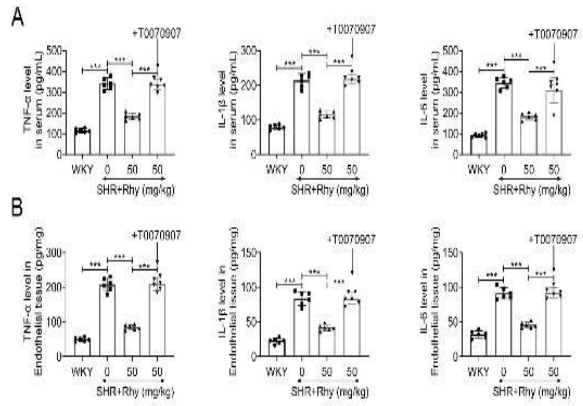

Figure 3: Rhy and alleviated pro-inflammatory factor secretion by inducing PPARγ expression**. (**A): Levels of TNF-a, IL-1b, IL-6 in each group; (B): The mRNA level of TNF-a, IL-1b, IL-6 in each group. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

Rhy alleviated oxidative stress by inducing PPARγ expression

To further reveal the role of Rhy in oxidative stress, the H_2O_2, MDA, GSH levels were analyzed in the rat groups. Elevation of MDA and H_2O_2 levels, and reduction in GSH levels in SHR group were found, but treatment with Rhy reversed the changes in their levels while treatment with PPARr inhibitor abolished the

effect of Rhy on oxidative stress (Figure 4 A, $p < 0.001$). Moreover, the level of Nrf2 and HO-1 were inhibited in SHR rats, but enhanced by Rhy treatment, and then abolished by PPARr inhibitor treatment (Figure 4 B, $p < 0.001$). These results suggest that Rhy is associated with reduced oxidative stress.

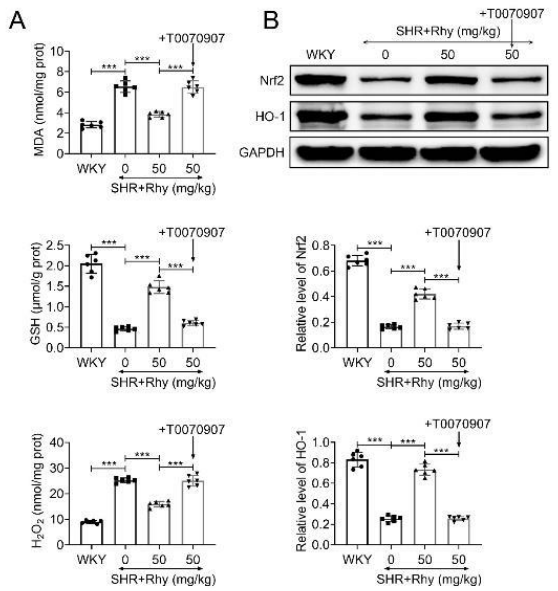

Figure 4: Rhy and alleviated pro-inflammatory factor secretion by inducing PPARγ expression**.** (A): The level of MDA, GSH and H2O2 in each group; (B): The level of of p-p65, p-IkBa in each group. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

DISCUSSION

Spontaneous hypertension is due to the long-term and repeated effects of environmental as well as genetic factors, resulting in elevated blood pressure [11]. In addition to genetic factors, essential hypertension is also closely related to environmental factors. The primary goal of essential hypertension treatment is to minimize the overall risk of cardiovascular death and disability [12]. To combat this disease, it is still necessary to further study its pathogenesis, find an effective treatment plan, and develop therapeutic drugs [13]. Interestingly, a pharmacological active substance isolated from Uncaria could serve as a promising drug for the treatment of spontaneous hypertension.

Elevated blood pressure in SHR rats is genetically determined by multiple genes, which is very similar to human hypertension. It is an ideal animal model for studying the pathogenesis of hypertension and screening antihypertensive drugs [14]. In this study, four-month-old male spontaneously hypertensive rats were given SHR at different concentrations of Rhy (0, 30 mg/kg, and 50 mg/kg) for two weeks, and WKY was used as the control. After blood pressure was measured, serum and aorta were collected for follow-up experiments. After performing Immunoblot, TUNEL, and ELISA assays, the data revealed that Rhy improved endothelial cell apoptosis, and alleviated pro-inflammatory factor secretion and oxidative stress by inducing the expression of PPARγ.

Rhy is a promising drug for spontaneous hypertension, while PPARγ is a ligand-activated transcription factor that regulates the transcription of several genes involved in fatty acids and energy metabolism [15]. It also has pleiotropic properties in the cardiovascular system [16]. The data confirmed that Rhy reduced hypertension and up-regulated PPARγ in SHR rats, and might affect the progression of spontaneous hypertension via PPARγ. Through the inhibition of PPARγ, T0070907 and its effects on oxidative stress were revealed.

In the present study, Rhy could also alleviate endothelial injury in hypertension by up-regulating PPARγ -mediated ferroptosis. In fact, the multiple biological activities of Rhy have been widely reported. Rhy mediates the calcium homeostasis by antagonizing the phosphorylation of ryanodine receptor 2, so as to ameliorate diabetic cardiomyopathy. Rhy treatment also ameliorate amyloid-β pathology, as well asthe inflammation in an Alzheimer's disease mouse model [17]. In addition, Rhy exerts significant neuroprotective effects against Aβ-stimulated oxidative stress, neurodegeneration, as well as memory impairment via the activation of Nrf2-ARE [18]. Similarly, Rhy suppresses oxidative stress in SHR model, and also improves trophocyte mobility potential by overexpressing ZEB1 via the inhibition of miR-141-3p. All these findings confirmed that Rhy is a promising drug in multiple types of diseases.

It has also been reported that Rhy reduced hypertension and up-regulated PPARγ in SHR rats, and inhibited ferroptosis [19]. Ferroptosis is a recently defined form of iron-dependent and non-apoptotic cell death, caused by lipid peroxidation at lethal levels. GPX4 (glutathione peroxidase 4), ACSL4 and SLC7A11 are key regulators of iron dead cell death. Previous studies have shown that the inhibition of iron death can significantly prevent myocardial remodeling, dysfunction, inflammation, hypertrophy, and fibrosis in hypertensive mice. Therefore, these studies confirm the key role of

ferroptosis in the progression of cardiovascular diseases.

CONCLUSION

Rhy aids the reduction of hypertension, up-regulates PPARγ in SHR rats, inhibits ferroptosis, and improves endothelial cell apoptosis. It also alleviates pro-inflammatory factor secretion and oxidative stress by inducing PPARγ expression. Therefore, Rhy is a potential drug for the treatment of spontaneous hypertension. However, there is a need for clinical studies to ascertain this.

DECLARATIONS

Acknowledgements

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None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhidong Zhu and Haiming Shi designed the study and carried out the experiments and data collection, analyzed and interpreted the data, and prepared the manuscript for publication. Both authors read and approved the manuscript.

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