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Small ubiquitin-like modifiers inhibitors lower blood pressure *via* ERK5/KLF2-dependent upregulation of the eNOS/NO pathway

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Abstract

Background: Small ubiquitin-like modifiers (SUMO)ylation is a dynamic and reversible post-translational modification playing pivotal roles in the regulation of cancer, diabetes, heart failure, and neurological diseases. However, whether SUMO inhibitors also have anti-hypertension effect remains yet to be explored. **Methods:** Blood pressure was monitored in spontaneously hypertensive rats (SHR) after Tannic acid (TA) administration for 4 weeks. The contents of nitric oxide (NO) and endothelin-1 (ET-1) in the serum of SHR were measured. Isolated endothelium-intact mesenteric artery rings were used to study relaxation effect of SUMO inhibitors. ERK5 SUMOylation was determined using co-immunoprecipitation (co-IP) and immunofluorescence (IF). NO levels were analyzed by IF. The expression levels of KLF2 and p-eNOS were semi-quantified by Western blot analysis. The transcriptional activity of eNOS promotor was assayed using ChIP-PCR. **Results:** Three SUMO inhibitors all reduced the phenylephrine (PE)-induced contraction of mesenteric artery rings in a concentration-dependent manner. Co-IP revealed that ponatinib promoted ERK5 SUMOylation, which was nulled following pre-treatment with the SUMO inhibitors. IF displayed that TA increased ERK5 accumulation and its co-localization with SUMO-1 in the nucleus. ChIP-PCR unveiled TA-induced enhancement of KLF2-dependent eNOS promoter activity and upregulation of eNOS/NO expression in HUVECs. *In vivo*, TA significantly lowered the blood pressure and improved the vascular reactivity by activating the KLF2/eNOS/NO pathway. Additionally, the level of NO was elevated along with decreased ET-1 levels in the serum of SHR. **Conclusions:** SUMO inhibitors inhibit ERK5 SUMOylation to promote KLF2-eNOS/NO signaling, indicating their therapeutic potential for the treatment of hypertension.

Keywords

hypertension; SUMO inhibitor; ERK5; SUMOylation; KLF2

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1 Introduction

Hypertension is a common public health issue causing significant morbidity and mortality worldwide[1]. Despite being the leading risk factor for stroke, ischemic heart disease, chronic kidney disease and other cardiovascular diseases, the rates of awareness, treatment, and control of hypertension remain very low[2]. Therefore, there is an urgent need to develop safer and more effective therapeutics for hypertension.

SUMOylation is an important post-translational modification in which small ubiquitin-like modifiers (SUMOs) are conjugated to specific substrates, altering their function, stability, subcellular localization, and interactions with partners. This process involves a cascade of enzymatic reactions mediated by SUMO-activating enzymes, the SUMO-conjugating enzyme UBC9, and several SUMO E3 ligases, with their activities correlating directly with disease prognosis and progression[3]. Consequently, there has been a significant emphasis on the discovery and development of SUMOylation inhibitors. Recently studies have found that SUMOylation is involved in several pathological processes, including endothelial cell dysfunction, dyslipidemia, and vascular smooth muscle cell

(VSMC) proliferation, which can ultimately lead to atherosclerosis. Hyper-SUMO2ylation impairs vascular endothelial function and increases vascular oxidative stress, whereas endogenous SUMO2 is essential for maintaining the normal physiological function of the vascular endothelium[4]. However, the role of SUMO inhibitors in endothelial function remains obscure.

The extracellular-signal-regulated kinase 5 (ERK5), also known as big MAP kinase-1 (BMK1), is the most structurally divergent member of the mitogen-activated protein kinase (MAPK) family and is ubiquitously expressed in various tissues. ERK5 is activated in response to a wide range of growth factors, such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor-2 (FGF-2), subsequently controlling cell differentiation, proliferation, and survival[5]. Importantly, ERK5 plays a major role in regulating endothelial function, eliciting a vasoprotective endothelial phenotype *via* its transcriptional activity[6]. Previous reports have shown that the covalent attachment of SUMOs at residues Lys6 and Lys22 of ERK5 modulates its transcriptional activity in cultured endothelial cells (EC)[7] and cardiomyocytes[8]. Several studies have proposed that ERK5 SUMOylation exerts a negative effect on endothelial protection by reducing ERK5 transcriptional activity. Erazo *et al.* demonstrated that SUMOylation is necessary for ERK5 nucleo-cytoplasmic shuttling, nuclear translocation, and transcriptional activation in cancer cell proliferation[9]. Notably, Paez-Mayorga *et al.* reported that ponatinib, an ERK5 SUMOylation trigger, accelerated inflammation and disrupted vascular homeostasis[10]. They also demonstrated that ERK5 SUMOylation *via* PIAS1 (SUMO E3 ligase) counteracted its transcriptional activity and diminished the expression of ERK5-responsive genes Krüppel-like factor 2 (KLF2) and endothelial nitric oxide synthase (eNOS). KLF2 is a laminar flow-inducible transcription factor primarily expressed by EC, and its expression is mediated by the phosphorylation of ERK5. KLF2 plays a crucial role in the regulation of endothelial function by inducing eNOS expression and increasing nitric oxide (NO) production[11]. This study aimed to investigate whether SUMO inhibitors exert a vasodilatory effect through the KLF2/eNOS/NO pathway by inhibiting ERK5 SUMOylation. Additionally, it explored whether the vasodilatory action of SUMO inhibition provides an anti-hypertensive property, which merits further investigation for the potential clinical applications in hypertension treatment.

2 Methods

2.1 Animal ethics

Spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). All animal experiments were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care International and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (Ethical approval number: IRB3017723).

2.2 Materials

Antibodies for eNOS (#32027), phospho-eNOS (Ser1177) (#9570), SUMO1 (#4930), and GAPDH (#5174) were purchased from Cell Signaling Technology (Beverly, MA, USA). The ERK5 antibody (#sc-398015) was purchased from Santa Cruz Biotechnology (CA, USA), and the KLF2 antibody (#PA5-40591) was purchased from Thermo Fisher Scientific (Waltham, USA). Tannic acid (TA; #HY-B2136), 2', 3', 4'-trihydroxy flavone (2-D08; #HY-114166), Ginkgolic acid (GA; #HY-N0077), and ponatinib (#AP-24534) were purchased from MedChemExpress (Shanghai, China). Phenylephrine (PE) and acetylcholine (ACh) were purchased from Aladdin (Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Manassas, VA, USA).

2.3 Cell culture

The cells were grown in DMEM (Hyclone, Utah, USA) containing 10% fetal bovine serum (FBS)(Biological Industries, Cromwell, CT, USA) and 1% penicillin/streptomycin (100 U/mL)(Hyclone, Utah, USA). The cells were cultured in a humidified incubator at 37°C with 5% CO_2.

2.4 Vascular tension of mesenteric artery

The vascular tension of mesenteric vessels was detected using a microvascular tension detection system. The mesentery was separated from WKY rats and placed in a physiological salt solution (PSS) that had been pre-oxygenated and maintained at 4°C. The PSS buffer contained (in mmol/L): NaCl 130, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$-$7H_2O$ 1.17, $NaHCO_3$ 14.9, glucose 5.5, EDTA 0.026, and $CaCl_2$ 1.6, with a pH of 7.4. The adipose tissue surrounding the mesenteric vessel was discarded, and the secondary mesenteric arteries were trimmed to 1.8 mm and suspended in the mesenteric artery measurement system. The mesenteric arterial ring was mounted between two wires and fixed in a bath filled with 5 mL PSS, continuously bubbled with a gas mixture of 95% O_2 + 5% CO_2 at 37°C. The functional integrity of the endothelium was checked using ACh. Vascular ring irrigation was restored to basal tension using PSS buffer and stabilized for 10 min.

The tension of the vascular ring was stimulated by PE or ACh. The vascular ring was then reinstated to basal tension with PSS buffer containing PE for 5 min. After this, vascular ring irrigation was restored to basal tension using PSS buffer and stabilized with different stimuli for 20 min. The tension of the vascular ring was stimulated by PE again. The percentage relaxation was calculated using the following equation:

Percentage relaxation = $(1-R_{PE\text{-}max}/R_{Base\text{-}max}) \times 100\%$, where $R_{PE\text{-}max}$ is the PE-induced relaxation of the artery ring after incubation with DMSO, TA, 2-D08 or GA, and $R_{Base\text{-}max}$ is the PE-induced relaxation of the artery under baseline conditions.

2.5 NO synthase activity assay

NO synthase activity was determined using a detection kit (Beyotime Biotechnology, Shanghai, China). Briefly, HUVECs were washed with PBS before adding 100 μL of NOS assay reaction solution (comprising 50%NOS assay buffer, 38.8%MilliQ water, 5% L-Arginine solution, 5% 0.1 mmol/L NADPH, and 0.2% DAF-FM DA). Samples were incubated at 37°C for 60 min and then images were captured by imaging microscope at an excitation wavelength of 488 nm and an emission wavelength of 515 nm. The relative fluorescence unit (RFU) was calculated using the following equation: $RFU = (RFU_{Stimulated} - RFU_{Blank}) / (RFU_{Unstimulated} - RFU_{Blank})$.

2.6 Western blot analysis

Tissues or cells were lysed in RIPA buffer (Biosharp, Hefei, China). Equal amounts of total protein were loaded into the electrophoretic apparatus. Following SDS-PAGE, the resolved proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk at room temperature for 1 h and then probed with primary antibodies at 4°C overnight. After incubation with secondary antibodies for 1 h, the membranes were visualized using the Odyssey infrared imaging system (LI-COR, Lincoln, Nebraska, USA) and analyzed with Odyssey v3.0 software.

2.7 Co-immunoprecipitation assay

HUVECs were lysed for co-immunoprecipitation (co-IP) experiments after different stimuli for 24 h. An appropriate amount of cell lysis buffer containing protease inhibitors was added, and the mixture left on ice for 30 min. Protein concentration was measured, and the protein samples were incubated with ERK5 antibodies at 4°C overnight. Protein A/G Magnetic Beads (MedChemExpress, Shanghai, China) were then added, and incubation was continued at 4°C overnight. The conjugates were collected using a magnetic separator. The immune-precipitates were subjected to Western blotting using the indicated antibodies.

2.8 Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the ChromaFlashOne-Step Magnetic ChIP Kit (Epigentek) following the manufacturer's instructions[12]. A ChIP-grade KLF2 antibody was used to precipitate the promoter fragment of eNOS, which was subsequently analyzed by PCR.

2.9 Quantitative RT-PCR

Total RNA was extracted using the RNA simple kit following manufacturer's instructions (TIANGEN). RNA amplification and quantification were carried out using SYBR Green SuperMix reagent (TaKaRa). The 2−ΔΔCt method was employed to calculate relative RNA expression levels, with GAPDH used as an endogenous reference control. A 237-bp region of the eNOS promoter, spanning from −597 to −360 was amplified by PCR using a primer pair: Forward: 5'-ACAGAGGAGTCATCCTGCGA-3' and Reverse: 5'-GTCTGTGGGCGTAACATCCC-3'. A 306-bp region of the GAPDH gene was amplified using the following primer pair: Forward: 5'-TTGATTTTGGAGGGATCTCG-3') and Reverse: 5'-CAATGACCCCTTCATTGACC-3'.

2.10 Blood pressure monitoring

SHR aged 8-10 weeks were randomly grouped based on the baseline blood pressure measured using a tail sphygmomanometer (BP2010, Softron, Beijing, China). Normal WKY rats served as the control group. The animals received daily administrations of TA (100 mg/kg) and Nifedipine (0.2 mg/kg) for 4 weeks. Blood pressure measurements were recorded weekly throughout the study period.

2.11 Measurement of ET-1 and NO

Serum samples from abdominal aorta of rat were immediately centrifuged for supernatant to collect the supernatant. Serum levels of ET-1 and NO were measured using ET-1 and NO Elisa assay kits respectively (Meimian Biotech Co., Ltd., Yancheng, China).

2.12 Statistical analysis

Statistical analysis was performed using GraphPad prism version 7.0. Data are presented as mean ± SEM and were analyzed using one-way ANOVA for multiple comparisons, followed by Tukey's test as post hoc test. *P*-values less than 0.05 were considered statistically significant.

3 Results

3.1 Dose-dependent vasorelaxant effect of SUMO inhibitors on mesenteric aortic rings with intact endothelium

To explore the pharmacological effects of SUMO inhibitors on vascular smooth muscle, we investigated the concentration-dependent vasorelaxant effects of GA, 2-D08, or TA (at concentrations ranging from 50 to 200 μmol/L) on endothelium-intact rat mesenteric aortic rings[13]. These rings were pre-contracted with PE. Compared to the control group, the PE-induced constriction of mesenteric arteries was significantly attenuated by TA (Fig. 1A & B), 2-D08 (Fig. 1C & D), and GA (Fig. 1E & F) in a dose-dependent manner (Fig. 1G-L). Specifically, the constriction of mesenteric arteries of TA, 2-D08 and GA decreased by 89.88 ± 8.74%, 90.85 ± 6.25% and 88.19 ± 9.33% at 200 μmol/L, respectively.

3.2 ERK5 sumoylation-dependent vasorelaxant effect of SUMO inhibitors on mesenteric aortic rings

SUMOylation plays crucial roles in a wide variety of physiological and pathological processes by modifying specific substrate proteins[14]. One such substrate, ERK5, is known to be involved in maintaining endothelial cell homeostasis[15]. To investigate whether the vasorelaxant effects induced by SUMO inhibitors are linked to ERK5, we initially validated the interactions between ERK5 and SUMOs through co-IP assays. Ponatinib, a multi-targeted kinase inhibitor, has been reported to trigger ERK5 SUMOylation and promote inflammation in EC[12]. In comparison to the control group, ponatinib increased ERK5 SUMO modification in HUVECs, which was significantly decreased by pre-treatment with TA, 2-D08, or GA for 24 h (Fig. 2A-C). Next, we examined the vasorelaxant effect of ponatinib on mesenteric aortic rings. As expected, ponatinib diminished the vasorelaxation induced by TA (Fig. 2D & E, 12.88 ± 2.97%), 2-D08 (Fig. 2F & G, 12.56 ± 4.36%), or GA (Fig. 2H & I, 13.23 ± 4.79%). These findings indicate that the vasorelaxant effects of SUMO inhibitors on mesenteric aortic rings are dependent on ERK5 SUMOylation.

3.3 SUMO inhibitors induce vasorelaxation *via* ERK5/KLF2-dependent upregulation of eNOS/NO pathway

NO, a vasodilator synthesized by eNOS, plays a crucial role in maintaining vascular homeostasis[16]. Here, we investigated the impact t of SUMO inhibitors on NO production in HUVECs. Immunofluorescence analysis revealed that NO production was significantly enhanced by SUMO inhibitors, an effect that was attenuated by ponatinib (Fig. 3A). Furthermore, SUMO inhibitors dose-dependently increased eNOS activity, whereas ponatinib markedly reduced eNOS activity in the presence of SUMO inhibitors The activity of eNOS increased by 3.12 ± 0.21, 3.09 ± 0.51 and 3.41 ± 0.40 with TA, 2-D08 and GA. (Fig. 3B-D). The expression and function of eNOS are regulated by various transcriptional factors, including KLF2[17]. Previous studies have identified KLF2 as a key transcriptional activator of ERK5[18]. To explore whether ERK5 SUMOylation affects the transcriptional activity of the eNOS promoter regulated by KLF2, we examined ERK5 and SUMO1 co-localization in HUVEC nuclei using confocal microscopy. Following treatment with TA, ERK5 exhibited a cytoplasm distribution pattern, while ponatinib increased ERK5 accumulation and its co-localization with SUMO-1 in the nucleus. As expected, pretreatment with TA decreased translocation of ERK5 into the nucleus and ERK5-SUMO1 co-localization compared to the ponatinib group (Fig. 3E). Western blot analysis demonstrated increased expression of KLF2 and phosphorylated eNOS (p-eNOS) proteins in HUVECs treated with TA for 24 h. Conversely, ponatinib significantly decreased the expression of KLF2 and p-eNOS proteins (Fig. 3F-H). In addition, ponatinib and TA group of the protein level of KLF2 increased by 117.02 ± 11.30% and the protein level of p-eNOS decreased by 102.90 ± 4.50% (Fig. 3G-H). Chromatin immunoprecipitation followed by PCR (ChIP-PCR) confirmed that TA enhanced KLF2-dependent eNOS promoter activity in HUVECs, and this effect was antagonized by ponatinib (Fig. 3I). Consistently, TA increased eNOS mRNA levels, which were reversed by ponatinib treatment (Fig. 3J). These results suggest that SUMO inhibitors suppress ERK5 SUMOylation, leading to increased transcriptional activities of KLF2 and eNOS genes, thereby promoting NO production and vascular homeostasis.

3.4 Tannic acid decreases blood pressure and improves vascular function in SHR

To determine the anti-hypertensive effect of SUMO inhibitors *in vivo*, TA was administered to SHR by gavage for 4 weeks. During the daily oral administration of TA and Nifedipine, the blood pressure in the TA and Nifedipine group continuously decreased compared to the non-treated control SHR group (Fig. 4A-D). The blood pressure of TA group decreased from 215.82 ± 8.64 to 172.36 ± 9.64 for 4 weeks. Additionally, serum NO levels were elevated following TA treatment compared to the SHR group (Fig. 4E), while serum ET-1 levels were reduced by TA group (Fig. 4F). TA treatment significantly diminished the PE-induced constriction of mesenteric arteries and enhanced the ACh-induced vasorelaxation in a dose-dependent manner compared to the SHR group (Fig. 4G-K). Furthermore, the expression levels of KLF2 and p-eNOS proteins were markedly increased in SHR treated with TA for 4 weeks (Fig. 4 L-O). These results suggest that TA effectively lowers blood pressure, enhances NO production, reduces ET-1 levels, and improves vascular function

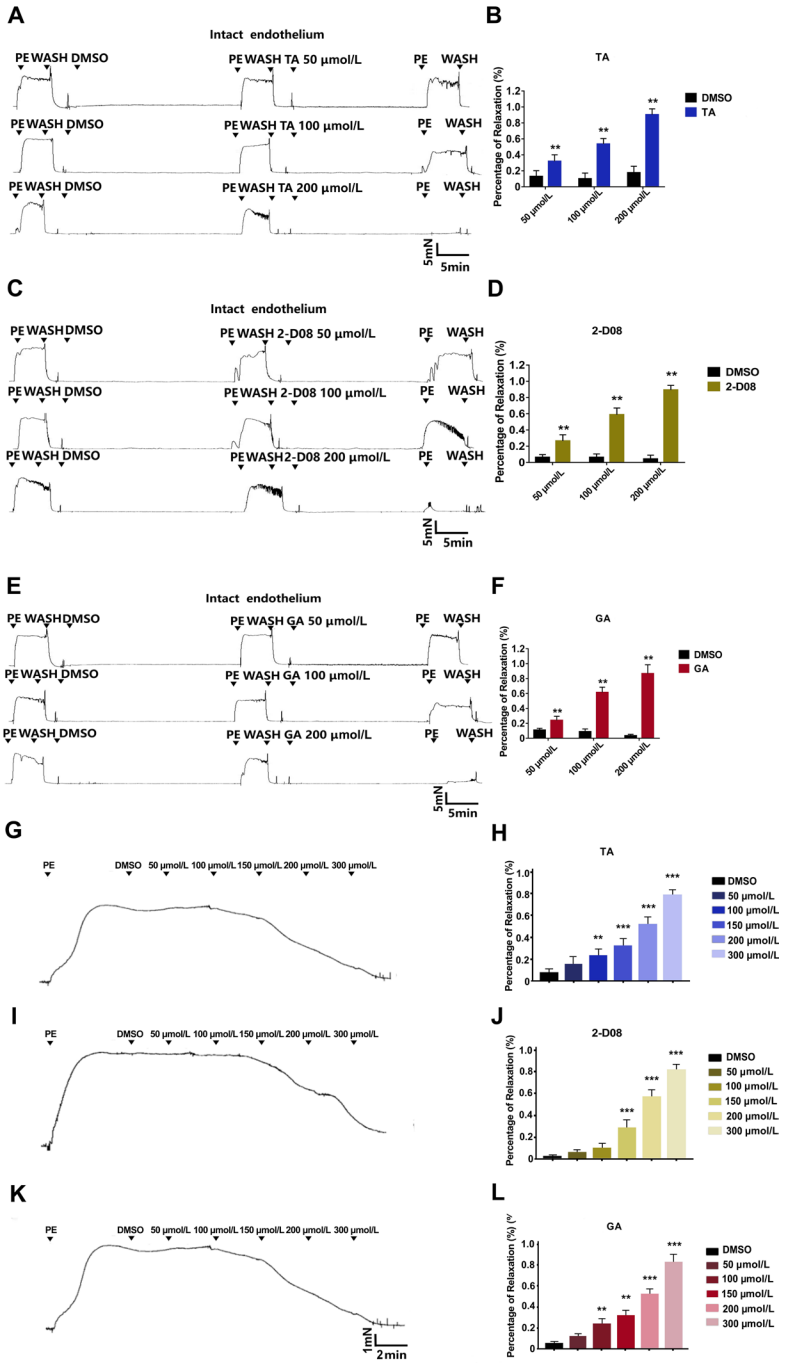

Fig. 1 SUMO inhibitors exert vasorelaxant effect on phenylephrine (PE)-preconstricted endothelium-intact mesenteric artery rings in a dose-dependent manner The effects of Tannic acid (TA) at different concentrations on PE-preconstricted mesenteric artery rings were measured by (A) the typical recording and (B) bar chart on statistical data. The effects of 2-D08 at different concentrations on PE-preconstricted mesenteric artery rings were measured by (C) the typical recording and (D) statistical data. The effects of Ginkgolic acid (GA) at different concentrations on PE-preconstricted mesenteric artery rings were measured by (E) the typical recording and (F) statistical data. The typical recording (G) and relative bar graph (H) showed PE-induced contraction in isolated artery after acute stimulation with TA for 2 min. The typical recording (I) and statistical data (J) showed PE-induced contraction in isolated artery after acute stimulation with 2-D08 for 2 min. The typical recording (K) and statistical data (L) showed PE-induced contraction in isolated artery after acute stimulation with GA for 2 min. $N = 6$. Data are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control.

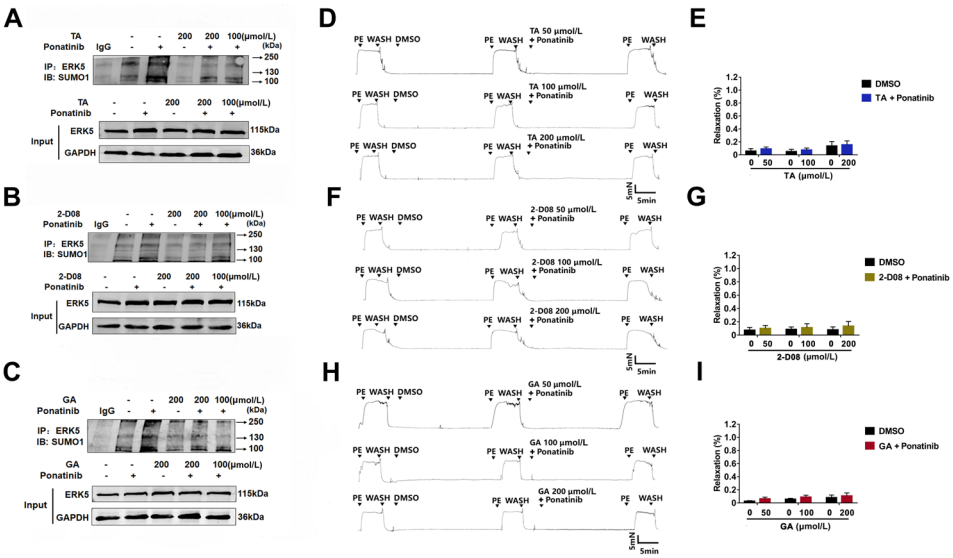

Fig. 2 Ponatinib eliminates the vasorelaxant effect of SUMO inhibitors on vasorelaxation of phenylephrine (PE)-preconstricted endothelium-intact mesenteric artery rings via extracellular signal-regulated kinase 5 (ERK5) SUMOylation

The interaction between ERK5 and SUMO was detected by co-immunoprecipitation (Co-IP) in HUVECs after incubation with (A) Tannic acid (TA), (B) 2-D08, and (C) Ginkgolic acid (GA) with or without ponatinib (200 nmol/L) for 24 h. $N = 3$. The contraction-responses of TA with pre-treatment of ponatinib (200 nmol/L) for 20 min to PE-induced constriction by (D) the typical recording and (E) bar chart on statistical data. The contraction-responses of 2-D08 with pre-treatment of ponatinib (200 nmol/L) for 20 min to PE-induced constriction by (F) the typical recording and (G) statistical data. The contraction-responses of GA with pre-treatment of ponatinib (200 nmol/L) for 20 min to PE-induced constriction by (H) the typical recording and (I) statistical data. $N = 6$. Data are expressed as mean ± SEM.

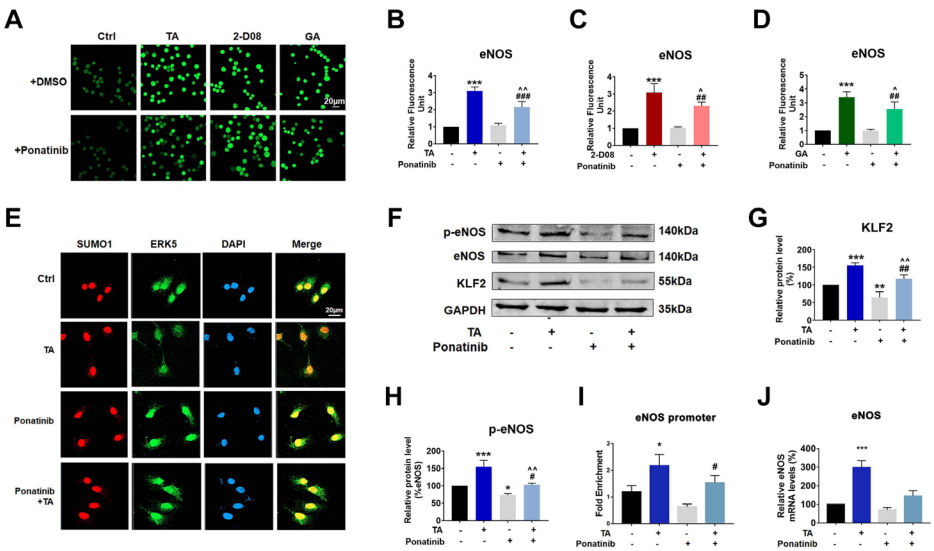

Fig. 3 SUMO inhibitors increase the expression of NO by activating the ERK5/KLF2/eNOS pathway in HUVECs

(A) The level of NO (green) was detected by immunofluorescence after treatment with SUMO inhibitors with or without ponatinib. Relative fluorescence unit of eNOS in HUVECs after incubation with (B) Tannic acid (TA), (C) 2-D08, and (D) Ginkgolic acid (GA) with or without ponatinib. $N = 5$. Data are expressed as mean ± SEM. $^{***}P < 0.001$, compared to the control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to the ponatinib group; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ compared to the TA, 2-D08 or GA group. (E) The co-localization of ERK5 (green) and SUMO1 (red) was observed under a confocal microscope. Scale bar: 20 μm. (F-H) The expression levels of KLF2 (F-G) and p-eNOS (H) measured by Western blot analysis and statistical data. (I) The transcriptional activity of eNOS promotor assayed by ChIP-PCR. (J) The relative mRNA levels of eNOS detected by real-time PCR; $N = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to the ponatinib group; $^{\wedge\wedge}P < 0.01$ compared to the TA group.

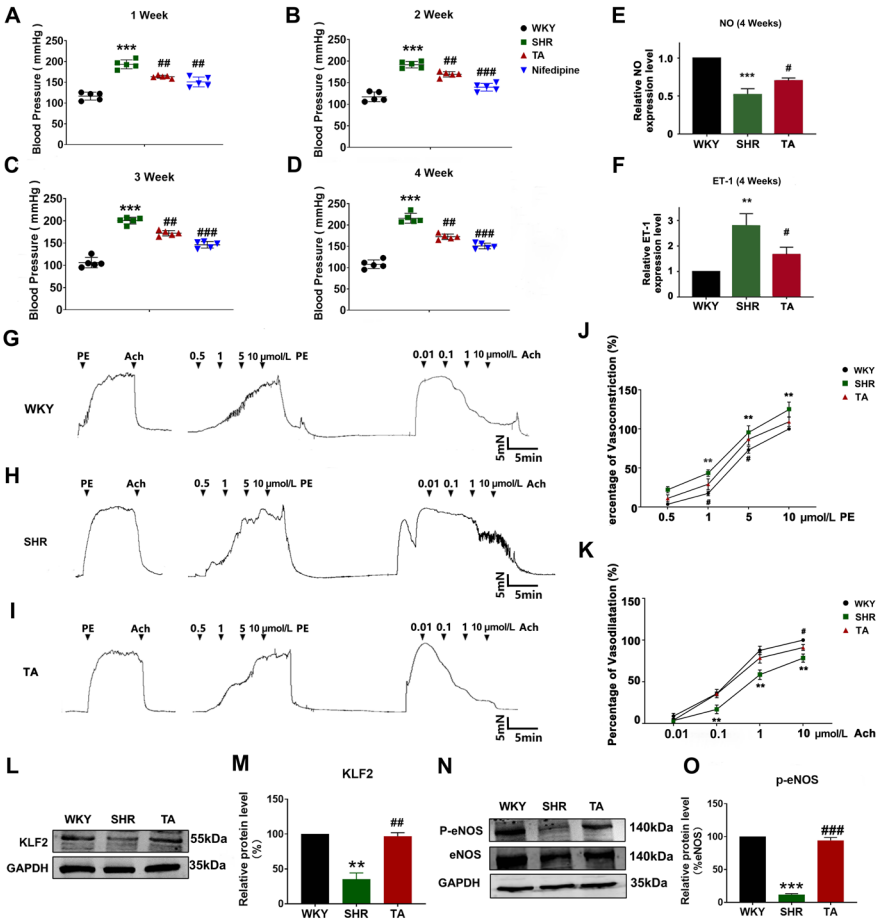

Fig. 4 Tannic acid decreases blood pressure and improves vascular dysfunction in WKY rats and spontaneous hypertension rat (SHR)

(A-D) Blood pressure was monitored in SHR after administration with (100 mg/kg) and Nifedipine (0.2 mg/kg) for 4 weeks, Nifedipine was used as a positive control drug. (E) The contents of NO and (F) ET-1 in serum of SHR after Tannic acid (TA) administration for 4 weeks. (G-I) Representative traces showed the effect of TA on acetylcholine (0.01 to 10 μmol/L)-induced dilation and phenylephrine (PE) (0.5 to 10 μmol/L)-induced contraction in isolated mesenteric artery rings. (J) Contraction-response curves of PE-induced in mesenteric artery rings in response to TA. (K) Dilation-response curves to ACh-induced in mesenteric artery rings in response to TA; $N = 7$. (L-O) The expression level of Krüppel-like factor 2 (KLF2) and p-eNOS in mesenteric artery were measured by Western blot analysis $N = 3$. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to WKY group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to SHR group.

by modulating the KLF2 and eNOS pathways.

4 Discussion

The present study provides strong evidence that the SUMO inhibitors decrease blood pressure and improve vascular dysfunction by inhibiting ERK5 SUMOylation. This inhibition represses the transcriptional activity of KLF2 and enhances the eNOS/NO pathway (Fig. 5).

The known risk factors for hypertension include heritable, lifestyle, and environmental factors[19]. Numerous epidemiological studies have shown that cold exposure is closely related to hypertension[20-21]. Jennifer *et al.* found that blood pressure was closely related to daily temperature among six geographically and climatically diverse US cities[22]. Similarly, Kim *et al.* demonstrated that the systolic blood pressure (SBP) and diastolic blood pressure (DBP) of cold-exposure workers were significantly increased[23]. Furthermore, a large-scale retrospective study showed that the mean SBP/DBP of Asian population increased by approximately 10/4 mmHg in winter[24]. Fregly *et al.* proved that chronic exposure to cold-stress stimulation (5°C) for 3-5 weeks increased BP in rats[25-26]. Pan *et al.* showed that the mean SBP/DBP of mice under 4°C cold-stress stimulation was 112.00 ± 2.14/102.86 ± 2.01 mmHg, while the mean SBP/DBP of mice kept at 25°C was 99.69 ± 2.51/93.00 ± 2.26 mmHg[27]. Specifically, Zhu *et al.* suggested that exposure to cold-stress stimulation (5 ± 2°C for 4 h daily)

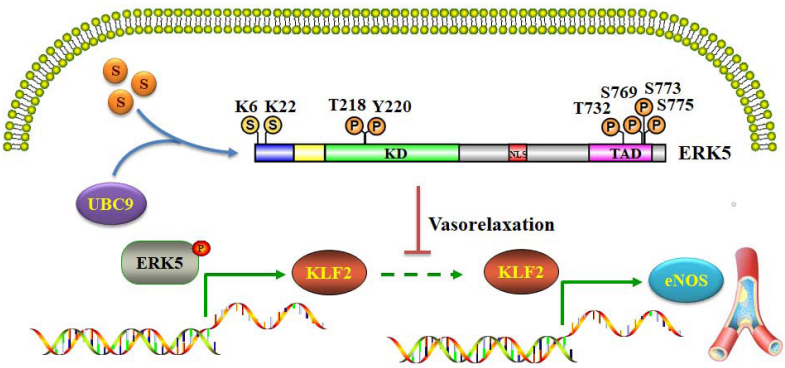

Fig. 5 A schematic illustration drawing the molecular mechanism of SUMO inhibitors decreased blood pressure and improved vascular dysfunction by inhibiting ERK5 SUMOylation via repressing the transcriptional activity of Krüppel-like factor 2 (KLF2) and enhancing the eNOS/NO pathway

significantly attenuated the ACh-induced vasorelaxation and increased SBP in rats by reducing eNOS in aortic vessels[28]. Chen *et al.* also demonstrated that knockdown of endothelin-1 (ET-1) significantly increased BP within 2 weeks of exposure to cold (6.7 ± 2°C) in male rats, reaching a peak level by 6 weeks[29]. Our study demonstrated that SUMO inhibitors exert a vasodilatory effect in SHR. However, whether SUMO inhibitors attenuate cold-induced hypertension needs to be further explored.

SUMOylation is a dynamic and reversible post-translational modification that requires a cascade of enzymes, including an E1-activating enzyme (SAE1/SAE2), an E2-conjugating enzyme (Ubc9), and multiple E3 ligases[30]. Given the dysregulation of SUMOylation implicated in many common diseases, efforts have been devoted to developing selective inhibitors targeting various enzymes involved in SUMOylation, including but not limited to SUMO-E1 inhibitors, SUMO-E2 inhibitors, SUMO-E3 inhibitors, and SENP inhibitors[4]. The first drug inhibiting the SUMO pathway, TAK-981, is currently being evaluated in clinical trials for metastatic solid tumors and non-Hodgkin's lymphoma[31]. Two natural compounds ginkgolic acid and anacardic acid have been found to have antiviral effects against coronavirus disease 2019[32]. Our group has previously revealed that ginkgolic acid alleviated cardiac fibrosis induced by myocardial infarction[33]. This study is the first to demonstrate that SUMO inhibitors significantly lower blood pressure by inhibiting ERK5 SUMOylation while activating the KLF2/eNOS/NO pathway. ERK5, acting both as a kinase and a transcription co-activator, is a key target of SUMOylation implicated in the pathogenesis of atherosclerosis[3]. ERK5's structure includes a large C-terminal domain with transcriptional activation capability in addition to an N-terminal canonical kinase domain[34-35]. Previous studies have demonstrated that SUMO modification of ERK5 at residues Lys6/Lys22 is required for ERK5 nucleo-cytoplasmic shuttling and its transcriptional activation. Paez-Mayorga1 *et al.* proposed that ERK5 SUMOylation counteracts its transcriptional activity in endothelial inflammation because ponatinib, an ERK5 SUMOylation trigger, diminishes the expression of ERK5-responsive genes such as KLF2/4 and eNOS[10]. Woo *et al.* reported that the expression of Ubc9 or PIAS1 (E3 ligase) significantly attenuates ERK5-induced KLF2 expression and subsequent eNOS expression, suggesting that ERK5 SUMOylation regulates transcriptional activity independently of ERK5 phosphorylation and kinase activity[9]. In contrast, mutation of these SUMO sites impairs ERK5's ability to translocate to the nucleus. Moreover, overexpression of SUMO protease SENP2 completely abolishes endogenous ERK5 nuclear localization in response to epidermal growth factor stimulation[11]. These results align with our data showing that incubation of EC with SUMO inhibitors significantly inhibited ERK5 SUMOylation and its nuclear distribution.

Considerable evidence supports the role of the MEK/ERK5/KLF2/eNOS pathway in promoting endothelial cell homeostasis[36]. Angolano *et al.* demonstrated that overexpression of the anti-inflammatory protein TNFAIP3 increased eNOS transcriptional activity in an ERK5-dependent manner, with subsequent upregulation of the eNOS transcriptional regulator KLF2 in EC, preventing EC dysfunction in atherosclerosis[37]. Li *et al.* indicated that Scutellarin attenuated vasospasm and neurological deficits *via* upregulating the expression of phosphorylated ERK5, KLF2, and eNOS after subarachnoid hemorrhage[38]. Lee *et al.* identified AMPK-dependent HDAC5/ERK5 signaling as a key mediator of eNOS and KLF2 expression in response to betulinic acid (BA), providing strong evidence for the use of BA to prevent endothelial dysfunction and treat vascular diseases[39]. Heo *et al.* demonstrated that disturbed flow-induced endothelial cell inflammation occurs *via* PKCζ-ERK5 interaction-mediated decrease in KLF2/eNOS stability, leading to PKCζ-mediated p53-SUMOylation and EC apoptosis[40].

To date, efforts have been focused on the developing selective inhibitors targeting proteins in the SUMOylation cascade, which

could be beneficial for treating various diseases. Small molecule SUMOylation inhibitors are typically derived from natural products, peptidomimetics, and synthetic derivatives. GA and TA, derived from natural products, inhibit SUMOylation by blocking the formation of the E1-SUMO complex. GA was the first identified SUMOylation inhibitor. TA, a nontoxic SUMOylation inhibitor, has been a useful tool for studying the SUMOylation pathway without affecting cell viability. 2-D08 inhibits the SUMOylation pathway by preventing the transfer of SUMO from UBC9 to the substrate. Compared to a nontoxic dose of GA (10 mmol/L) or 2-D08, TA is more efficient in decreasing levels of SUMOylated substrates and shows little cytotoxicity in HepG2 cells after longer exposure times[41]. These studies suggest that TA has greater potential for clinical applications. Thus, in this study, we focused on the anti-hypertensive effect of TA *in vivo*.

In this study, we employed the SHR model to investigate the impact of TA on blood pressure. Given the multifactorial etiology of hypertension, which includes factors such as environmental temperature and dietary influences, it is imperative to utilize a diverse array of animal models to comprehensively assess the therapeutic efficacy of TA in managing hypertension. Furthermore, a range of cellular models, including primary endothelial cells, should be utilized for *in vitro* investigations. In short, translational research focusing on disease modeling and drug discovery is essential to fully elucidate the therapeutic potential of small molecule SUMOylation inhibitors in both *in vitro* and *in vivo* contexts.

Our study showed that TA at a concentration of 100 mg/kg lowered blood pressure in SHR with appreciable effectiveness and safety. While considerable effort has been devoted to developing SUMO-E1 and SENP inhibitors, there has been less focus on developing SUMO-E2 inhibitors. Due to the lack of substrate specificity, many SUMO inhibitors suffer from low potency or poor isoform selectivity and have the potential to disrupt normal physiologic processes. Furthermore, many inhibitors exhibit activities in the micromolar concentration range, indicating the needs and opportunity to develop more selective and potent SUMOylation inhibitors for clinical applications.

Author contributions

Yang B F, Zhao D and Liu Y designed the experiments and supervised the project. Tang N N, Li J T and Wang Z were responsible for the manuscript writing. Zuo J L and Zhang Z F analyzed the data. Sun Y L, Li X, Mu R X, Huang D, Han Y N and Chen Y Q performed the *in vitro* experiments. Ma Q X, Zhang J, Wu J Y, Wang H, Zhao H X and Dong X L conducted the in vivo experiments. Wang Z G revised the manuscript.

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Ethics approval

All animal experiments were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care International and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (Ethical approval number: IRB3017723).

Conflict of interest

Yang B F is the editor-in-chief of Frigid Zone Medicine. The article was subject to the journal's standard procedures, with peer review handled independently of him and his research groups.

Data availability statement

All data in this studyare available from from the corresponding author, on request.

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