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RESEARCH PAPER

Ginsenoside Rb3 attenuates oxidative stress and preserves endothelial function in renal arteries from hypertensive rats

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BACKGROUND AND PURPOSE

Panax ginseng is commonly used to treat cardiovascular conditions in Oriental countries. This study investigated the mechanisms underlying the vascular benefits of ginsenoside Rb3 (Rb3) in hypertension.

EXPERIMENTAL APPROACH

Rings of renal arteries were prepared from spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats and were cultured *ex vivo* for 8 h. Contractile responses of the rings were assessed with myograph techniques. Expression of NADPH oxidases was assessed by Western blotting and immunohistochemistry. Reactive oxygen species (ROS) were measured using dihydroethidium fluorescence imaging and production of NO was determined using the fluorescent NO indicator DAF-FM diacetate in human umbilical vein endothelial cells.

KEY RESULTS

Ex vivo treatment with Rb3 concentration-dependently augmented endothelium-dependent relaxations, suppressed endothelium-dependent contractions and reduced ROS production and expressions of NOX-2, NOX-4 and $p67^{phox}$ in arterial rings from SHR. Rb3 treatment also normalized angiotensin II (Ang II)-stimulated elevation in ROS and expression of NOX-2 and NOX-4 in arterial rings from WKY rats. Rb3 inhibited Ang II-induced reduction of NO production and phosphorylation of endothelial NOS in cultures of human umbilical vein endothelial cells. Rb3 also inhibited oxidative stress in renal arterial rings from hypertensive patients or in Ang II-treated arterial rings from normotensive subjects.

CONCLUSION AND IMPLICATIONS

Ex vivo Rb3 treatment restored impaired endothelial function in arterial rings from hypertensives by reversing over-expression of NADPH oxidases and over-production of ROS, and improved NO bioavailability. Our findings suggest that medicinal plants containing Rb3 could decrease oxidative stress and protect endothelial function in hypertension.

Abbreviations

Ang II, angiotensin II; DHE, dihydroethidium; DMSO, dimethyl sulphoxide; DPI, diphenylene iodonium; HUVEC, human umbilical vein endothelial cells; L-NAME, N^G-nitro-L-arginine methyl ester; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; SNP, sodium nitroprusside

Introduction

Panax ginseng is used clinically to alleviate cardiovascular disorders in traditional Oriental medicines and most of the pharmacological effects of ginseng have been attributed to the bioactive constituents, ginsenosides. Approximately 30 ginsenosides have been chemically identified, many of which exert a wide spectrum of pharmacological actions, particularly in protection of neuronal function on animal models of cerebral ischaemia and neuro-degeneration (Ye *et al.*, 2011; Fang *et al.*, 2012; Zhu *et al.*, 2012). Several ginsenosides protect against homocysteine-induced impairment of endothelium-dependent relaxations (Zhou *et al.*, 2005) and inhibit platelet aggregation (Lee *et al.*, 2010). These studies suggest therapeutic potentials of ginsenosides, possibly as adjuncts to existing therapies for vascular diseases.

A number of ginsenosides including Rb1, Rbg, Re and two ginsenoside metabolites, protopanaxadiol and protopanaxatriol stimulated NO production in human endothelial cells (Chen, 1996; Leung *et al.*, 2007; 2009). Ginsenoside Rb1 inhibits H_2O_2-induced senescence of endothelial cells through preserving NO bioavailability (Liu *et al.*, 2012), whereas *Panax notoginseng* lowers blood pressure in spontaneously hypertensive rats (SHRs) and its active constituents, Rb1 and Rg1 dilate arteries through an endothelial NOS (eNOS)-dependent mechanism (Pan *et al.*, 2012; nomenclature follows Alexander *et al.*, 2013). Both ginsenosides Rb1 and Rb3 (Figure 1) contain the same glycone as protopanaxadiol. Rb3 exerted an anti-diabetic effect (Bu *et al.*, 2012), suppressed angiotensin II (Ang II)-stimulated proliferation of vascular smooth muscle cells (Wang *et al.*, 2010b) and inhibited experimentally induced myocardial dysfunction (Wang *et al.*, 2010a). However, the potential benefits of Rb3 against vascular dysfunction have not been explored. Therefore the present study aimed to examine whether *ex vivo* treatment with Rb3 could restore the impaired endothelial function in arteries from hypertensive rats and humans through limiting oxidative stress as the key cellular mechanism.

Methods

Animals and artery preparation

All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996) and were approved by CUHK Animal Experimentation Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 22 animals were used in the experiments described here.

SHRs and normotensive Wistar-Kyoto (WKY) rats of ~6 months old were purchased from the Chinese University of Hong Kong (CUHK) Animal Service Centre. Rats were killed by CO_2 inhalation, and intralobal renal arteries from both sides were rapidly dissected out and placed in Krebs solution containing (in mmol·L^{-1}) 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 11 D-glucose. Each artery was cleaned of adhering connective tissues and cut into four ring segments with length ~1.6 mm for functional studies in a Multi Myograph System (Danish Myo Technology, Aarhus, Denmark). Briefly, ring segments were suspended between two stainless steel wires passing through the lumen and fixed to the jaws of the myograph, which was filled with 5 mL Krebs solution oxygenated with 95% O_2 and 5% CO_2 and maintained at 37°C, (pH of ~7.4). All rings were initially stretched to an optimal resting tension of 2.5 mN and equilibrated at 37°C for 60 min before the start of experiments. Changes in isometric force were continuously recorded (Wong *et al.*, 2010a).

Vascular reactivity assay

Each ring was first contracted by phenylephrine (Phe, 1 μmol·L^{-1}). Once a sustained tension was reached, either ACh (3 nmol·L^{-1}–10 μmol·L^{-1}) or sodium nitroprusside (SNP; 1 nmol·L^{-1}–10 μmol·L^{-1}) was added cumulatively to evoke endothelium-dependent or endothelium-independent relaxations respectively. In the first set of experiments, SHR renal

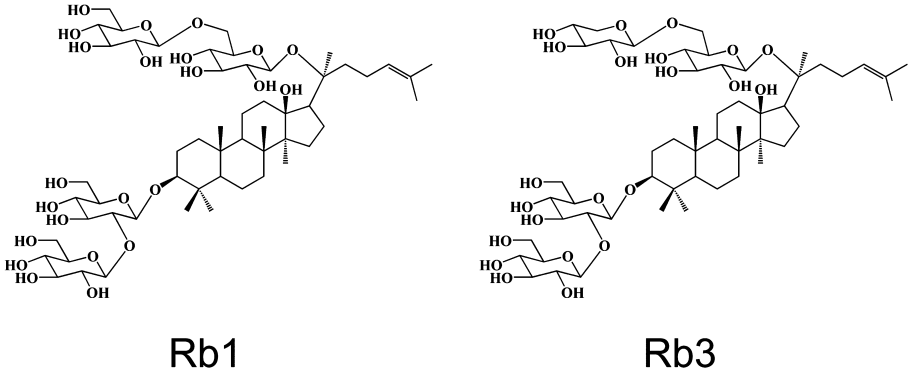

Figure 1
Chemical structure of ginsenosides Rb1 and Rb3.

arteries with intact endothelium were exposed to Rb3 at three concentrations (0.1–1 μmol·L^{-1}) for 8 h. After *ex vivo* treatment, rings were transferred to myograph chambers for evaluation of ACh-induced relaxations. In the second series of experiments, after 8 h treatment of WKY renal arteries with Ang II (100 nmol·L^{-1}), rings were collected for assessment of vascular reactivity and changes in marker proteins for oxidative stress. Losartan (angiotensin receptor antagonist, 3 μmol·L^{-1}), tempol [reactive oxygen species (ROS) inhibitor, 100 μmol·L^{-1}] or diphenylene iodonium (DPI; putative NADPH oxidase inhibitor, 100 nmol·L^{-1}) were used as positive controls to improve endothelial function in comparison with the effect of Rb3. For the last set of experiments studying endothelium-dependent contractions, SHR renal arteries or Ang II-treated WKY renal arteries were treated for 30 min with N^{G}-nitro-L-arginine methyl ester (L-NAME, 100 μmol·L^{-1}) to inhibit the basal or ACh-stimulated production of NO, and then contractions were triggered by ACh (30 nmol·L^{-1}–100 μmol·L^{-1}) in control or in the presence of Rb3 (in three concentrations). For *ex vivo* tissue culture, SHR renal arteries were cultured in DMEM (Gibco, Gaithersgerg, MD, USA) in the presence of Rb3, or WKY renal arteries were exposed to Ang II (100 nmol·L^{-1}) with and without co-treatment with Rb3. *Ex vivo* treatment refers to 8 h tissue incubation.

Human arteries

The use of human renal arteries complied with the principles outlined in the Declaration of Helsinki and was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. Arteries were isolated from nephrectomy specimens of normotensive subjects or hypertensive patients (having systolic and diastolic pressure >140 mmHg and 90 mmHg respectively) with their consent. The mean age was 61.2 years (range between 43 and 75 years) for hypertensive patients and 51 years (range between 27 and 70 years) for normotensive subjects. Renal arteries from hypertensive patients were incubated overnight in the presence or absence of Rb3 (1 μmol·L^{-1}) in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco) at 37°C. Renal arteries from normotensive patients were incubated with or without Rb3 before the addition of Ang II (1 μmol·L^{-1}). Due to the limited supply of human specimens, all arteries were used for immunohistochemistry and Western blotting.

Western blot analysis

After drug treatment, renal arteries were homogenized in ice-cold RIPA lysis buffer (1 μg·mL^{-1} leupetin, 5 μg·mL^{-1} aprotonin, 100 μg·mL^{-1} PMSF, 1 mmol·L^{-1} sodium orthovanadate, 1 mmol·L^{-1} EGTA, 1 mmol·L^{-1} EDTA, 1 mmol·L^{-1} NaF and 2 mg·mL^{-1} β-glycerolphosphate). The homogenates were centrifuged at 20 000× *g* for 20 min at 4°C. The supernatant was collected, and the protein concentration was determined using the Lowry method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein in samples were separated by electrophoresis on 10% SDS-PAGE and then transferred onto an immobilon-P PVDF membrane (Millipore, Billerica, MA, USA). Non-specific binding sites were blocked by 5% non-fat milk or 1% BSA in 0.05% Tween-20 PBST, and then incubated for 8 h at 4°C with primary antibodies against NADPH oxidase subunits (NOX-2, NOX-4, p67^{phox}), nitrotyrosine, phospho-eNOS and eNOS at a dilution of 1:500. The blots were incubated with appropriate secondary antibodies at a 1:3000 dilution for 1 h at room temperature, and then washed three times for 20 min in PBST. The membranes were finally developed with an enhanced chemiluminescence detection system (ECL reagents, Amersham Pharmacia, Piscataway, NJ, USA) and finally exposed to X-ray films. Equal protein loading was verified with use of a housekeeping anti-GAPDH antibody (Ambion, Austin, TX, USA).

Immunofluorescence microscopy

The expression of NAD(P)H oxidase subunits in human arteries were visualized by immunofluorescence microscopy (Dong *et al.*, 2012). Briefly, arteries were embedded in OCT compound (Sakura Finetek, Torrance, CA, USA), snap frozen and cut into 10 μm thick cryostat sections that were fixed in 4% paraformaldehyde for 30 min and treated with 0.05% Triton X in PBS for 1 min. The sections were blocked with 5% normal donkey serum for 1 h at room temperature. Primary antibodies against NOX-2, NOX-4 and p67^{phox} (Abcam, Cambridge, MA, USA) were incubated overnight at 4°C. After several washes in PBS, the sections were incubated with appropriate Alexa Fluor 546 IgG (Invitrogen, Molecular Probes, Carlsbad, CA, USA) for 1 h at room temperature. After cover-slipping, the sections were observed under the confocal microscope Olympus Fluoview FV1000 (Olympus America Inc., Melville, NY, USA). Images were first acquired with Olympus Fluoview software (version 1.5; FV10-ASW1.5) and then merged using the SPOT advanced software (Version 4.6, Diagnostic, Instruments, Sertling Heights, MI, USA).

Detection of ROS by dihydroethidium (DHE) fluorescence

ROS in arteries was detected using DHE fluorescent dye. Renal arteries were embedded in OCT compound (Sakura Finetek), snap frozen in liquid nitrogen and cut into 10 μm thick cryo-sections. After 15 min incubation in DHE (5 μmol·L^{-1}: Molecular Probes, Eugene, OR, USA) at 37°C, sections were imaged by confocal microscopy system of Olympus Fluoview FV1000 with excitation and emission wave lengths of 515 nm and 585 nm respectively (Dong *et al.*, 2012). Results are shown as percentage fluorescence intensity of the control.

NO measurement

Human umbilical vein endothelial cells (HUVECs) was purchased from Lonza (Walkersville, MD, USA) and cultured in endothelial growth medium. These cells seeded on glass coverslips were loaded with 1 μmol·L^{-1} fluorescent NO indicator DAF-FM diacetate (Molecular Probes) in the dark at 24°C for 10 min, washed for 20 min and placed in a chamber for fluorescence imaging. Fluorimetric measurements of intracellular NO production were performed using the Olympus Fluoview laser scanning confocal system (FV1000, Olympus, Tokyo, Japan) mounted on an inverted IX81 Olympus microscope. The amount of NO produced in response to 1 μmol·L^{-1} A23187 was recorded by measuring the fluorescence intensity excited at 488 nm and emitted at 515 nm (Wong *et al.*, 2011). Changes in intracellular NO in HUVECs are shown as a ratio of fluorescence relative to the intensity (F_1/F_0) and analysed by the Fluoview software (Olympus).

Materials

ACh, phenylephrine, L-NAME, SNP, tempol, losartan, DPI, actinomycin D dimethyl sulphoxide (DMSO) and A23187 were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Ang II was from Tocris Bioscience (Avonmouth, UK), and Rb3 was from the National Institutes for Food and Drug Control, China. Rb3, ACh, L-NAME, phenylephrine, tempol and Ang II were dissolved in distilled water and other drugs in DMSO. DMSO 1% (v/v) did not modify ACh-induced relaxations or contractions in arteries.

Data analysis

Results represent means ± SEM of n separate experiments. Relaxations were expressed as percentage reduction in phenylephrine-induced contraction. ACh-induced contractions were expressed as the percentage of KCl (60 mmol·L^{-1})-induced contraction, which was comparable among different treatment groups. Data were analysed by one-way ANOVA followed by Bonferroni *post hoc* tests whenever appropriate (GraphPad Software, San Diego, CA, USA). $P < 0.05$ indicates a statistical difference between group means.

Results

Rb3 improves endothelial function in SHR renal arteries

To examine the protective effect of Rb3 on endothelial function, both endothelium-dependent relaxation and endothelium-dependent contraction were studied. Representative traces in Figure 2A show that rings of renal arteries from SHR relaxed significantly less in response to ACh than arteries from WKY. The NOS inhibitor L-NAME (100 µmol·L^{-1}) abolished ACh-induced relaxations without affecting SNP-induced relaxations (data not shown). *Ex vivo* treatment with Rb3 (0.1–1 µmol·L^{-1}) improved ACh-induced relaxations in SHR arteries in a concentration-dependent manner (Figure 2C). In the presence of L-NAME (100 µmol·L^{-1}) to block production of endothelium-derived NO, cumulative addition of ACh triggered contractions in SHR arteries but not in WKY arteries (Figure 2B). Such contractions were absent in arteries without endothelium (data not shown). Rb3 (0.1–1 µmol·L^{-1}) concentration-dependently inhibited ACh-induced contractions in SHR arteries after the 8 h *ex vivo* treatment (Figure 2B and D). By contrast,

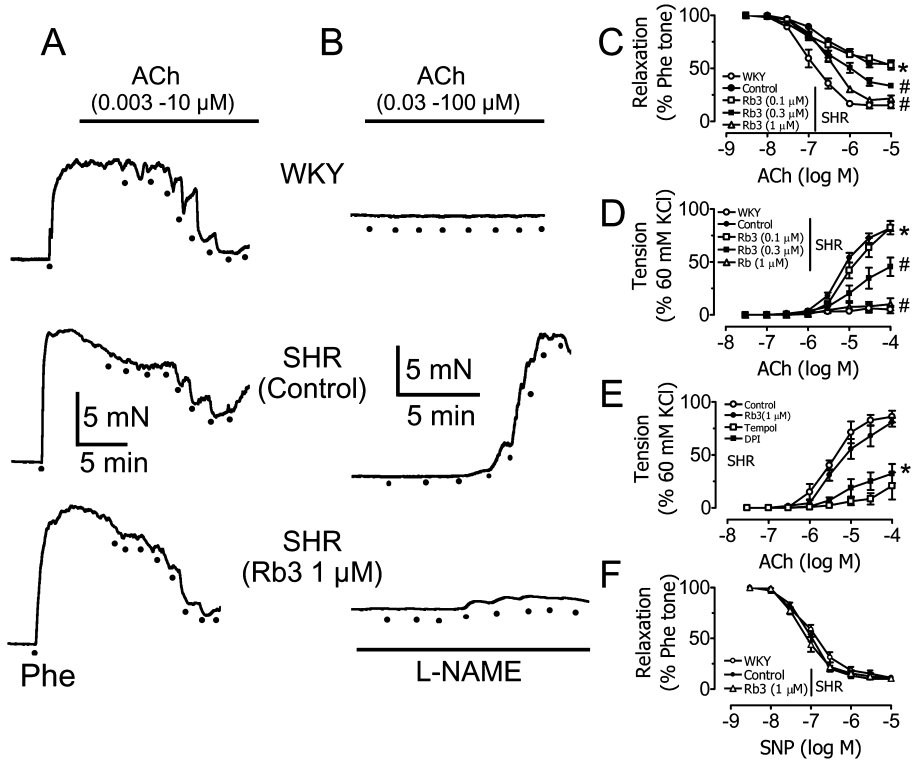

Figure 2

ACh-induced relaxations in phenylephrine (Phe)-contracted SHR renal arteries (A & C) and ACh-induced contractions (B & D) in L-NAME-treated SHR renal arteries with and without *ex vivo* 8 h treatment with Rb3 (0.1–1 µmol·L^{-1}). Acute treatment (30 min) with Rb3 did not affect ACh-induced contractions, whereas 100 µmol·L^{-1} tempol or 100 nmol·L^{-1} DPI inhibited the contractions (E). SNP-induced relaxations were similar between SHR and WKY and unaffected by *ex vivo* treatment with 1 µmol·L^{-1} Rb3 (F). Results are means ± SEM of four to six rings from different rats. *$P < 0.05$, significant difference between WKY and SHR; #$P < 0.05$, significant difference between SHR control and Rb3 group.

30 min incubation with 1 μmol·L^{-1} Rb3 did not inhibit ACh-induced contractions (Figure 2E). ACh-induced contractions were reversed by co-treatment with the ROS inhibitor tempol (100 μmol·L^{-1}) or the NADPH oxidase inhibitor DPI (100 nmol·L^{-1}) (Figure 2E). By contrast, SNP-induced endothelium-independent relaxations did not differ between SHR and WKY arteries and not affected by Rb3 treatment (Figure 2F).

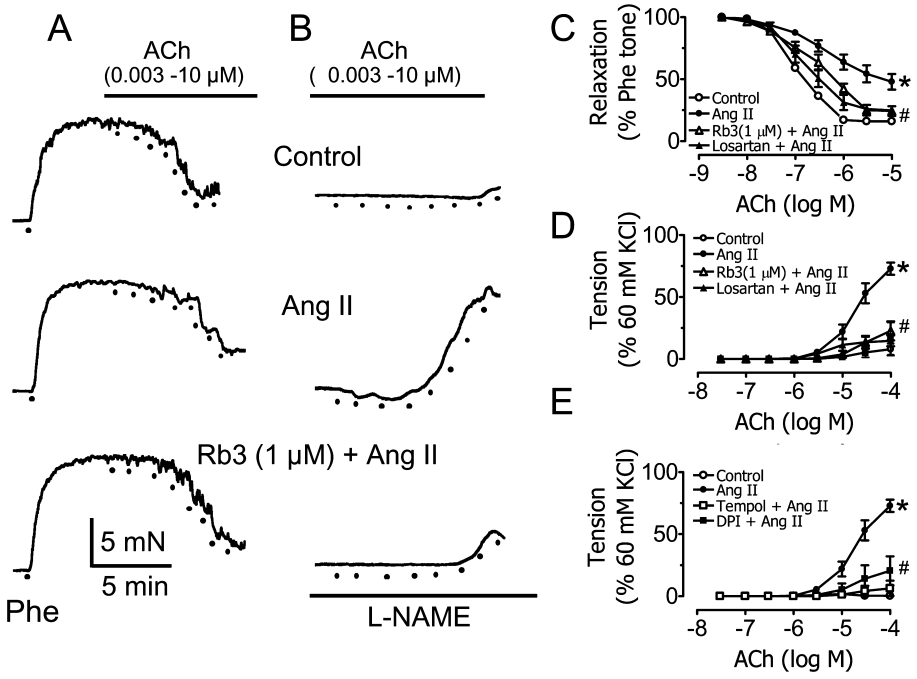

Figure 3

ACh-induced relaxations (A) or contractions (B) in WKY renal arteries following 8 h exposure to 100 nmol·L^{-1} Ang II with and without co-treatment with Rb3. The contraction was obtained in the presence of L-NAME. Concentration-response curves for ACh-induced relaxations (C) and contractions (D and E) in Rb3-treated renal arteries with losartan, tempol or DPI as positive control. Results are means ± SEM of four to six rings from different rats. *P < 0.05, significant difference between WKY control and Ang II group; #P < 0.05, significantly different from Ang II alone.

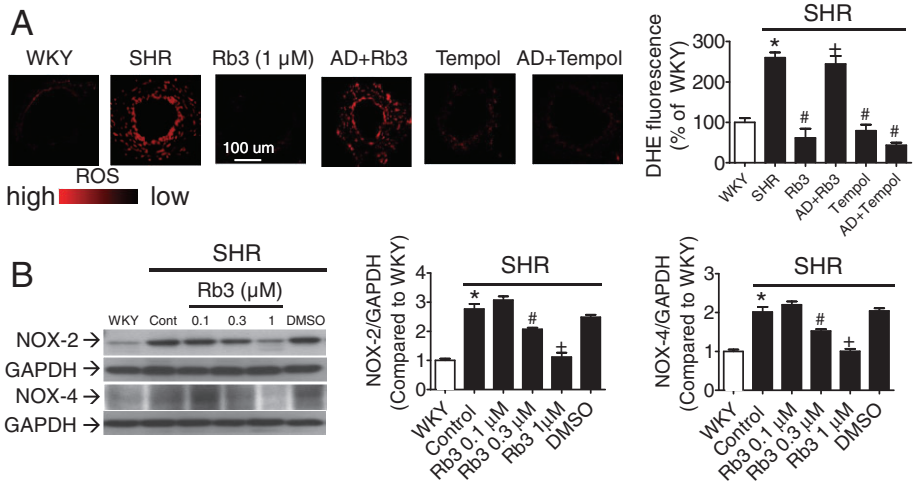

Figure 4

ROS over-production revealed by DHE fluorescence imaging in SHR renal arteries was reversed by *ex vivo* treatment with 1 μmol·L^{-1} Rb3; this effect was prevented by co-treatment with actinomycin D (AD, 2 μmol·L^{-1}) (A). *Ex vivo* incubation with Rb3 (0.1–1 μmol·L^{-1}) concentration-dependently reduced the over-expression of NADPH subunits, NOX-2 and NOX-4 in SHR arteries (B). Losartan (3 μmol·L^{-1}) used as positive control. Results are means ± SEM of four to five rings from different rats. *P < 0.05, significantly different from control; #P < 0.05, significantly different from SHR; +P < 0.05, significantly different from Rb3.

Rb3 reverses Ang II-induced endothelial dysfunction in WKY renal arteries

Incubation for 8h of WKY renal arterial rings with Ang II (100 nmol·L^{-1}) attenuated ACh-induced relaxations (Figure 3A) and unmasked endothelium-dependent contractions (Figure 3B). Co-treatment with Rb3 (0.1–1 μmol·L^{-1}) concentration-dependently rescued the impaired relaxations (Figure 3C) and prevented contractions (Figure 3D). Losartan, tempol and DPI also inhibited ACh-induced contractions in Ang II-treated arteries (Figure 3D and E).

Rb3 normalizes the elevated vascular expression of NADPH oxidases and ROS over-production

Raised levels of ROS inhibits endothelium-dependent relaxations and partly mediate endothelium-dependent contrac-

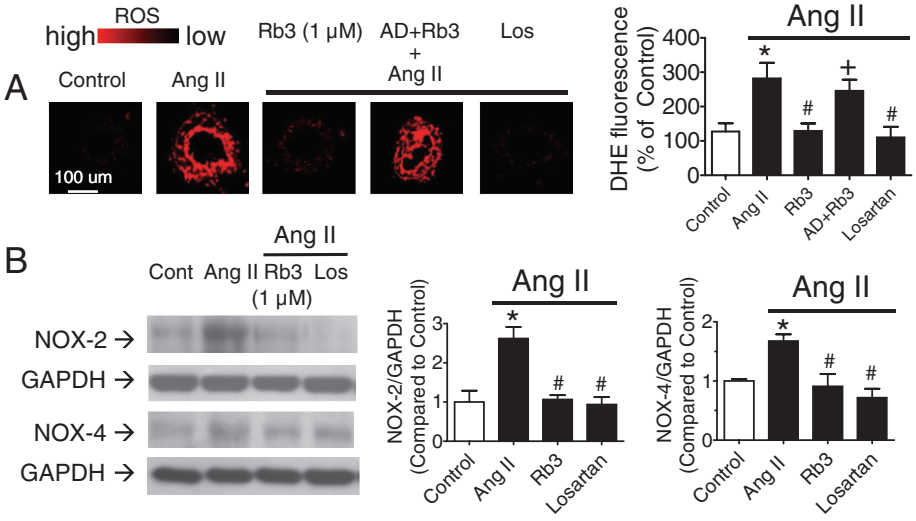

Figure 5

ROS over-production shown by DHE fluorescence imaging in Ang II (100 nmol·L^{-1})-treated WKY renal arteries was reversed by *ex vivo* treatment with 1 μmol·L^{-1} Rb3; this effect was prevented by actinomycin D (AD, 2 μmol·L^{-1}) (A). *Ex vivo* incubation with Rb3 (0.1–1 μmol·L^{-1}) concentration-dependently inhibited the Ang II-induced up-regulation of NOX-2 (B) and NOX-4 in WKY renal arteries (B). Losartan at 3 μmol·L^{-1} used as positive control. Results are means ± SEM of four to five rings from different rats. *P < 0.05 versus control; #P < 0.05 versus Ang II group; +P < 0.05 versus Rb3.

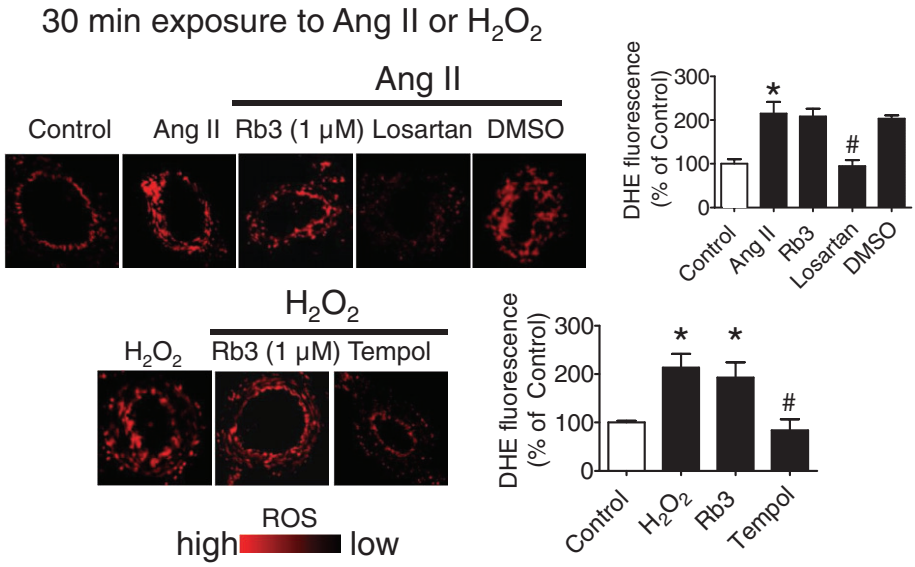

Figure 6

Lack of effect of acute exposure (30 min) to Rb3 on Ang II- or H_2O_2-stimulated ROS elevation. Results are means ± SEM of four rings from different rats. *P < 0.05, significantly different from control; #P < 0.05, significantly different from Rb3.

tions in hypertensive rats (Wong *et al.*, 2010a; Tian *et al.*, 2012). The ROS inhibitor tempol and DPI suppressed ACh-induced contractions in SHR arteries (Figure 2E), suggesting a significant role of ROS. Staining with DHE showed that *ex vivo* treatment of SHR arteries with Rb3 normalized the elevation of both ROS accumulation and expression of NADPH oxidase subunits, NOX-2 and NOX-4 (Figure 4). Similar effects were observed in normotensive WKY renal arteries in which Rb3 prevented Ang II-stimulated ROS production (Figure 5A) and the expression of NOX-2 and NOX-4 (Figure 5B). The angiotensin receptor antagonist losartan (3 μmol·L^{-1}) antagonized the effect of Ang II (Figure 5). By contrast, 30 min exposure to 1 μmol·L^{-1} Rb3 did not affect the ROS production acutely stimulated by Ang II or H_2O_2 in WKY arteries (Figure 6). Finally, the ROS-reducing effect of *ex vivo* Rb3 treatment was reversed by the mRNA synthesis inhibitor actinomycin D (2 μmol·L^{-1}) in arteries from both rat strains (Figures 4A and 5A), whereas actinomycin D did not affect tempol-induced ROS reduction (Figure 4A).

Rb3 inhibits oxidative stress in arteries of hypertensive patients

The expression of NADPH oxidase subunits and associated ROS production was greater in renal arteries from hypertensive patients compared with those from normotensive subjects (Figure 7). Measurement of DHE fluorescence showed that *ex vivo* treatment with 1 μmol·L^{-1} Rb3 normalized the elevated levels of both ROS (Figure 7A) and NOX-2, NOX-4 and p67^{phox} in arteries of hypertensive patients (Figure 7B).

Rb3 inhibits Ang II-stimulated oxidative stress in arteries of normotensive subjects

Ang II (1 μmol·L^{-1}, 8 h incubation) increased ROS level (Figure 8A), elevated oxidative stress marker nitrotyrosine (Figure 8B) and enhanced the expression of NOX-2, NOX-4 and p67^{phox} (Figure 8C and D) in renal arteries from normotensive subjects. These effects were reversed by co-treatment with 1 μmol·L^{-1} Rb3 (Figure 8).

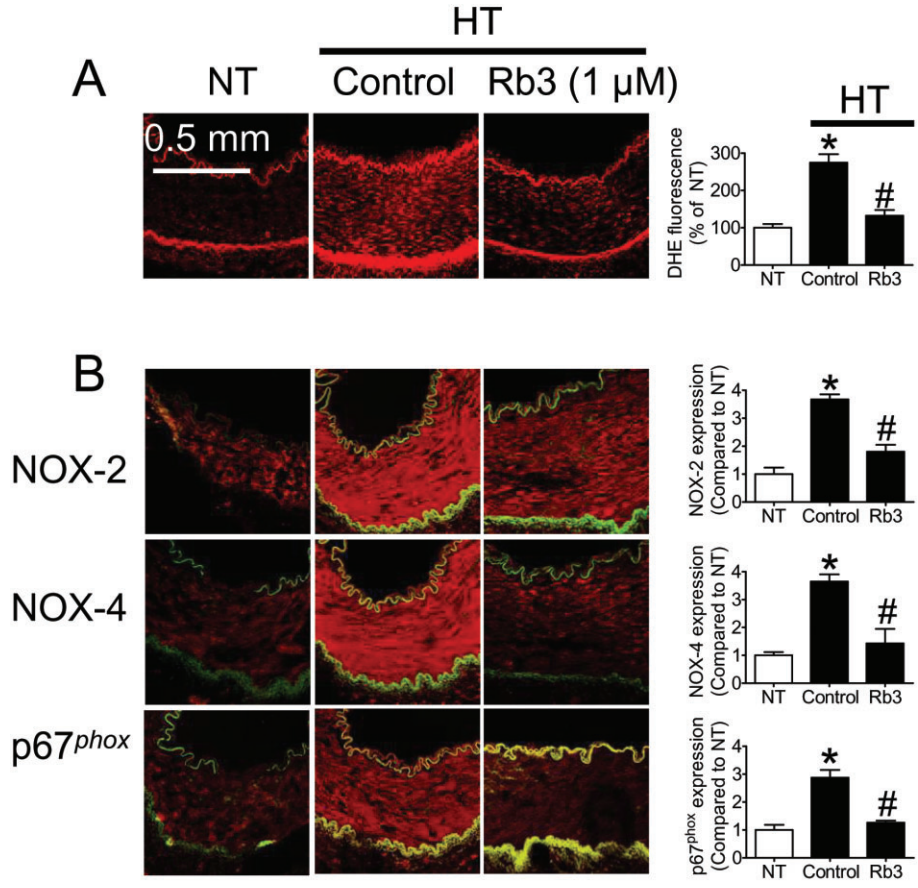

Figure 7

ROS over-production (A) and oxidative stress markers, NOX-2, NOX-4 and p67^{phox} (B) in renal arteries from hypertensive patients (HT) compared with normotentive patients (NT) was reduced by *ex vivo* treatment with Rb3 as revealed by immunofluorescence microscopy. Yellowish-green autofluorescence indicates the elastin of the internal and external elastic laminae; the former separates the luminal endothelium from the medial smooth muscle layer while the latter separates the smooth muscle layer from the adventitia. Signals from Alexa Fluor 546-conjugated secondary antibodies attached to primary antibodies against NOX-2, NOX-4 and p67^{phox} appeared reddish orange. Photomicrographs are representative images from experiments performed on samples from four different patients and scale bar applies to all images. Results are means ± SEM of four rings *P < 0.05, significantly different from NT; #P < 0.05, significantly different from HT control.

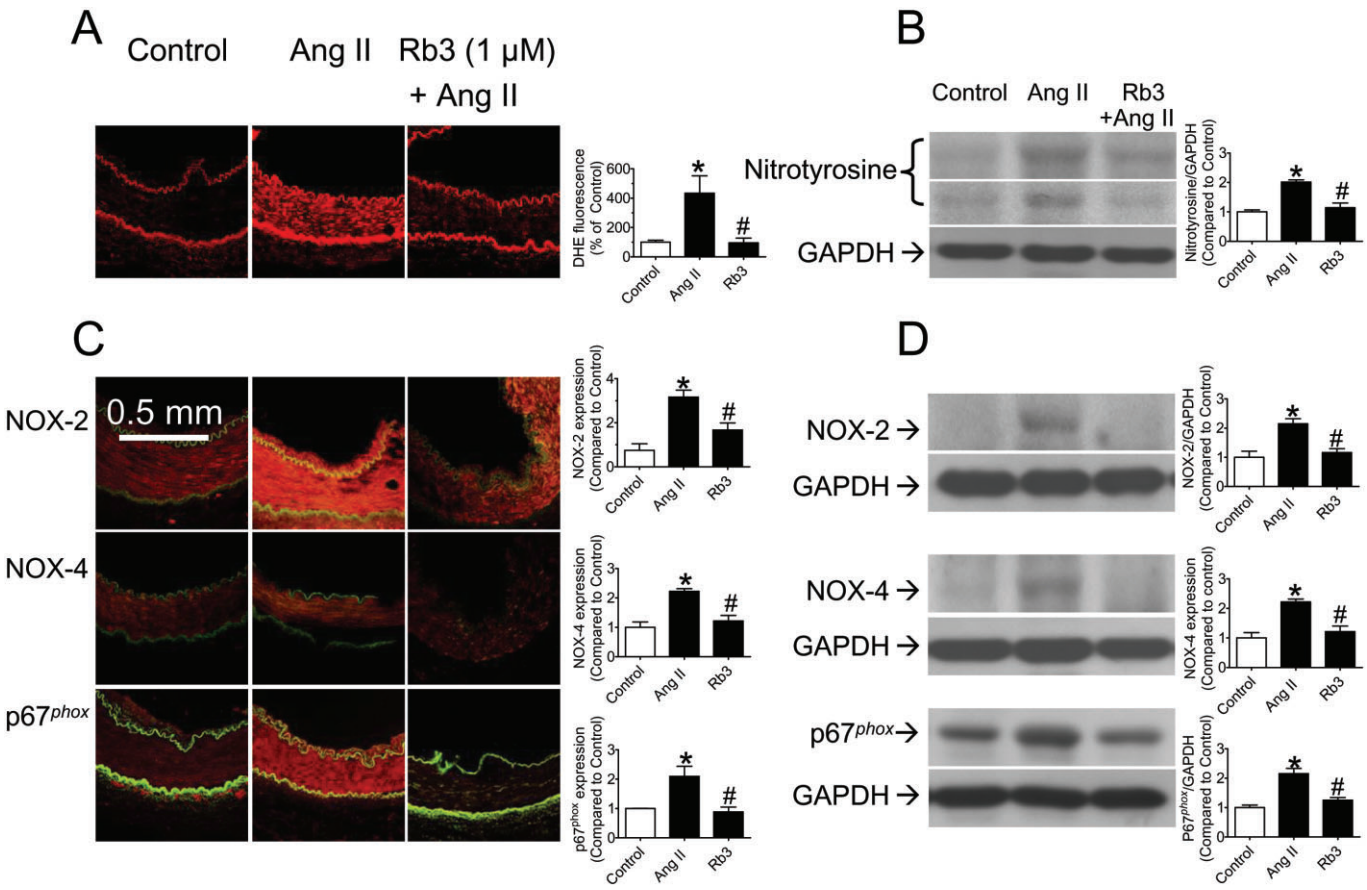

Figure 8

Ex vivo 8 h exposure to 1 μmol·L^{-1} Rb3 prevented the Ang II (1 μmol·L^{-1})-stimulated ROS production (A), increase in nitrotyrosine levels (B) and up-regulation of NOX-2, NOX-4 and p67^{phox} as measured using DHE fluorescence dye (C) and Western blotting (D) in renal arteries from normotensive patients. Photomicrographs and blots are representative images from experiments performed on samples from different patients. Results are means ± SEM of four rings. *P < 0.05, significantly different from control; #P < 0.05, significantly different from Ang II.

Rb3 reverses Ang II-induced reduction of NO production in HUVECs

The calcium ionophore A23187 (1 μmol·L^{-1}) stimulated time-dependent rises in NO production in HUVECs, and this increase was inhibited by 8 h culture with Ang II (Figure 9A–C). The Ang II-induced attenuation of NO production (Figure 9B and C) and phosphorylation of eNOS at Ser^{1177} (Figure 10) was reversed by co-treatment with Rb3 (0.1–1 μmol·L^{-1}) and also by losartan and tempol.

Discussion

Although the ginsenoside Rb3 was reported to ameliorate ischaemia-reperfusion injury in rat cardiac myocytes (Shi *et al.*, 2011), its potential vascular benefit is largely unknown. The present study provides novel evidence showing the protective benefits of *ex vivo* treatment with ginsenoside Rb3 to restore the impaired endothelial function in hypertensive rats. The major results include (i) Rb3 concentration-dependently reversed the attenuated endothelium-dependent relaxations and inhibited the exaggerated endothelium-dependent contractions in renal arteries from SHRs, and (ii) Rb3 inhibited oxidative stress, protected endothelium-dependent relaxations and suppressed endothelium-dependent contractions in Ang II-treated arteries of normotensive WKY rats. In addition, Rb3 reversed the Ang II-induced reduction of NO production in HUVECs. These beneficial effects against oxidative stress in rat arteries were also demonstrated in human arteries. Rb3 normalized the elevated ROS generation and inhibited the increased expression of NADPH oxidases in renal arteries from hypertensive patients. Likewise, *ex vivo* treatment with Rb3 also abolished Ang II-stimulated rises in ROS production, the level of nitrotyrosine (an indicator of increased oxidative stress) and NADPH oxidase expressions in renal arteries from normotensive subjects.

Oxidative stress or elevated ROS are associated with vascular dysfunction and subsequent development of hypertension (Wong *et al.*, 2010b). ROS production is mediated mainly by NADPH oxidase and ROS scavengers inhibit the development of endothelial dysfunction in hypertensive rats (Wong *et al.*, 2010a). Excessive ROS impair endothelium-dependent relaxations and trigger endothelium-dependent contractions in hypertension (Wong *et al.*, 2010a; Félétou *et al.*, 2011; Tian

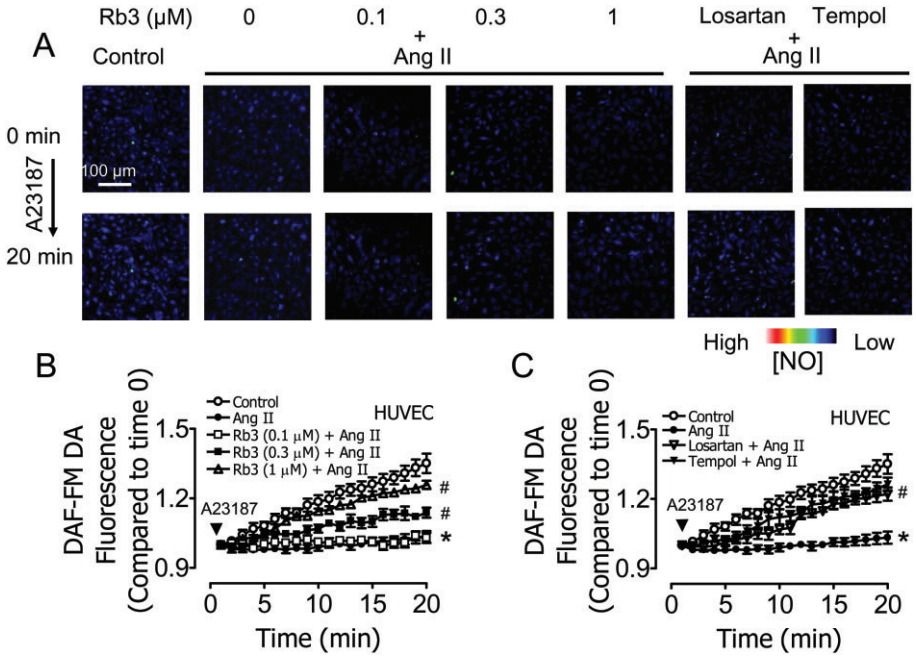

Figure 9

A23187 (1 μmol·L^{-1})-stimulated rise of NO in cultured HUVECs using NO-sensitive fluorescence dye. (A) Ang II (1 μmol·L^{-1})-induced reduction of NO production was concentration-dependently reversed by *ex vivo* treatment with Rb3 (0.1–1 μmol·L^{-1}) (B) and by losartan or tempol (C). Results are means ± SEM of rings from four to five. *P < 0.05, significantly different from control; #P < 0.05, significantly different from Ang II.

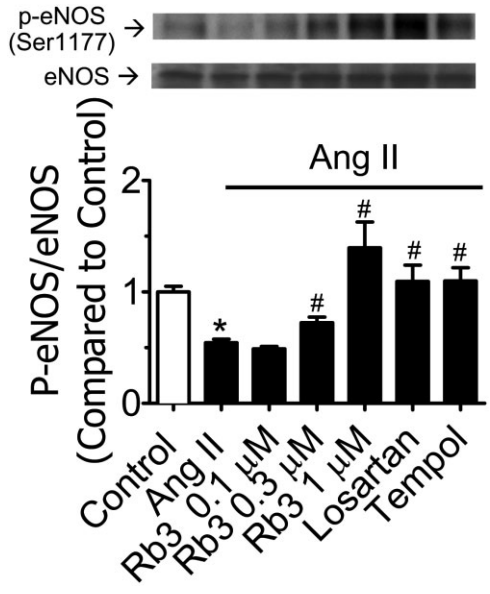

Figure 10

Ang II (100 μmol·L^{-1}, 8 h) treatment reduced the phosphorylation of eNOS at Ser^{1177} and this effect was reversed by Rb3 (0.1–1 μmol·L^{-1}), losartan and tempol. Results are means ± SEM of rings from four to five. *P < 0.05, significantly different from control; #P < 0.05, significantly different from Ang II.

et al., 2012). The present results support a critical role of oxidative stress in maintenance of hypertension-related endothelial dysfunction as treatment with tempol (a superoxide dismutase mimic) reversed endothelium-dependent contractions and ROS over-production in SHR arteries and in Ang II-treated WKY arteries. The present study suggests that Rb3 would be highly effective in reducing oxidative stress through inhibiting the expression and activity of NADPH oxidases because Rb3 treatment reversed the Ang II-induced over-expression of NOX2 and NOX-4 in WKY arteries. As observed with the angiotensin receptor antagonist losartan, Rb3 also abolished Ang II-stimulated ROS production in WKY renal arteries. The beneficial effects of Rb3 in SHR or Ang II-treated WKY arteries were not seen following co-treatment with actinomycin D (mRNA synthesis inhibitor), suggesting an involvement of expression of genes and associated proteins in Rb3-induced effects. By contrast, acute exposure (30 min) to Rb3 did not inhibit Ang II-induced ROS elevation in WKY arteries (Figure 6) or the endothelium-dependent contractions in SHR arteries (Figure 2E). Furthermore, 30 min exposure to tempol, but not to Rb3, inhibited H_2O_2-induced ROS accumulation in WKY arteries (Figure 6). These results indicated that Rb3 is unlikely to be either a direct ROS scavenger or an angiotensin receptor antagonist. The increased oxidative stress was not only observed in SHR arteries but also in renal arteries from hypertensive patients. These human arteries expressed high levels of ROS, NOX-2, NOX-4 and p67^{phox}. Up-regulated expression of these oxidative stress

markers in human arteries was normalized by *ex vivo* treatment with Rb3 in a concentration-dependent manner. In addition, Rb3 prevented Ang II-triggered elevation in ROS, NADPH oxidases and nitrotyrosine in human arteries from normotensive subjects. The present results demonstrated that Rb3 restored the impaired endothelial function through limiting oxidative stress in the vascular wall. Further experiments show that Rb3 treatment reversed Ang II-induced reduction of NO production and eNOS phorsphorylation in HUVECs. As tempol produces the same effect as Rb3, it is probable that increased vascular ROS accumulation reduced the eNOS activity in SHR arteries while Rb3 limited oxidative stress as the primary mechanism to restore the diminished NO bioavailability in arteries from hypertensive rats.

The concentration–response studies showed that Rb3 exhibited similar potency in improving endothelium-dependent relaxation, inhibiting endothelium-dependent contractions, reducing ROS over-production and NADPH oxidase expression in renal arteries and increasing NO production and eNOS phosphorylation in human endothelial cells.

In summary, *ex vivo* treatment with Rb3 confers protection against oxidative stress and endothelial dysfunction in arteries from hypertensive rats and similar benefits were observed in renal arteries from hypertensive patients. The findings in human renal arteries are consistent with *ex vivo* results in rat arteries. Rb3-induced vascular benefits are mostly likely mediated by down-regulation of the expression and/or activity of NADPH oxidase and subsequent reversal of ROS over-production. Because clinical studies report that the direct ROS scavengers are largely ineffective to improve cardiovascular events (Koh *et al*., 2009), the present findings strengthen the prospect of the potential use of extracts of medicinal herbs rich in Rb3 as a therapeutic alternative in cardiovascular dysfunction. Targeting NADPH oxidases with Rb3 to ameliorate hypertension-associated vascular inflammation and dysfunction provides a mechanism for the common clinical application of ginseng products particularly in Oriental populations.

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Author contributions

Youhua Wang, Jinghui Dong, Chi Wai Lau and Zhen Gao performed the experiments; Yu Huang and Ping Liu designed the study and prepared the manuscript; Duan Zhou and Jingyi Tang provided Rb3 and fund support and were involved in data presentation; Chi Fai Ng provided human specimens.

Conflict of interest

Authors declare that they have not any conflict of interest.

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