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INVOLVEMENT OF PROLYLCARBOXYPEPTIDASE IN THE EFFECT OF RUTAECARPINE ON THE REGRESSION OF MESENTERIC ARTERY HYPERTROPHY IN RENOVASCULAR HYPERTENSIVE RATS

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SUMMARY

1. Previous studies indicate that rutaecarpine blocks the elevation of blood pressure and inhibits vascular hypertrophy in experimental hypertensive rats. The present study is to explore whether the effect of Rut is related to the activation of prolylcarboxypeptidase (PRCP).

2. Renovascular hypertensive rats (Goldblatt two-kidney, one-clip (2K1C)) were developed using the male Sprague-Dawley rats. Chronic treatment with rutaecarpine (10 or 40mg/kg per day) or losartan (20mg/kg per day) for 4 weeks to the hypertensive rats caused a sustained dose-dependent repression of blood pressure, increased the lumen diameter and decreased the medium thickness, which was companied by a similar reduction in the media cross-sectional area: lumen area ratio in mesenteric arteries compared with untreated hypertensive rats.

3. Angiotensin (Ang) II expression was significantly increased in the mesenteric arteries of hypertensive rats compared with sham-operated rats. No significant differences in plasma Ang II levels were observed between untreated hypertensive and sham-operated rats. The hypertensive rats treated with rutaecarpine showed a decreased trend of Ang II levels and the high-dose rutaecarpine decreased significantly Ang II levels in both plasma and mesenteric arteries.

4. Expression of PRCP protein or kallikrein mRNA expression was significantly inhibited in the right kidneys and mesenteric arteries of hypertensive rats. However, expression of PRCP protein or kallikrein mRNA was significantly increased after treatment with rutaecarpine or losartan (20 mg/kg per day).

5. The data suggest that the repression of increases in systolic blood pressure and reversal of mesenteric artery remodeling by rutaecarpine may be related to the increased expression of PRCP in the circulation and small arteries in the 2K1C hypertensive rats.

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Keywords

prolylcarboxypeptidase; rat; renohypertension; rutaecarpine; vascular remodeling

INTRDUCTION

Rutaecarpine [8, 13 – dihydroindolo - (2′, 3′:3, 4) pyrido (2, 1 - b) quinazolin – 5 (7H)-one] is a quinazolinocarboline alkaloid that can be extracted from a well-known Chinese herbal drug Wu-Zhu-Yu, the dried, unripe fruit of *Evodia rutaecarpa* (Juss) [1]. Recent studies have shown that rutaecarpine has inhibitory effects on the vasoconstriction induced by anaphylaxis in guinea-pigs, as well as on the increases in systolic blood pressure and artery hypertrophy in hypertensive rats. [2-4] It has been reported that the intracellular Ca^{2+}-nitric oxide (NO)-cGMP signal pathway is involved in the dilator effects of rutaecarpine on endothelial cells and vascular smooth muscle cells (VSMC),[5] but the precise mechanism by which rutaecarpine causes hypotension remains unclear.

The renin-angiotensin system (RAS) has an important role in the development and maintenance of hypertension. Angiotensin (Ang) II, a member of RAS, not only causes vasoconstriction but also regulates cytological characteristics of VSMC, leading to vascular remodeling. The kallikrein-kinin system (KKS) has been implicated in the regulation of renal function, blood flow and blood pressure. Degradation of the arterial KKS contributes to the pathogenesis of cardiovascular diseases.[6,7] Arterial kallikrein is decreased in Goldblatt renovascular and salt-induced hypertensive rat.[8,9] Kallikrein gene delivery attenuates cardiac remodeling and promotes neovascularization in spontaneously hypertensive rats.[10]

Prolylcarboxypeptidase (angiotensinase C, PRCP), a prekallikrein (PK) activator and a degrading enzyme of angiotensin, may serve as a physiological counterbalance to the RAS (Fig.1). Recently, losartan is shown to increase bradykinin levels in hypertensive humans.[11] This increase is likely to alter the expression of the KKS and contribute bradykinin to a therapeutic action in hypertension. In the present study, we used losartan as a control drug to investigate whether the hypotensive effects of rutaecarpine, as well as inhibition of vascular hypertrophy inhibitory, are related to the increased expression of PRCP or kallikrein-kinin axis in the 2K1C hypertensive rats.

METHODS

Drugs and regents

Losartan was kindly provided by Merck Company (Wilmington, NJ, USA). Rutaecarpine was producted by the School of Pharmaceutical Sciences of Central South University. Primers for the polymerase chain reaction (PCR) were synthesized Takara (Dalian, P.R. China), who also supplied the reverse transcription-polymerase chain reaction (RT-PCR) kits. Trizol reagent was obtained from Molecules Research Centre (Cincinnati, OH, USA). The radioimmunoassay kit for Ang II measurement was purchased from the Immunity Institute of Dongya (Beijing, P.R.China). The phosphorylated (p-)extracellular signal-regulated kinase (ERK)1/2 monoclonal antibody of was obtained from Santa Cruz biotechnology (Heidelberg, Germany). The PRCP polyclonal antibody was purchased from Boster (Wuhan, P.R. China).

Animal and surgery

Sprague-Dawley rats (185 ± 20 g) were obtained from the Animal Center of Hunan Agriculture University (Hunan China) and allowed to accommodate to environmental conditions for 1 week. All rats were cared and used in compliance with the Guide for the Care and Use of

Laboratory Animals published by the National Institutes of Health (NIH publication 86-23, revised 1986) (http://iacuc.med.miami.edu/x13.xml). Surgical procedures were performed on anaesthetized rats (pentobarbital sodium, 60 mg/kg, i.p.). The left kidney artery was exposed and one silver clip was applied to the artery, as described previously.[4] Sham-operated (control) rats underwent the same procedure, but the arteries were not clipped. One week after recovery from surgery, systolic blood pressure (SBP) was measured using the tail-cuff method. Rats with an SBP ≥ 140 mmHg at the end of the 4 week period after surgery were considered as hypertensive. Rats were randomly divided into five groups (n = 12 for each group) as follow: (i) sham-operated rats; (ii) hypertensive rats; (iii) hypertensive rats treated with low-dose rutaecarpine (10 mg / kg per day); (iv) hypertensive rats treated with high-dose rutaecarpine (40 mg / kg per day); and (v) hypertensive rats treated with losartan (20mg / kg per day). Rutaecarpine or losartan was initially administered at the 11 weeks after surgery by oral gavage twice a day and treatment lasted for 4 weeks. Systolic blood pressure was measured in conscious state 1 h before the administration of rutaecarpine or losartan in the morning every 6 days.

Vascular morphology

Four weeks after drug treatment started, rats were weighed and anesthetized with pentobarbital sodium (60 mg/kg, i.p.). Left kidney arteries of six rats from each group were fixed by perfusion with phosphate-buffer saline (PBS) followed by 4% paraformaldehyde for 10 min. The third-order branches of the superior mesenteric arteries were freed of connective tissue and preserved in 10% formalin for morphological analysis. After dehydration and embedding, the transverse sections were made and stained with Haematoxylin and eosin (HE). The internal diameter of the lumen (LD), media thickness (MT), media cross-sectional area (MSCA) and lumen area (LA) of each section were measured using an image-analysis system (BI-2000, Taimong, Sichuan, China).

The remaining six rats in each group were used for radioimmunoassay, western blotting analysis, RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR).

Radioimmunoassay of Ang II in plasma and mesenteric artery

At the end of the experiment, rats were anesthetized with pentobarbital sodium as above described. Exsanguination was performed by inserting an angiocatheter into the carotid artery. Blood samples (3 mL) were collected in tubes with a solution containing 30 μL of 0.3 mol/L EDTA, 30 μl of 0.34 mol/L 8-Hydroxyquinoline, and 15 μl of 0.32 mol/L dimercaprol. Plasma was collected by centrifugation at 1500 g for 10 min at 4 °C, and then frozen at −20 °C until assay. After the exsanguination, a segment of the third mesenteric artery was freed of connected tissues, snap frozen in liquid nitrogen, and homogenized in a lysis buffer (50 mmol / L Tris, 150 mmol / L NaCI, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), sodium orthovanadate 1 mmol / L, sodium fluoride 1 mmol / L, EDTA 1 mmol / L, leupeptin 2 μg/mL, and PMSF 1 mmol / L). Samples were centrifuged at 10000 g for 5 min and the supernatant was collected and stored at −20 °C for radioimmunoassay. Concentrations of Ang II in the plasma or mesenteric arteries were determined using antisera raised against rat Angiotensin □kit. This antibody (Dongya, Beijing, China) does not cross-react with other angiotensins or angiotensinogens.

RNA preparation and RT-PCR

A section of mesenteric arteries was removed rapidly from the rats, dissected and frozen in liquid nitrogen for subsequent analysis of kallikrein mRNA levels. Total RNA isolation and semiquantitative RT-PCR were performed according to standard methods. Briefly, The cDNA was used as a template for polymerase chain reaction (PCR), the primers and the size of the expected products are as follows: kallikrein: forward 5′-

CCTGATCCTATTCCTCGACCTGTCCCTG-3′, and reverse 5′-GTAGATGGCTGGCATGTTGGTTTTGG-3′ (721bp); [12] *β*-actin: forward 5′-GAGACCTTCAACACCCCAGCC-3′ and reverse 5′-TCGGGGCATCGAACCGCTCA-3′ (422bp). The PCR amplification profiles consisted of denaturation at 94°C 5 min, then 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 60 s. The linear exponential phases for kallikrein and *β*-actin PCR were 30 cycles. Equal amounts of corresponding kallikrein and *β*-actin RT-PCR products were loaded on 1.5 % agarose gels. Optical densities of ethidium bromide-stained DNA bands were quantitated and the results were expressed as kallikrein /*β*-actin ratios.

Western blotting

After collection of blood samples, a segment of mesenteric artery and a portion of the right kidney were removed rapidly and snap-frozen in liquid nitrogen. Tissues from individual rats were homogenized separately in lysis buffer (50 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1% NP-40; 0.5% Sodium Deoxycholate; 0.1% SDS; 1mmol/L EDTA; 1mmol/L PMSF; 2 μg/ml Leupeptin) and centrifuged at 3000 g for 15min. The supernatant was collected and stored at −20°C for western blotting. Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford IL, USA). Equal amount of proteins (60 μg) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin (BSA), membranes were incubated with primary antibodies against PRCP (1:333 dilution; 2 h) and p-ERK1/2 (p-ERK1 and p-ERK2; 1:400 dilution; 2 h) at 37°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; 0.75 h) at 37°C followed by western blotting luminal reagent. Expression levels were determined by densitometry.

Statistical Analysis

All data were analysed using ANOVA and multiple comparison tests (Newman-keuls' test). Data are presented as mean ± SEM. $P < 0.05$ was considered significant.

RESULTS

Blood pressure

No difference in the baseline SBP was observed between any of the groups before operation. After the renal artery had been clipped in 2K1C hypertensive group, there was a significant increase in SBP observed. Systolic blood pressure values at 10 and 14 weeks are given in Table 1. During the treatment period, a reduction in SBP was observed in the treated groups. Hypertensive rats treated with high-dose of rutaecarpine showed a significant reduction in the SBP (ANOVA, $P<0.01$) compared with untreated hypertensive group at the end of treatment period. No significant reduction in SBP was seen in rats treated with low-dose rutaecarpine. The losartan-treated hypertensive rats showed the similar reduction in SBP as the high dose of rutaecarpine treated.

Morphological analysis of mesentery artery

Obvious hypertrophy was observed in mesenteric arteries from the hypertensive rats (Table 2). In the untreated hypertensive rats, changes in the vascular structure were evident compared with the sham-operated rats ($_{ANOVA, P} < 0.01$), as indicated by the in creased MCSA / LA and MT / LD ratios. Treatment of hypertensive rats with losartan or high-dose of rutaecarpine for 4 weeks resulted in a significant and similar reduction of MCSA / LA and MT / LD ratios. No significant effect was found with low-dose rutaecarpine.

Plasma and mesenteric arteries concentrations of Ang II

Plasma and the mesenteric artery concentrations of Ang II are shown in Fig. 2. Plasma Ang II concentrations in untreated hypertensive rats at Week 14 were slightly, but not significantly, higher than those in control rats. However, the Ang II content of mesenteric arteries was significantly increased in untreated hypertensive rats compared with control rats (171.9 ± 16.1 *vs* 121.6 ± 12.5 pg / mL, respectively, $P<0.01$). Treatment with losartan significantly increased plasma Ang II concentrations ($P<0.01$), but had no effect on Ang II content in mesenteric arteries compared with untreated hypertensive rats. However, treatment with high-dose rutaecarpine resulted in a significant reduction in Ang II in both the plasma and mesenteric arteries.

Activity of ERK1/2 in mesenteric arteries

The ERK1/2 activity was determined on the basis of phosphorylation (p-ERK1/2). As shown in Fig. 3, the expression of p-ERK1/2 was significantly increased in mesenteric arteries of the untreated hypertensive rats. Treatment with losartan or high-dose rutaecarpine significantly decreased ERK1/2 activity. No significant decrease in p-ERK1/2 level was observed in rats treated with low-dose rutaecarpine.

Expression of PRCP in kidney and mesenteric artery

The expression of PRCP in kidneys and mesenteric arteries is shown in the Fig. 4 and Fig. 5. The PRCP protein content was significantly decreased in kidneys and mesenteric arteries from untreated hypertensive rats compared with sham-operated rats. Hypertensive rats treated with losartan or high-dose rutaecarpine exhibited a significant increase in PRCP protein expression in the kidney and mesenteric artery.

Expression of kallikrein mRNA in mesenteric artery

The expression of kallikrein mRNA was significantly inhibited in mesenteric arteries from untreated hypertensive rats. Treatment with losartan or both low- and high-dose rutaecarpine significantly increased the expression of kallikrein mRNA (Fig. 6).

DISCUSSION

The novel and important finding of the present study is that chronic administration of rutaecarpine inhibits the hypertrophic remodeling of small arteries in 2K1C hypertensive rats. This inhibition is related to the increased expression of PRCP in the cardiovascular system, resulting in significant conversion of PK to kallikrein, which reduces the Ang II levels in the circulation and small arteries.

Hyperactivity of RAS can be induced by narrowed renovascular arteries and contributes to the onset and development of hypertension. Angiotensin II, one of main effectors of RAS, is a multifunctional peptide. Its overexpression not only alters hemodynamic states but also causes cell growth, apoptosis, migration and extra cellular matrix deposition resulting in cardiovascular remodeling in pathological conditions.[13] The results of present study showed that the Ang II content was significantly increased in mesenteric arteries, but not in plasma, in the chronic hypertensive rats, which is consistent prior report.[14,15] Losartan significantly elevated Ang II level in plasma, but not significantly in mesenteric arteries. More importantly, we found that rutaecarpine reduced Ang II levels in both the plasma and mesenteric arteries, which differs from the effects of losartan,[16] At this stage, the mechanism by which rutaecarpine produces these changes is unclear.

The Ang II type 1 (AT1) receptor is one of the effectors of Ang II, its signals transmitted via G-protein couple mechanisms to excite several intracellular signal pathways. Extracellular

signal-regulated kinase 1/2 (p44/42 mitogen-actived protein kinase (MAPK)), one main members of MAPK family, is a central signaling protein that relates to the hyperplasia and migration of VSMC [17]. The ERK1/2 cascade serves as one of the main lines of communication that bridges the gap between the receptors and the intracellular targets in distinct cellular compartments and organelles. The results of the present study showed that both rutaecarpine and losartan inhibited ERK1/2 activation in the small arteries in hypertensive rats. It was postulated that the effects of rutaecarpine in reducing blood pressure and inhibiting mesenteric arterial hypertrophy may be due to reduced of Ang II expression and inhibition of ERK1/2 activity.

The KKS has been implicated in the regulation of renal function, blood flow and blood pressure. Kallikrein converts kininogen into bradykinin, which binds to bradykinin B_2 receptor and modulates activities of several vasodilators such as eicosanoids and NO.[18] It has reported that inhibition of brain KKS increases blood pressure.[15] Studies have shown that kallikrein mRNA and protein are present in blood vessels.[19] Arterial kallikrein is decreased in Goldblatt renovascular and salt-induced hypertensive rats [8, 9]. The results of present study indicated that kallikrein mRNA expression was decreased in the hypertrophic mesenteric arteries of Goldblatt 2K1C hypertensive rats. Treatment with rutaecarpine increased kallikrein mRNA expression in mesenteric arteries of hypertensive rats, suggesting that the antihypertensive effect of rutaecarpine may be associated with the activation of the KKS.

The RAS interacts with KKS at several levels. In addition to angiotensin-converting enzyme (ACE, also named kininase II), PRCP is a notable junction between RAS and KKS. Not only does PRCP activate PK to increase the production of kallikrein, but it also converts Ang I and Ang II into angiotensin (1-7).[20] The dual function of PRCP suggests that a balance exists between KKS and RAS. Prolylcarboxypeptidase is present in kidney, heart, blood vessel and testis.[21] The function of PRCP in increasing bradykinin and converting Ang I and Ang II into angiotensin(1-7) correlates with the enhanced vasodilation function of NO, bradykinin and prostaglandin [22-24]. The results of the present study showed that the decreased expression of PRCP in kidney or mesenteric artery in the untreated hypertensive rats was marked increased after treatment with rutaecarpine, suggesting that stimulation of PRCP expression may be a possible future antihypertensive therapeutic approach. Both PK and Ang II are substrates of PRCP. However, the k_m values of the PK and Ang II for PRCP are 0.2 mmol / L [25] and 6.7 nmol / L,[26] respectively, indicating that the affinity of Ang II binding to PRCP is much greater than that of PK. Because hyperactivity of RAS causes a marked increase in Ang II expression in the cardiovascular system in the hypertensive rats, the mechanism of inhibition of Ang II by rutaecarpine is likely to be related to the enhanced the expression of PRCP in this situation.

The mechanisms underlying the induction of PRCP by rutaecarpine or losartan remain to be determined. It has reported that losartan increases bradykinin levels in hypertensive humans.[11] The present results of study showed that losartan also increased the expression of PRCP and kallikrein mRNA in hypertensive rats, which supported the notion that the hypotensive effect of losartan is not due only to the blockade of Ang II type I receptor, but also the activation of the KKS. In contrast, rutaecarpine increased the expression of PRCP and kallikrein mRNA, but decreased the expression of Ang II in plasma and mesenteric arteries, suggesting that the hypotensive action of rutaecarpine may be related to modulation of the balance between KKS and RAS. Marcic and Erdos reported that ACE inhibitors regulated the function of bradykinin not only by inhibiting bradykinin metabolism but also by a process depends on the crosstalk between ACE and the B_2 receptor.[27] Previous studies have indicated that the effect of rutaecarpine, losartan or perindopril in the hypertensive rats are related to increase the synthesis and release of calcitonin gene-related peptide (CGRP).[4,28] Furthermore, a recent study has shown that rutaecarpine increases the synthesis and release of CGRP via vanilloid receptor

subtype (VR1) [3]. These data suggested that a crosstalk may exist between Ang II receptors, VR1 and the KKS. These pathways require further investigation.

In conclusion, the onset and development of renal hypertension is correlated with the decreased PRCP in circulation and main tissues of the cardiovascular system. The effects of rutaecarpine in reducing the SBP and reversing vascular remodeling may involve increased expression of PRCP, which further affects the balance between the RAS and the kallikrein-kinin axis.

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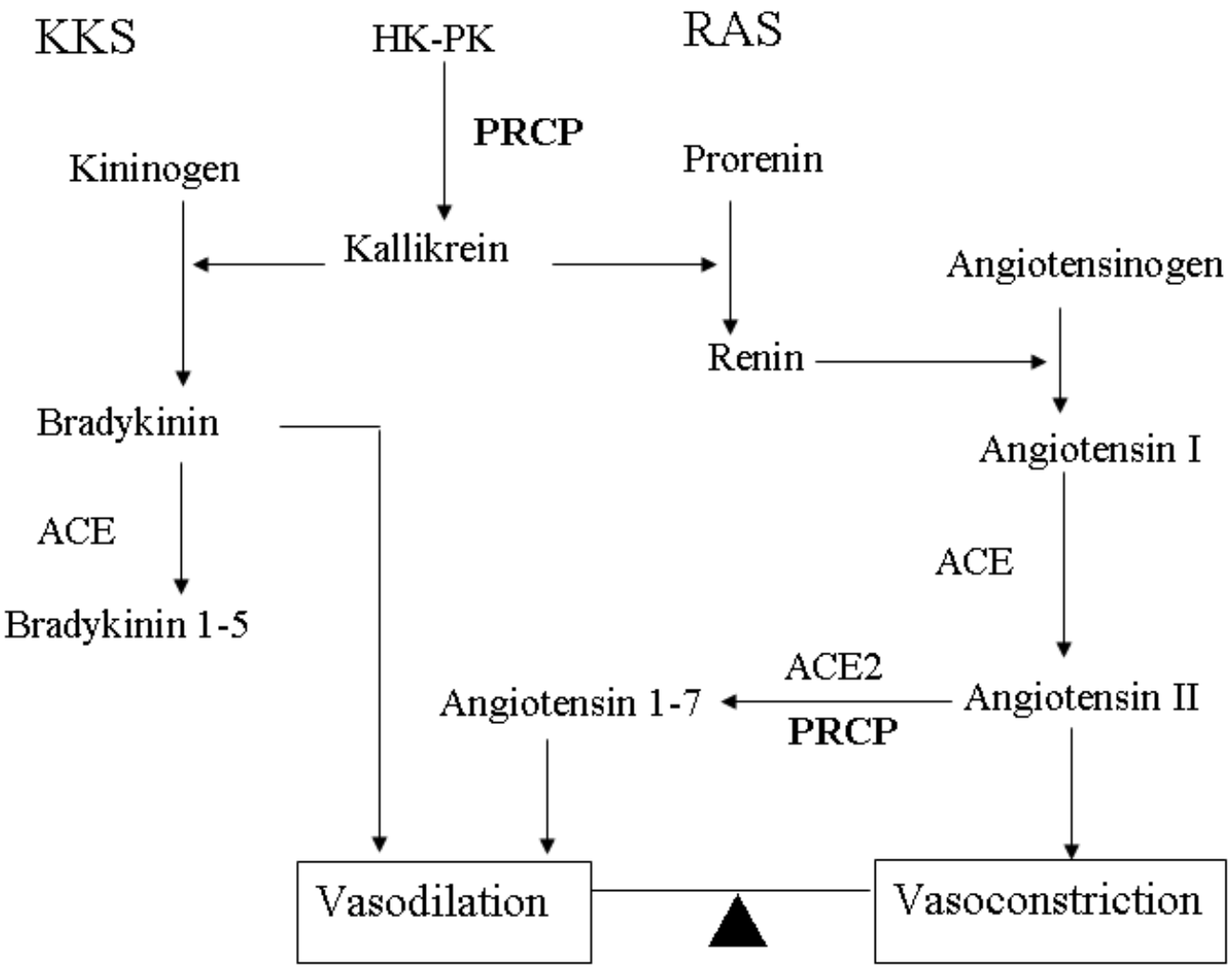
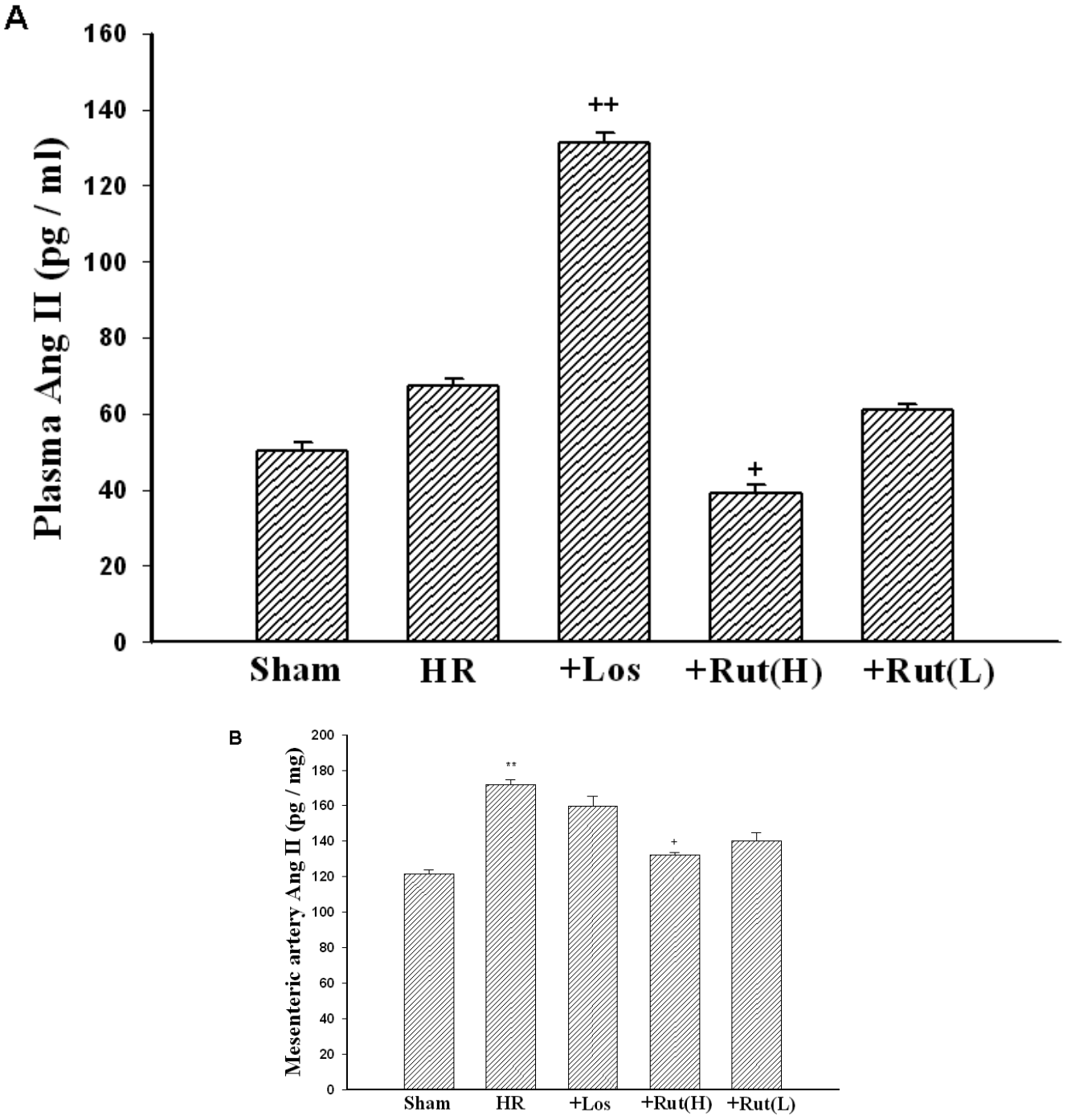


Figure 1.



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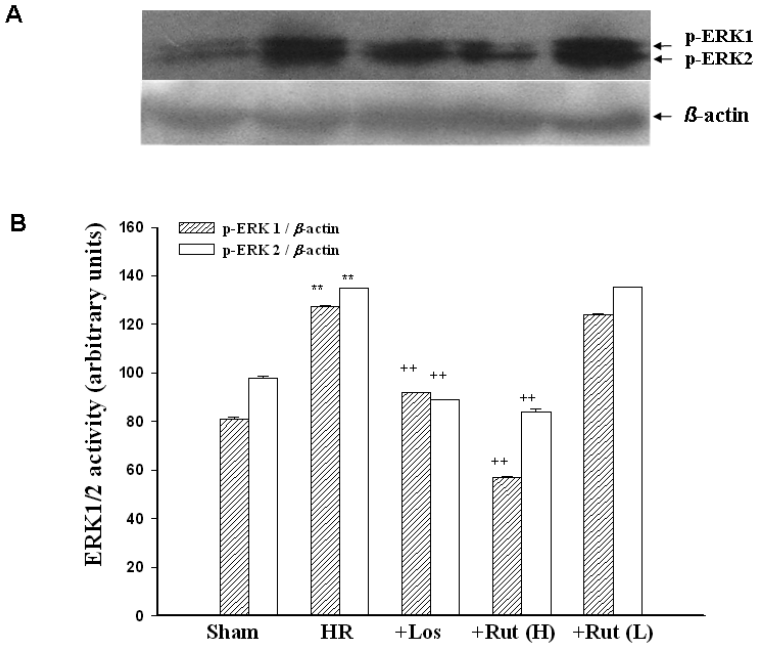

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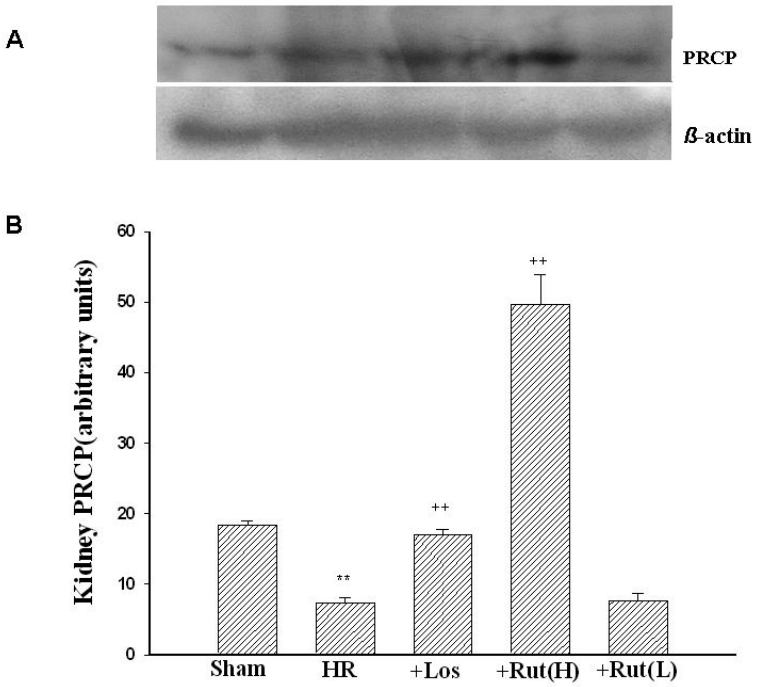

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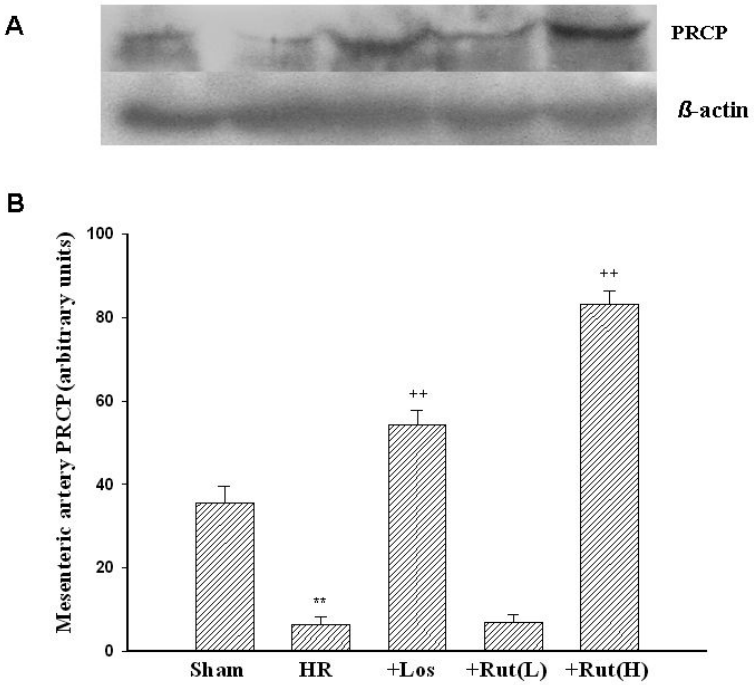

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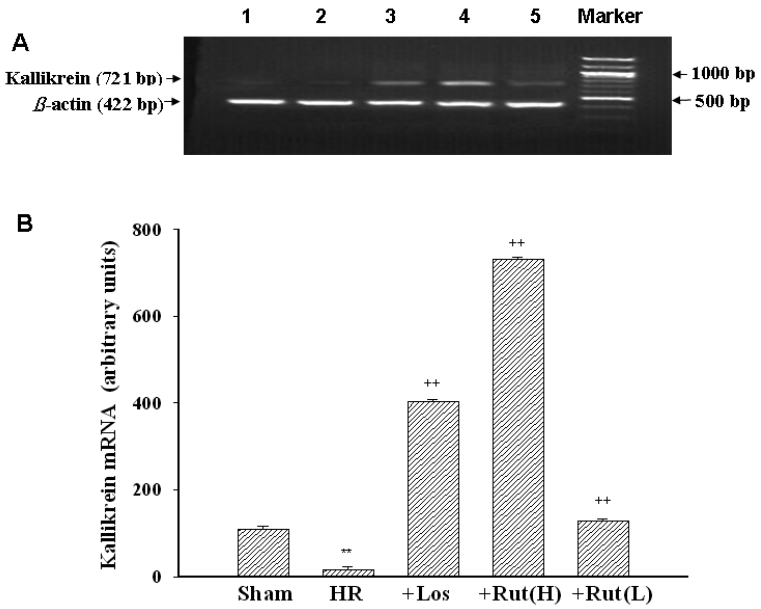

Figure 6.

Table 1

Systolic blood pressure (mmHg) (n = 12 in each group)

Groups	The primary SBP before operation	The SBP after operation and or before treatment	The SBP after treatment
Sham & Control	116 ± 3	118 ± 3	122 ± 5
Hypertensive rats	121 ± 5	164 ± 6**	164 ± 5
+ Rut (10 mg/kg. d)	114 ± 5	159 ± 5**	148 ± 5
+ Rut (40 mg/kg. d)	120 ± 4	162 ± 5**	126 ± 4++
+Losartan (20mg/kg.d)	114 ± 5	157 ± 5**	122 ± 3++

+Rut: hypertensive rats treated by Rut; +Losartan: hypertensive rats treated by Losartan. Data are expressed as mean ± SEM

** $P < 0.01$ *vs* Sham-operated rats (Sham) or Control

++ $P < 0.01$ *vs* hypertensive rats.

Table 2

Morphological Characteristics of mesenteric arteries

Groups	LD (μm)	MCSA–to–LA ratio	MT–to–LD ratio
Sham & Control (n=5)	175.3 ± 18.0	1.11 ± 0.23	0.22 ± 0.02
Hypertensive rats (n=6)	119.3 ± 18.4**	4.94 ± 0.41**	0.71 ± 0.05**
+ Rut (10 mg/kg. d) (n=4)	137.6 ± 24.6	3.80 ± 0.55	0.65 ± 0.07
+ Rut (40 mg/kg. d) (n=6)	166.0 ± 22.9 ++	1.36 ± 0.15 ++	0.24 ± 0.02 ++
+Losartan (20mg/kg.d) (n=6)	165.0 ± 22.8 ++	1.54 ± 0.19 ++	0.29 ± 0.03 ++

MCSA:Media cross-sectional area; LA:Lumen area; MT:Medium thickness; LD:Lumen diameter. +Rut: hypertensive rats treated by Rut; +Losartan: hypertensive rats treated by Losartan; Data are expressed as mean ± SEM

** $P<0.01$ *vs* Sham-operated rats (Sham) or Control

++ $P<0.01$ *vs* hypertensive rats.