

Original Article

# Recombinant adeno-associated virus-mediated human kallikrein gene therapy protects against hypertensive target organ injuries through inhibiting cell apoptosis

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**Aim:** Overexpression of human tissue kallikrein (HK), mediated by recombinant adeno-associated virus (rAAV), decreased blood pressure in spontaneous hypertensive rats (SHRs) and reduced injury to the heart, aorta and kidney. In this study, we used both an *in vivo* animal model and *in vitro* cell culture system to investigate whether rAAV-mediated HK gene therapy protects against organ damage by inhibiting cell apoptosis.

**Methods:** rAAV encoding HK (rAAV-HK) or LacZ (rAAV-lacZ) were delivered as a control to spontaneously hypertensive rats (SHRs) and cultured human embryonic kidney (HEK) 293 cells.

**Results:** Treatment with rAAV-HK decreased cell apoptosis in the target organs of SHRs and also inhibited lipopolysaccharide (LPS)-induced HEK 293 apoptosis. The rAAV-HK delivery system also increased the levels of apoptosis-inhibiting proteins bcl-2 and bcl-x<sub>L</sub>, and decreased the level of Bax and the activity of caspase 3, two promoters of apoptosis. In addition to its role in the inhibition of apoptosis, rAAV-HK also activated the cell survival and proliferation signaling pathways ERK1/2 and PI3K/AKT.

**Conclusion:** rAAV-mediated HK gene delivery has multiple therapeutic possibilities for treating hypertension, not only by decreasing blood pressure, but also by directly inhibiting end-organ damage.

**Keywords:** kallikrein; recombinant adeno-associated virus; gene therapy; hypertension; apoptosis; target organs injuries

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## Introduction

Hypertension is a chronic disease affecting approximately 1 billion individuals worldwide and causing approximately 7.1 million deaths per year<sup>[1]</sup>. Hypertension damages major organs, eventually leading to heart failure, atherosclerosis and kidney diseases. Several clinical trials have demonstrated that although some special outcomes differ between the classes, the different drugs available for lowering blood pressure have similar mechanisms of cardiovascular prevention<sup>[1]</sup>.

Gene therapy may provide long-term control of blood pressure for hypertensive patients<sup>[2]</sup>. Recombinant adeno-associated virus (rAAV) is an appealing vector for gene therapy protocols<sup>[3]</sup>, possibly mediating stable transduction and long-term expression of target genes *in vivo*. Data accumulated from

thousands of animal studies and hundreds of human patients have demonstrated that rAAV vectors can be effectively and safely used for gene therapy<sup>[4]</sup>.

The kallikrein/kinin system (KKS) plays an important role in cardiovascular disease and prevention. Tissue kallikrein cleaves the low molecular weight kininogen substrate to produce the kinin peptide, which binds to bradykinin B2 receptor to trigger a broad spectrum of biological effects, such as vasodilation, inflammation, pain, and cell proliferation<sup>[5]</sup>. The kallikrein gene has become a popular target gene for the treatment of cardiovascular diseases.

Our previous studies showed that overexpression of human tissue kallikrein (HK) mediated by rAAV caused a long-term decrease in blood pressure in spontaneous hypertensive rats (SHRs) and high salt-induced hypertensive rats and lessened injuries to the heart, aorta and kidney<sup>[6–8]</sup>. We hypothesized that in addition to its blood pressure-lowering effects, KKS could prevent end-organ injury by inhibiting apoptosis. In this study, we delivered rAAV encoding HK (rAAV-HK) to

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SHRs and cultured human embryonic kidney 293 cells, using rAAV encoding LacZ (rAAV-lacZ) as a control, to determine whether overexpression of HK can block apoptosis.

## Materials and methods

### Production and measurement of titers of rAAV serotype-2 vectors

The rAAV vector plasmid pXXUF<sub>1</sub>, the rAAV plasmid containing a reporter gene, pdxII-LacZ (control), the packaging plasmid pXX2 and the adenovirus helper plasmid pXX6 have been described previously<sup>[9]</sup>. The pXXUF<sub>1</sub> vector contains two inverse terminal repeats, a cytomegalovirus promoter (CMV) and a poly-A tail. An 860-bp HK fragment (*NotI/NotI*) containing an open reading frame was subcloned into pXXUF<sub>1</sub> downstream of CMV to establish the pUF1-HK plasmid. The rAAV-HK and rAAV-LacZ viruses were produced as described previously<sup>[6]</sup>. The titer of rAAV-HK and rAAV-lacZ was determined by dot blot hybridization.

### Animal treatment and gene delivery

Animal treatment and gene delivery were described previously<sup>[6]</sup>. Twelve three-month-old male SHRs weighing 250–280 g were randomly divided into two groups (six rats in each group). A single injection of rAAV-HK (about  $1 \times 10^{11}$  transducing units in 1 mL of saline solution) into the tail of the SHRs was administered in the treatment group. For the control group, the SHRs were injected with rAAV-lacZ. Nineteen weeks after rAAV delivery, all animals were sacrificed using an intraperitoneal injection of pentobarbital at a dose of 50 mg/kg body weight. Part of the heart, kidney and aorta was collected from each rat in liquid nitrogen and stored at -80 °C. The other part of each organ was embedded in paraffin and cut into sections (4 μm) for the TdT-mediated dUTP nick end-labeling (TUNEL) assay.

All animal experimental protocols were approved by the Institutional Animal Research Committee of Tongji Medical College and carried out according to the guidelines of the National Institutes of Health.

### TdT-mediated dUTP nick end-labeling (TUNEL) assay

To investigate apoptosis in the organs of hypertensive rats, we conducted a TdT-mediated dUTP nick end-labeling (TUNEL) assay on paraffin-embedded tissue sections of the heart, kidney and aorta of the SHRs. The TUNEL assay kit was obtained from R&D Systems (Minneapolis, USA), and the *in situ* apoptosis analysis was performed according to the manufacturer's instructions. The TUNEL-positive (apoptotic) cells were counted in 10 microscopic fields of each tissue section.

### Treatments of human embryonic kidney 293 cells

Human embryonic kidney (HEK) 293 cells, which express the bradykinin B2 receptor, were cultured in DMEM containing 10% FBS<sup>[10]</sup>. Apoptosis was induced by incubating the HEK 293 cells with 200 μmol/L LPS in non-FBS DMEM medium for 24 h. In order to investigate the effect of HK overexpression on apoptosis, the HEK 293 cells were treated with  $1 \times 10^8$  pfu. rAAV-HK or rAAV-lacZ (control) for 3 d before LPS-induced

apoptosis. To demonstrate the effect of kinin on cell apoptosis, HEK 293 cells were treated with human bradykinin ( $1 \times 10^{-6}$  mmol/L) or bradykinin+Hoe140 (a Bradykinin B2 receptor inhibitor,  $1 \times 10^{-4}$  mmol/L) for 2 h before LPS-induced apoptosis. The positive control was only treated with LPS in non-FBS DMEM medium, and the negative control received no treatment or LPS. Apoptotic responses were assessed by three independent methods, including Acridine orange/Ethidium Bromide staining analysis, annexin V and PI FACS analysis, and caspase 3 activity assay, as described below.

### Acridine orange/Ethidium bromide (AO/EB) staining analysis

To detect nuclear DNA condensation, cell nuclear staining (AO/EB staining) was performed. A dye mixture containing 100 μg/mL of both acridine orange and ethidium bromide (AO/EB) was added to the cell culture dish at a final concentration of  $3 \times 10^5$ /mL. Cells with intact nuclear membranes stained green, while nuclei that had lost membrane integrity stained orange. Apoptotic cells could be distinguished from nonapoptotic cells on the basis of the presence of nuclear condensation and fragmentation.

### Annexin V and PI FACS analysis

HEK 293 apoptosis was assayed by annexin V and propidium iodide (PI) staining and analyzed by fluorescence-activated cell sorting (FACSVantage<sup>TM</sup>, BD Biosciences, USA).

### Caspase 3 activity assay

To determine caspase 3 activity, HEK 293 cells were washed twice with ice-cold phosphate-buffered saline and then lysed in the Cell Lysis Buffer provided in the Caspase 3 Colorimetric Assay Kit (R&D Systems). The cell lysates were concentrated with Centricons (mol wt cut-off 3 000; Millipore) to obtain protein concentrations of 2–4 mg/mL. The enzymatic reaction for caspase 3 activity was carried out using the p-nitroanilide-conjugated DEVD peptide (DEVD-pNA) substrate, as described by the manufacturer. The results were quantitated by spectrophotometry at a wavelength of 405 nm.

### Western blot analysis

Total tissue protein was extracted from SHR tissues and cultured HEK 293 cells using TRIzol reagent (Invitrogen Life Technologies) and protein concentrations were detected by the Bradford method. Protein samples (20 μg per lane) were separated by 10% SDS/PAGE, electrophoretically transferred onto PVDF membranes, and the membranes were blocked for 2 h at room temperature with 5% nonfat dried milk in 10 mmol/L Tris-Cl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween-20 (TBS-T). The membranes were then incubated overnight at 4 °C with rabbit anti-β-actin polyclonal antibody, followed by a 2 h incubation at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase. For some experiments, rabbit anti-PI3K (1:500 dilution), rabbit anti-AKT (1: 1000 dilution), rabbit anti-phospho-AKT(1: 1000 dilution), rabbit anti-ERK1/2 (1: 1000 dilution), rabbit anti-Phospho-ERK1/2 (1: 1000 dilution), rabbit anti-bcl-2 (1:1000 dilution), rabbit anti-

bcl-x<sub>L</sub> (1: 1000 dilution) or mouse anti-bax (1: 1000 dilution) was used instead of the anti-β-actin antibody.

#### Statistical analysis

Data are expressed as means ± SDs. Differences were analyzed for significance by one-way repeated-measures ANOVA and further analyzed with the use of the Newman-Keuls test for multiple comparisons between treatment groups. The results were considered significant at  $P < 0.05$ .

## Results

### Kallikrein gene delivery attenuates cell apoptosis in target organs of SHRs

First, we used the TUNEL staining assay to detect apoptotic cells in target organs of SHRs that were treated as described above. Our data showed that the heart, kidney and aorta of rAAV-HK-treated SHRs had significantly fewer TUNEL-positive cells than rAAV-LacZ-treated SHRs (Figure 1A), and kallikrein gene delivery significantly reduced the ratio of TUNEL-positive cells to total tissue cells compared with the control group ( $n=6$ ,  $P < 0.01$ ) (Figure 1B). These results suggest that kallikrein delivery attenuates cell apoptosis in the target organs of SHRs.

### Kallikrein gene delivery significantly activates ERK1/2 and PI3K/AKT signaling pathways in SHRs

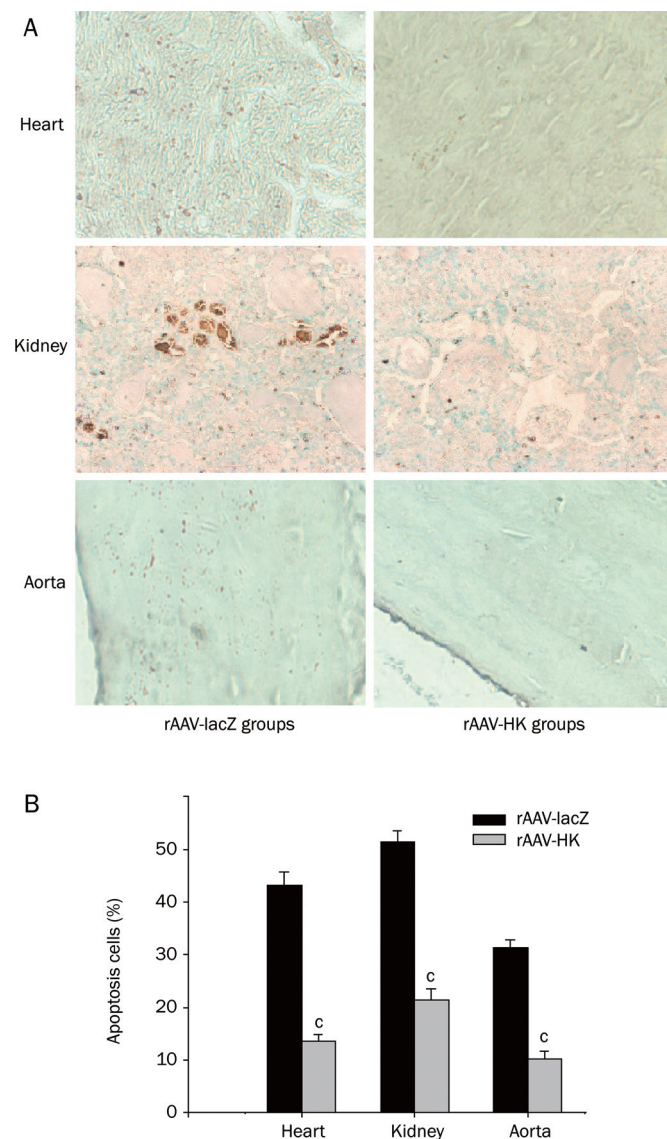
We also examined whether HK gene delivery could change the balance between cell death and survival by altering critical cell proliferation signaling pathways. Thus, we examined the effects of rAAV-HK on the PI3K/AKT and ERK1/2 signaling pathways in SHRs using Western blotting. The protein levels of PI3K, phosph-AKT and phosph-ERK1/2 were significantly increased in rAAV-HK-treated SHRs compared with those treated with rAAV-lacZ (Figure 2). These increased protein levels indicated that overexpression of kallikrein stimulated phosphorylation of ERK1/2 and PI3K/AKT signaling molecules in the target organs of SHRs, which might also contribute to the anti-apoptotic effects of kallikrein gene delivery in SHRs.

### Kallikrein gene delivery inhibits apoptosis by affecting the bcl-2 protein family

In order to understand the mechanisms underlying the anti-apoptotic effects of HK gene delivery on the target organs of SHRs, we examined the expression levels of different genes in the bcl-2 protein family using Western blotting. Kallikrein gene delivery significantly increased the protein levels of bcl-2 and bcl-x<sub>L</sub>, two known anti-apoptosis proteins, and decreased the protein level of Bax, a pro-apoptosis protein (Figure 3). These observations suggested that the overexpression of kallikrein gene inhibited hypertension-induced cell apoptosis by modulating the expression of bcl-2 protein family members.

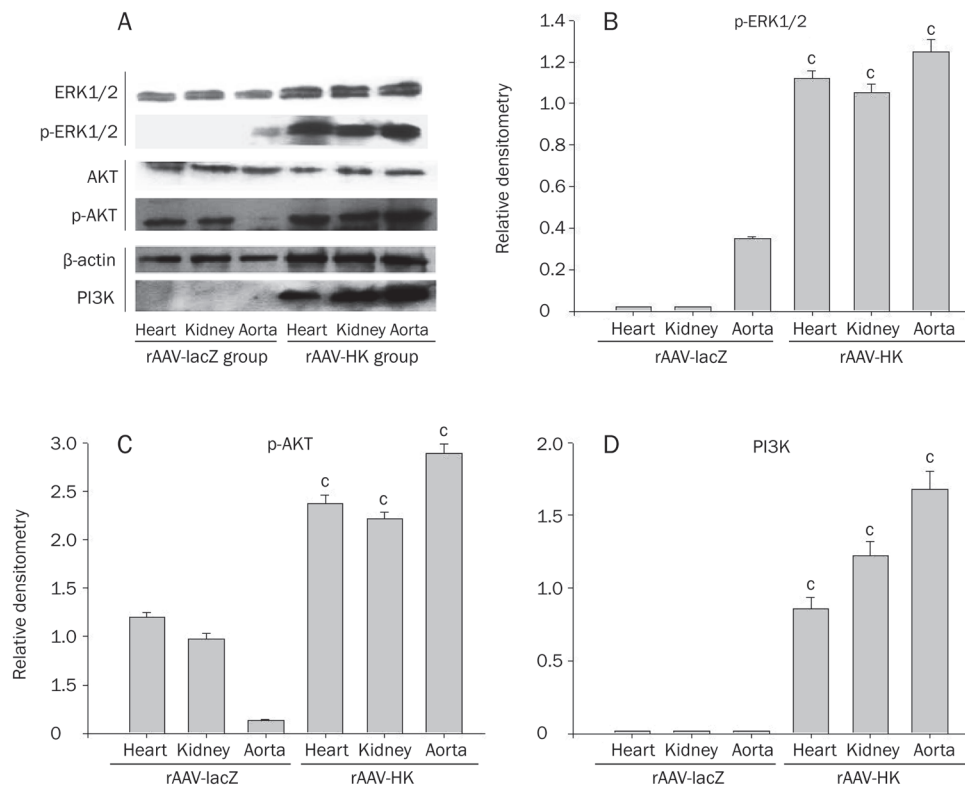
### Kallikrein/kinin system inhibits HEK 293 LPS-induced apoptosis

Having determined the protective effects of kallikrein against apoptosis *in vivo*, we then sought to confirm these effects in an *in vitro* cell culture system. The effects of the kallikrein/

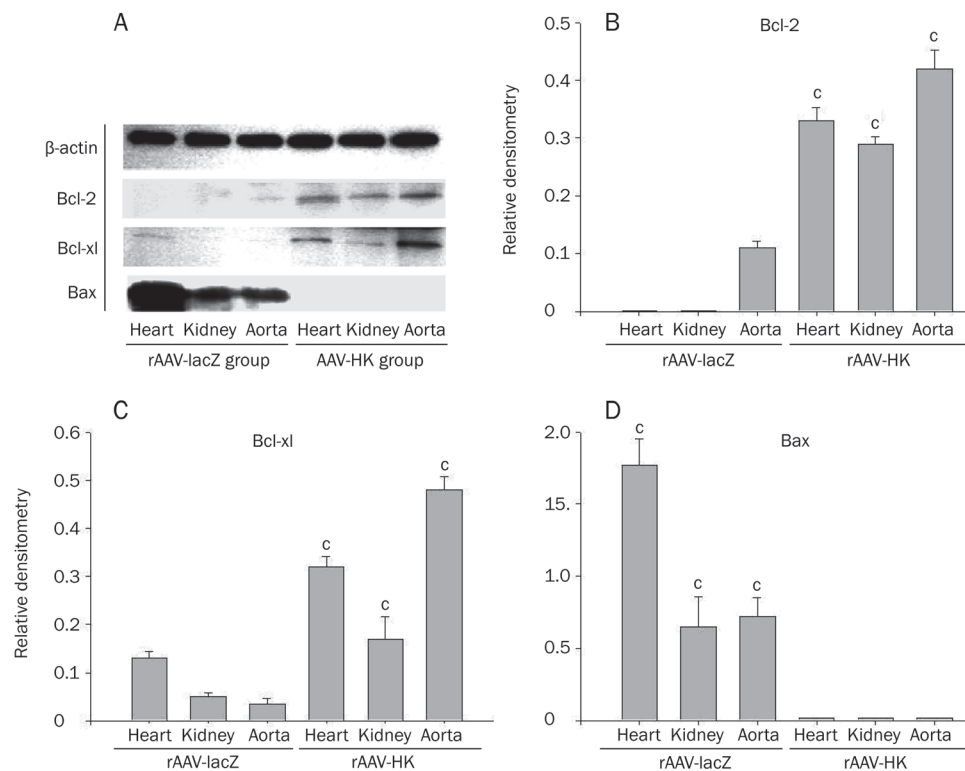


**Figure 1.** Kallikrein gene delivery attenuates apoptosis in organs of SHRs. TUNEL-positive (apoptotic) cells were counted in 10 microscopic fields for each tissue section. TUNEL-positive staining of all tissues was significantly reduced in the rAAV-HK-treated SHRs compared with the rAAV-LacZ-treated SHRs (Figure 1A). As seen in the histogram, Kallikrein gene delivery significantly reduced the ratio of TUNEL-positive cells to total tissue cells compared with the control group ( $n=6$ ,  $^{\circ}P < 0.01$  vs rAAV-LacZ group, Figure 1B).

kinin system on HEK 293 cell apoptosis were detected by acridine orange/ethidium bromide staining and flow cytometry (double staining with annexin V and PI). rAAV-HK and Kinin treated HEK 293 cells had markedly reduced LPS-induced apoptosis ( $P < 0.05$ ), while rAAV-lacZ had no effect on LPS-induced apoptosis. Hoe 140, a B2 receptor inhibitor, attenuated the anti-apoptotic effect of kinin (Figures 4, 5). These data indicate that the kallikrein/kinin system has a direct anti-apoptotic effect by activating the B2 receptor.



**Figure 2.** Kallikrein gene delivery significantly activates cell proliferation signaling pathways in SHRs. The protein levels of phosph-ERK1/2, phosph-AKT, and PI3K were detected by Western blotting (A), and the expression levels, as determined by densitometry, were normalized to total ERK1/2, AKT, and  $\beta$ -actin (B, C, D). The histogram displays the significant increase of p-ERK1/2, p-AKT, and PI3K in rAAV-HK-treated SHR compared with rAAV-lacZ control group.  $^{\circ}P < 0.01$

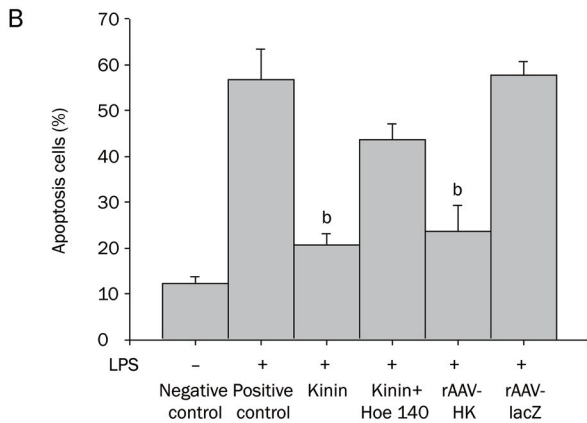
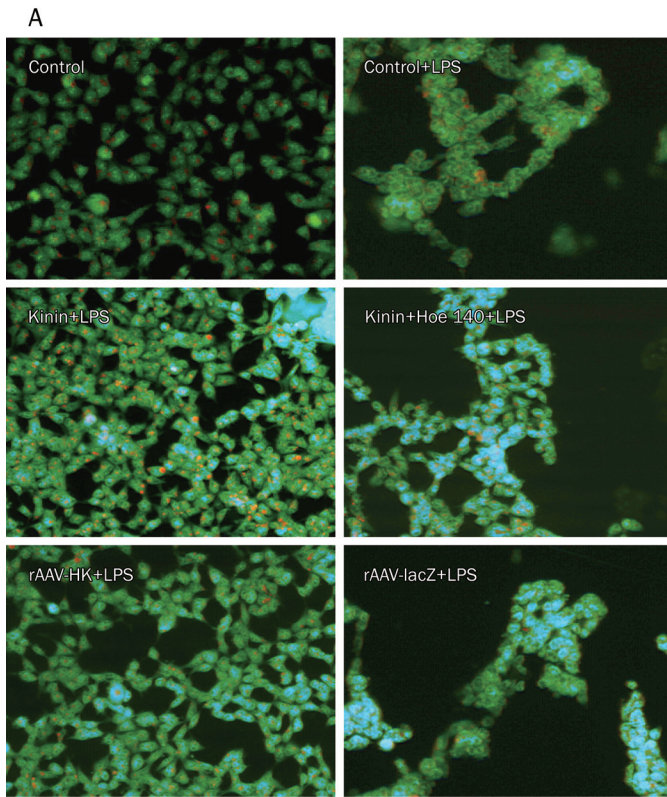


**Figure 3.** The kallikrein/kinin system inhibits apoptosis by modulating the bcl-2 protein family. Protein expression levels in the tissue samples were detected by Western blot analysis (A). Blots were scanned and the relative expression levels, as determined by densitometry, were normalized to total  $\beta$ -actin as shown in the histogram (B, C, D). Kallikrein gene delivery significantly increased the protein levels of bcl-2, and bcl-xl, two anti-apoptotic proteins, and decreased the protein level of Bax, a pro-apoptotic protein.  $^{\circ}P < 0.01$  vs rAAV-LacZ group.

### Kallikrein/kinin system reduces caspase 3 activity

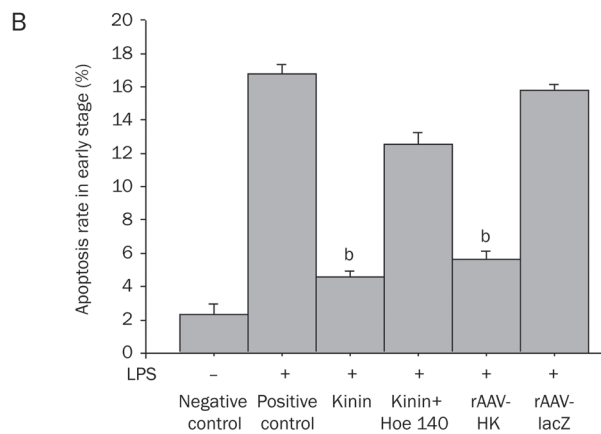
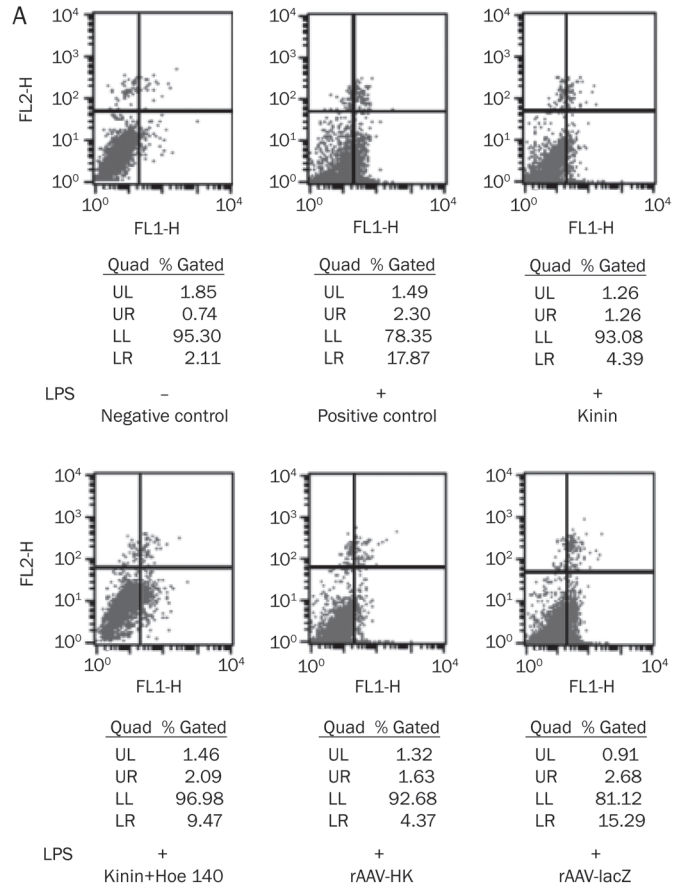
Caspase 3 is a central executor of apoptosis signaling. We tested whether rAAV-HK delivery could alter downstream

signaling of the apoptosis pathway by affecting caspase 3 activity. The results of the caspase 3 activity analysis showed that with non-FBS medium, the activity of caspase 3 increased



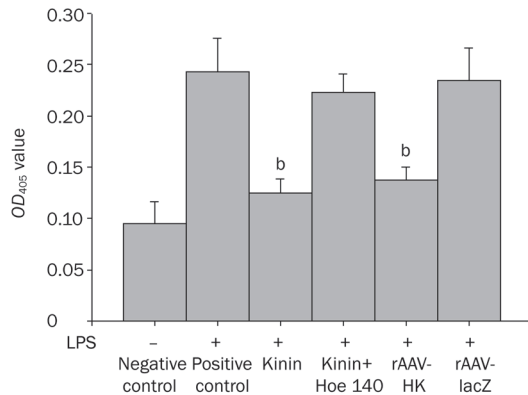
**Figure 4.** The kallikrein/kinin system reduces the ratio of apoptotic-to-live cells after apoptosis induction. HEK 293 cells were examined under a fluorescent microscope after acridine orange/ethidium bromide staining (A). Bright green cells with condensed chromatin are live apoptotic cells. Bright orange cells with condensed chromatin are dead apoptotic cells. Fainter green and orange cells with diffuse chromatin are non-apoptotic live and dead cells, respectively. The results showed that apoptosis increased when cells were exposed to LPS in non-FBS medium (positive control group) compared to that in medium without LPS (negative control group). Treatment with kinin or rAAV-HK markedly reduced LPS-induced HEK 293 apoptosis ( $P < 0.05$ ), while rAAV-lacZ had no effect on apoptosis. Hoe 140, a B2 receptor inhibitor, attenuated the effects of kinin on apoptosis(B). <sup>°</sup> $P < 0.01$  vs rAAV-LacZ.

significantly upon LPS exposure compared with the negative control group. Treating cells with rAAV-HK and kinin, but



**Figure 5.** The kallikrein/kinin system reduces the ratio of apoptotic-to-live cells after apoptosis induction. Annexin V and PI FACS analysis showed that the early-stage apoptotic rate (positive for annexin V and negative for PI) increased in non-FBS medium with LPS (positive control group) compared to that in medium without LPS (negative control group). Treatment with kinin and rAAV-HK markedly decreased apoptosis compared to the positive control group, the kinin+Hoe140 group and the rAAV-lacZ group (<sup>b</sup> $P < 0.05$ ), and these cells had no obvious differences compared to the negative control group.

not rAAV-lacZ, inhibited the effect of LPS on caspase 3 activity ( $P < 0.05$ ). Kinin+Hoe140 did not affect caspase 3 activity after



**Figure 6.** The kallikrein/kinin system reduces apoptosis by inhibiting the caspase-related signaling pathways. In non-FBS medium, LPS exposure significantly increased the activity of caspase 3 compared to the negative control group. Treatment with rAAV-HK and kinin, but not rAAV-lacZ, inhibited the effects of LPS on caspase 3 activity ( $P < 0.05$ ). Kinin+Hoe140 had no effects on the LPS-induced increase in caspase 3 activity.

LPS exposure (Figure 6). These data suggest that overexpression of the kallikrein gene inhibits LPS-induced apoptosis by modulating the activity of caspase 3 through activation of the B2 receptor.

#### Kallikrein/kinin system activates ERK1/2 and PI3K/AKT signaling pathways in HEK 293 cells

We also confirmed that the kallikrein/kinin system activates

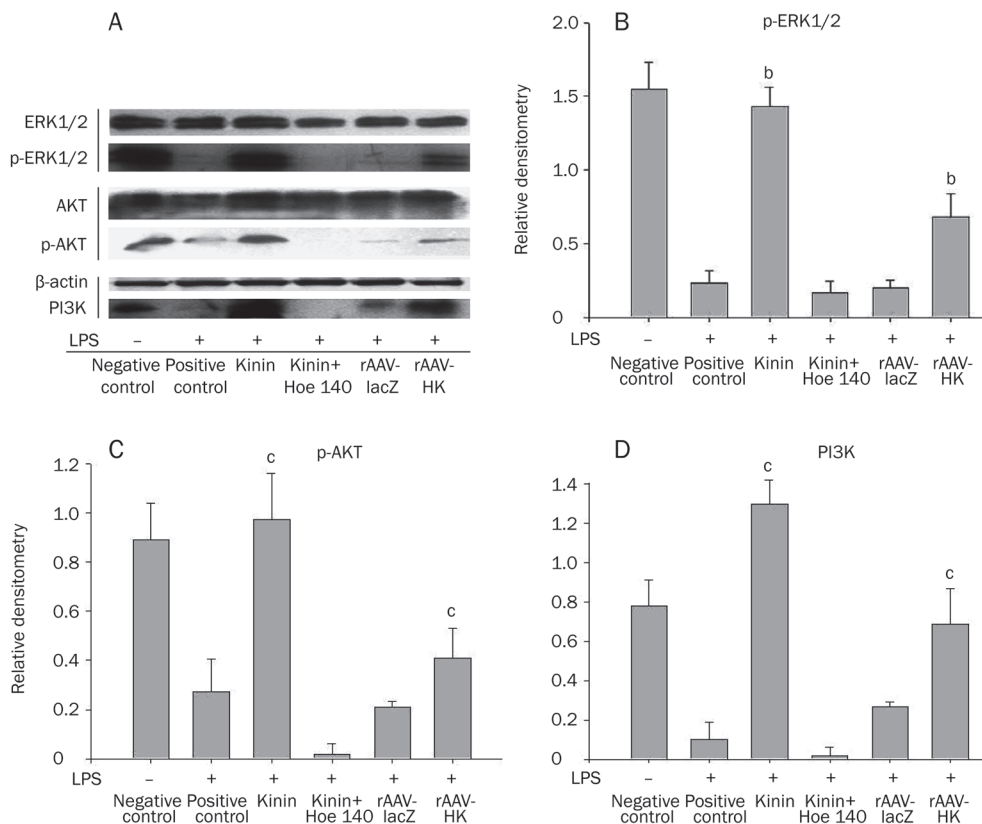
the PI3K/AKT and ERK1/2 signaling pathways in HEK 293 cells using Western blotting. Expression of PI3K, phospho-AKT and phospho-ERK1/2 decreased when HEK 293 cells were exposed to LPS in non-FBS medium. Treatment with rAAV-HK and kinin, but not rAAV-lacZ, inhibited the effect of LPS on PI3K, phospho-AKT and phospho-ERK1/2 expression ( $P < 0.05$ ). Kinin+Hoe140 did not counteract the decreased expression of PI3K, phospho-AKT and phospho-ERK1/2 induced by LPS (Figure 7). These data indicate that the kallikrein/kinin system activates the PI3K/AKT and ERK1/2 signaling pathways by activating the B2 receptor, which may contribute to the observed anti-apoptotic effects of the kallikrein/kinin system in HEK 293 cells.

#### Kallikrein/kinin system inhibits apoptosis in HEK 293 cells by affecting the bcl-2 protein family

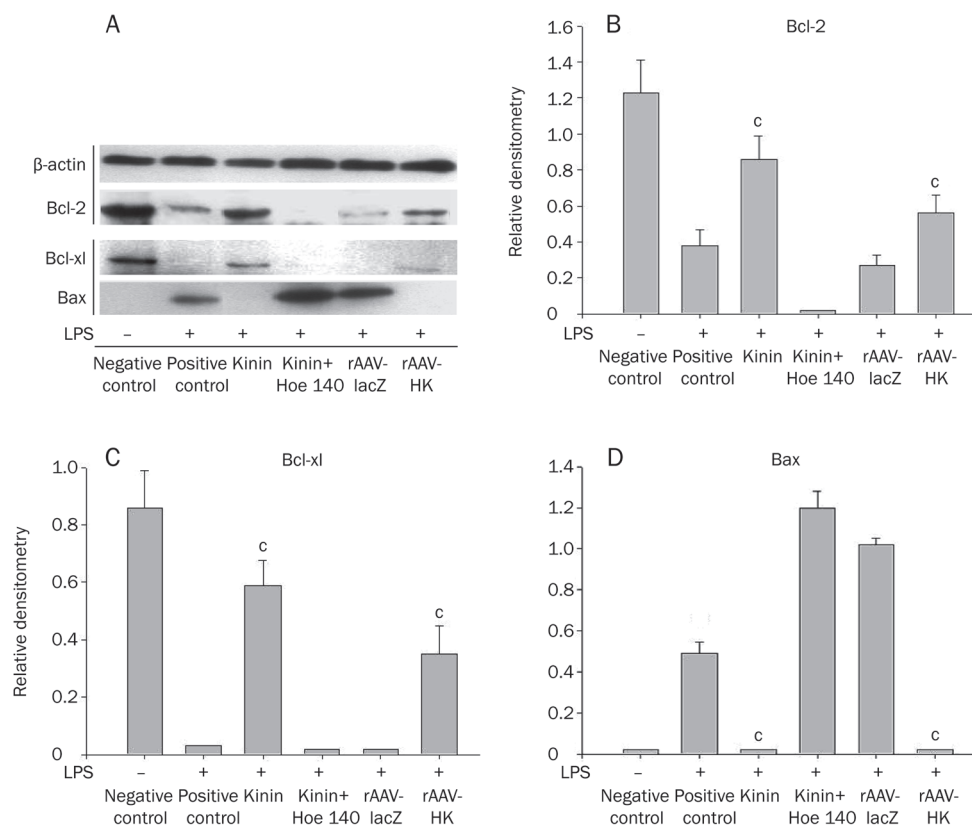
Finally, we examined the effects of the kallikrein/kinin system on the expression of different proteins in the bcl-2 protein family in HEK 293 cells using Western blotting. In non-FBS medium, LPS exposure decreased the levels of bcl-2 and bcl-xl and increased the expression of bax in HEK 293 cells. However, treatment with rAAV-HK and kinin, but not lacZ or kinin+Hoe140, attenuated the effects of LPS on the expression of the bcl-2 family of proteins (Figure 8). These data indicate that the kallikrein/kinin system directly protects against apoptosis by affecting the activity of the bcl-2 protein family.

#### Discussion

Accumulating evidence indicates that apoptosis is increased in



**Figure 7.** The kallikrein/kinin system activates cell proliferation signaling pathways in HEK 293 cells. Western blot analysis was performed on cells in non-FBS medium exposed to LPS (A), and the relative expression levels of p-ERK1/2, P-AKT, and PI3K as determined by densitometry, were normalized to total ERK1/2, AKT, and  $\beta$ -actin, as shown in the histograms (B, C, D). The expression levels of PI3K, phospho-AKT and phospho-ERK1/2 decreased upon LPS exposure. Treatment with rAAV-HK and kinin, but not rAAV-lacZ, inhibited the effect of LPS on the expression of PI3K, p-AKT and p-ERK1/2. Kinin+Hoe140 had no effects on the LPS-induced decrease in PI3K, phospho-AKT and phospho-ERK1/2 expression. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs positive control.



**Figure 8.** The kallikrein/Kinin system inhibits apoptosis by affecting the bcl-2 protein family in HEK 293 cells. Western blot analysis was performed on lysates from cells in non-FBS medium exposed to LPS (A), and the relative expression levels, as determined by densitometry, were normalized to total  $\beta$ -actin, as shown in the histograms (B, C, D). When exposed to LPS in non-FBS medium, the protein levels of bcl-2 and bcl-x<sub>L</sub> in HEK 293 cells decreased while the protein level of bax increased. However, treatment with rAAV-HK and kinin, but not lacZ or Kinin+Hoe140, attenuated the effects of LPS on the expression levels of the bcl-s family. <sup>c</sup>*P*<0.01 vs rAAV-LacZ group.

the heart, kidney and aorta of hypertensive patients, resulting in heart failure, renal dysfunction and vascular remodeling<sup>[11]</sup>. The present study demonstrated that a single intravenous injection of rAAV-HK could protect against apoptosis in these organs of SHR. Furthermore, *in vitro* assays showed that kallikrein/kinin activates the Bradykinin B2 receptor to inhibit LPS-induced apoptosis in HEK 293 cells. We also demonstrated that rAAV-HK modulates the expression level of the bcl-2 protein family to exert its anti-apoptotic effects, which consequently leads to decreased activation of caspase 3. In addition to modulating the apoptotic pathway, rAAV-HK also activates the PI3K/AKT and ERK1/2 pathways, which are critical to cell proliferation, both *in vivo* and *in vitro*. In summary, these data suggest that in addition to reducing blood pressure, rAAV-mediated HK gene delivery also protects against hypertension-induced apoptosis in organs affected by this chronic disease.

Increased cardiomyocyte apoptosis has been recently demonstrated in SHR and patients with essential hypertension<sup>[12]</sup>. Although apoptosis could be induced by pressure overload, *in vitro* and *in vivo* findings indicated that angiotensin II could be critical in cardiac apoptosis<sup>[13,14]</sup>. A study of patients with essential hypertension by González and his colleagues indicated that the efficacy of antihypertensive treatment in reducing blood pressure does not predict its capacity to reduce myocardial apoptosis in hypertensive patients<sup>[15]</sup>. They also found that chronic AT1 blockade is associated with reduced myocardial apoptosis in essential hypertension<sup>[15]</sup>. The ben-

eficial effects of angiotensin-converting enzyme inhibition in hypertension, cardiovascular and renal disease can be partially attributed to kinin accumulation resulting from the activation of the B2 receptor<sup>[16,17]</sup>. Previous animal studies suggested that kallikrein/kinin protects against myocardial apoptosis after ischemia/reperfusion injury<sup>[18,19]</sup>. In our study, delivery of the kallikrein gene protected against cardiomyocyte apoptosis in SHR; therefore, this gene could potentially be used to treat hypertension.

Enhanced apoptosis is present in the kidneys of SHR. Our previous study indicated that delivery of the kallikrein gene could attenuate renal tubule injury and decrease the level of urinary microalbumin in SHR<sup>[6]</sup>. The present study demonstrated that kallikrein/kinin protects against hypertensive renal injury by inhibiting apoptosis in the kidney. Kallikrein/kinin directly improves renal function, independent of its effects on blood pressure, by inhibiting apoptosis in multiple animal models, including salt-sensitive rats and gentamicin-induced renal injury<sup>[20,21]</sup>. Our previous study demonstrated that rAAV-mediated HK delivery upregulates the B2 receptor and downregulates angiotensin II receptor 1, endothelin receptor A and vasopressin receptor 2<sup>[22]</sup>, which could also contribute to the anti-apoptotic effects of the kallikrein/kinin system in hypertensive kidney injuries.

Vascular remodeling contributes to end-organ damage in hypertension, meaning prevention of this phenomenon should be a therapeutic aim of antihypertensive therapy. Growth, apoptosis, inflammation and fibrosis all contribute to arterial

remodeling in hypertension<sup>[23]</sup>. As shown in our study, aortas of DOCA-salt rats, SHRs and angiotensin-infused rats exhibit apoptosis<sup>[23]</sup>. Numerous inflammatory modulators are also present in the vasculature, including reactive oxygen species<sup>[24]</sup>, NO<sup>[25]</sup>, angiotensin type 2 receptors<sup>[26]</sup> and the endothelin system<sup>[27]</sup>. The present study suggests that kallikrein/kinin regulates apoptosis in the aorta through countervailing influences on anti-apoptotic and pro-apoptotic systems.

The present study also demonstrated that rAAV-HK or kinin delivery has a direct anti-apoptotic effect in HEK 293 cells, suggesting that the kallikrein/kinin system protects against hypertension-induced organ injury by inhibiting apoptosis and not just by lowering blood pressure.

Apoptosis is a cellular self-destruction mechanism involved in a variety of biological events. Two cellular pathways trigger apoptosis: the intrinsic pathway, for which the mitochondrion is the central organelle governed by the pro- and anti-apoptotic Bcl-2 family of proteins, and the extrinsic pathway, consisting of the TNF-related family of cell surface receptors<sup>[28]</sup>. The Bcl-2 family can be divided into the anti-apoptotic members, such as Bcl-2 and Bcl-x<sub>L</sub>, and pro-apoptotic members, such as Bax<sup>[28]</sup>. The present study demonstrated that overexpression of the kallikrein gene in both hypertensive rats and cultured cells upregulates Bcl-2 and Bcl-x<sub>L</sub> expression and downregulates Bax expression, and the effects of kinin on the Bcl-2 family could be inhibited by a bradykinin B2 receptor inhibitor. These results suggest that kallikrein/kinin regulates the Bcl-2 protein family through the bradykinin B2 receptor to protect cells from hypertension-induced apoptosis.

The apoptotic process is executed by a family of cysteine proteases, which specifically cleave their substrates at aspartic acid residues<sup>[29]</sup>. These proteases, known as caspases, are activated through the extrinsic and/or intrinsic pathways. In the present study, delivery of the kallikrein gene decreased the activity of caspase 3, an important pro-apoptotic factor<sup>[29]</sup>, thus inhibiting apoptosis in hypertensive organs. In addition to inhibiting the apoptosis pathway, kallikrein/kinin activates the PI3K/Akt and ERK1/2 signaling pathways, which are involved in cell survival and proliferation.

In summary, delivery of the kallikrein gene protected against hypertension-induced organ damage by inhibiting cell apoptosis and enhancing cell survival. These effects were achieved by activating the PI3K/Akt and ERK1/2 signaling pathways and decreasing caspase 3 activity. This study suggests that rAAV-mediated HK gene delivery could be used as a novel hypertension treatment that not only decreases blood pressure, but also inhibits apoptosis in affected organs.

#### Acknowledgements

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#### Author contribution

Dao-wen WANG designed research; Jiang-tao YAN, Tao WANG performed research; Tao WANG contributed new analytical tools and reagents; Tao WANG analyzed data; Jiang-tao YAN wrote the paper.

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