Online Data Supplement

Bradykinin stimulates renal Na^+ and K^+ excretion by inhibiting the K^+ channel (Kir4.1) in the distal convoluted tubule.

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Running Title: Bradykinin and NCC

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Material and Methods

Preparation of the DCT

Mice were sacrificed by CO_2 inhalation plus cervical dislocation. After mice were sacrificed, the abdomen was opened to expose the left kidney, we perfused the left kidney with 2 ml L-15 medium (Life Technology) containing Type 2 collagenase (250 unit/ml). The collagenase-perfused kidney was then removed for further dissection. The renal cortex was separated and cut into small pieces for additional incubation in collagenase-containing L-15 media for 30-50 min at 37^{o}C. The tissue was then washed three times with fresh L-15 medium and transferred to an ice-cold chamber for dissection. The isolated DCT tubules were placed on a small cover glass coated with poly-lysine and the cover glass was placed on a chamber mounted on an inverted microscope. For continuous perfusion of bradykinin (4 μg/min/kg BW) or vehicles for 3 days, the mice were implanted subcutaneously with an osmotic mini-pump (Model. 1007D, Alzet). The animal use protocol was approved by independent animal usage committee in both NYMC and Harbin Medical University.

Generating KS-Kir4.1 knockout (KO) mice

Mice expressing Pax8-rtTA and tet-on LC-1 transgene were crossed with *Kcnj10*-floxed mice to generate inducible kidney-specific *Kcnj10* knockout mice (KS-*Kcn10*$^{-/-}$). *Kcnj10* deletion was carried out in 8-week-old male and/or female mice homozygous for floxed *Kcnj10* gene and heterozygous for Pax8-rtTA/LC-1 transgene by providing doxycycline (5mg/ml, 5% sucrose) in the drinking water for 2 weeks. This was followed by at least 2 additional weeks without doxycycline treatment, before performing experiments. Littermate mice of the same age and genetic background drinking 5% sucrose were used as controls (*Kcnj10*$^{+/+}$).

Genotyping

Tail DNA was PCR amplified with the following primers: *kcnj10* forward 5'-TGATGTATCTCGATTGCTGC-3' and reverse 5'-CCCTACTCAATGCTCTTAAC-3' yielding a 550 bp product from the floxed Kcnj10 gene and a 420 bp product from the wild-type allele; Pax8rtTA forward 5'CCATGTCTAGACTGGACAAGA-3' and Pax8rtTA reverse 5'-CAGAAAGTCTTGCCATGACT-3' which yields a 220 bp product; and LC1-CRE forward 5'-TTTCCCGCAGAACCTGAAGATG-3' and reverse 5'-TCACCGGCATCAACGTTTTCTT-3' which yields a 190 bp product.

Single channel recording

Single channel patch-clamp experiments were performed in the basolateral membrane of the DCT. Single K^+ channel currents were recorded with an Axon200B amplifier (Axon), low-pass filtered at 1 KHz, and digitized by an Axon interface (Digidata 1332) with sampling rate of 4 KHz. For the calculation of channel numbers, we selected a channel recording at least 10 min long. We determine the channel open probability (P_o) from the channel number (N) and NP_o (a product of channel number and open probability) which was calculated from data samples of 60 seconds duration in the steady state. NP_o was determined using the following equation:

$$NP_o = \Sigma(t_1 + 2t_2 + ...it_i)$$

where t_i is the fractional open time spent at each of the observed current levels. The channel conductance was determined by measuring the current amplitudes over several voltages.

Whole cell recording

Whole-cell patch-clamp experiments were performed in the early portion of the DCT (DCT1). An Axon 200A amplifier was used for the measurement of K^+ current (I_K) reversal potential and Ba^{2+}–sensitive K^+ currents. For measuring I_K reversal potential, the tip of the pipette was filled with pipette solution containing (in mmol/l) 140 KCl, 2 $MgCl_2$, 1 EGTA, and 5 HEPES (pH 7.4). The pipette was then back-filled with the pipette solution containing amphotericin B (20 μg/0.1 ml). The bath solution is the same as the one we used to perform the single channel recordings. For the measurement of whole-cell Ba^{2+}–sensitive K^+ currents, the bath solution contains (in mmol/L) 140 KCl, 2 $MgCl_2$, 1.8 $CaCl_2$ and 10 HEPES (pH=7.4). After forming a high resistance seal (>2 GΩ), the membrane capacitance was monitored until the whole-cell patch configuration was formed. The currents were low-pass filtered at 1 KHz, digitized by an Axon interface with 4 KHz sampling rate (Digidata 1440A). Data were analyzed using the pClamp software system 9.0 (Axon).

Immunoblotting

Whole kidney protein extract was obtained from frozen kidney homogenized in a buffer containing (in mmol/L) 250 sucrose, 50 Tris-HCl pH 7.5, 1 EDTA, 1 EGTA, 1 DTT supplemented with phosphatase and protease inhibitor cocktails (Sigma). Protein (40-60 μg) was separated on 7% (wt/vol) Tris-Glycine gel and transferred to nitrocellulose membrane. The membranes were incubated 2 hours with 5% milk in TBS-T and then incubated overnight at 4ºC with primary antibodies (NCC, pNCC at Thr^{53} , and NKCC2) with 1:1000 dilution. The membrane was then examined with Protein Simple infrared imaging system (FlourChem R).

Procedures for renal clearance

Animal were anesthetized by 2-4% isoflurane through inhaling mask. The mice were placed on a heated small blanket to maintain body temperature at 37°C. The trachea was canulated to clear any mucus that may be produced during the experiment. A carotid artery was catheterized with PE10 tubing for blood collection, jugular vein was also cannulated for iv infusion. The bladder was exposed and catheterized via a suprapubic incision with a 10 cm piece of PE-10 tubing for urine collections. After completion of surgery, isotonic saline was given intravenously for 4 hr (0.3 ml/1 hr 0.9% saline) to replace surgical fluid losses and to maintain hemodynamics. To study the effect of bradykinin on urinary Na^+ and K^+ excretion, one time perfusion of bradykinin (0.5 ng/kg BW) was used. Urine collections started one hr after infusion of 0.3 ml saline and total 6 collections (every 30 minutes) were performed (2 for controls and 4 for experiments). After renal clearance experiment, the mice were sacrificed by IV somnasol.

Result

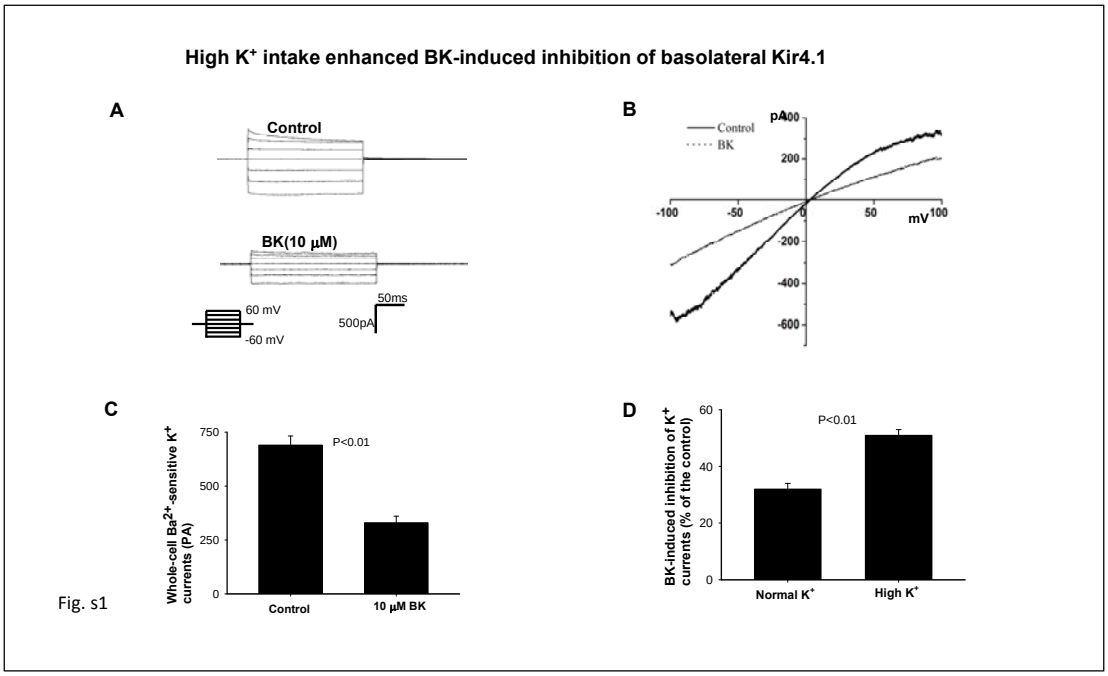

Fig. s1 HK intake enhanced the inhibitory effect of BK on the basolateral K^+ conductance. (A) A whole-cell recording shows the Ba^{2+}-sensitive K^+ currents in the DCT of the mice on HK diet for 7 days and currents was measured from -60 mV to 60 mV at a 20 mV step. (B) A recoding demonstrating the whole-cell K^+ currents measured in the DCT with RAMP protocol from -100 mV to 100 mV in the mice on a HK diet for 7 days. (C) A bar graph summarizes the results of 7 experiments showing that BK treatment decreased the K^+ currents. (D) A bar graph showing that HK intake enhanced the inhibitory effect of BK on the basolateral K^+ channels in the DCT in comparison to the group with normal K^+ diet (n=7).

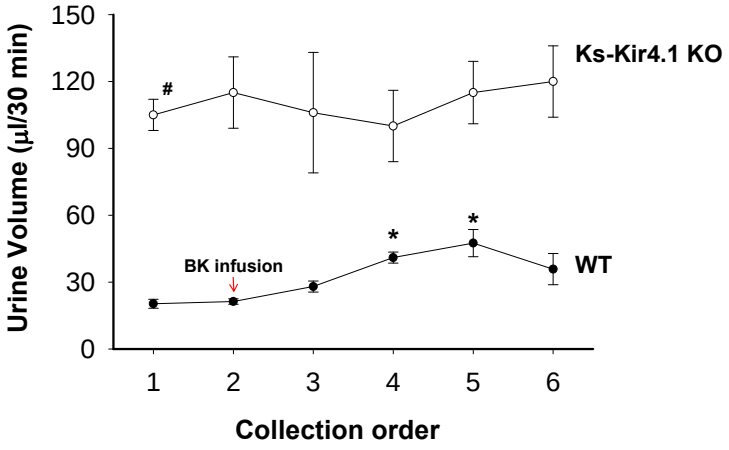

Fig. s2

Fig.s2 **Effect of BK infusion on urine volume of WT and Ks-Kir4.1 KO mice**. For the clearance study, the mice were intravenously perfused with isotonic saline for 4 hr (0.3 ml/1 hr containing 140 mM Na^+ and 5 mM K^+) and urine collections started one hr after saline infusion. After two collections (1 and 2) before BK injection (control), 100 µl saline containing 100 µM BK (indicated by a red arrow) was infused and 4 collections (every 30 min) were made. "*" indicates the value is significantly different from the control (1st and 2nd collections). "#" indicates the difference in urine volume of Ks-Kir4.1 KO mice is significantly different from WT (determined by two-way *ANOVA*).

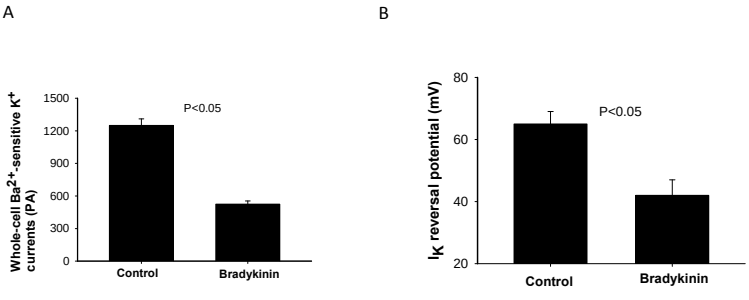

Fig. s3

Fig.s3 **Effect of BK infusion on whole-cell K^+ currents and I_K reversal potential** (A) A bar graph summarizes the results of experiments in which Ba^{2+} -sensitive K^+ currents were measured at -60 mV in the DCT from control (vehicle) and BK-treated mice (n=6). BK was infused through an osmotic pump (4 g/min/kg BW) for three days. (B) A bar graph summarizes the results of experiments in which I_K reversal potential of the DCT was measured from control (vehicle) and BK-treated mice (n=7). The I_k reversal potential was measured with whole-cell recording with 140 mmol/L K^+ in the pipette (intracellular solution) and 140 mmol/L Na^+ /5 mmol/L K^+ in the bath.

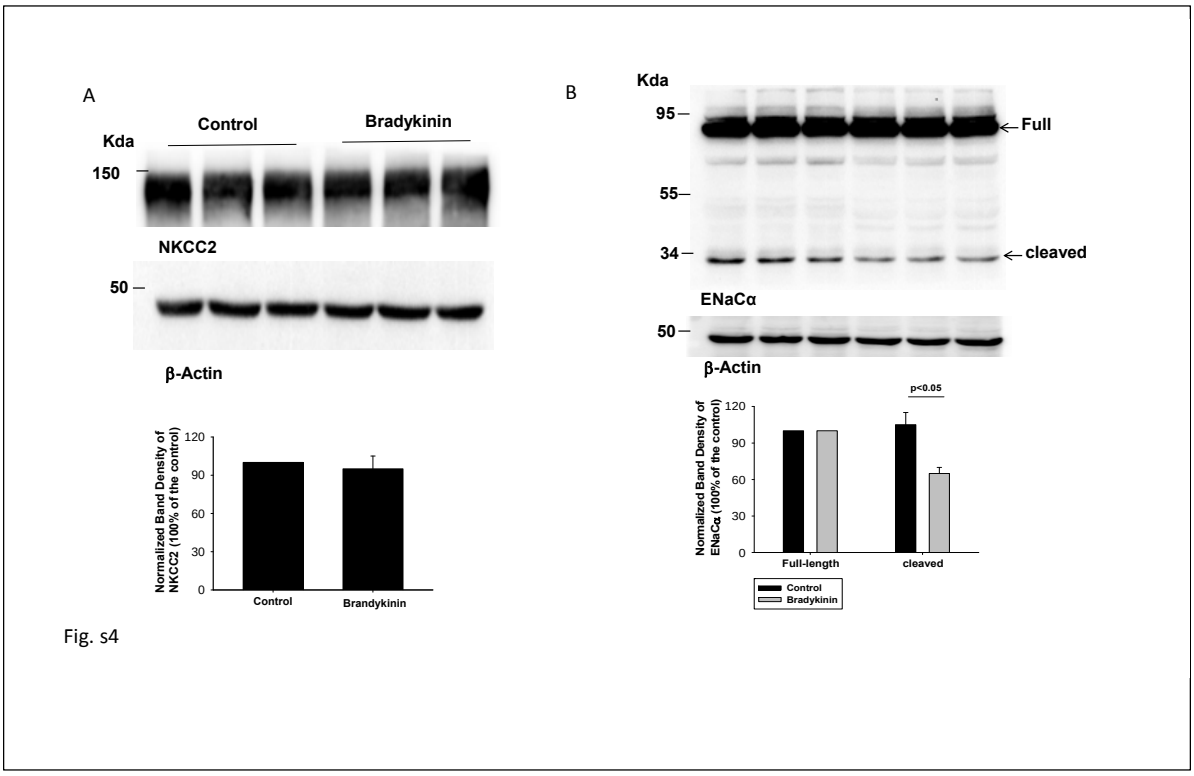

Fig. s4

Figs4 BK infusion does not affect the expression of NKCC2 but decreases the expression of cleaved ENaCα. Western blot showing the expression of NKCC2 (A) and ENaCα (B) in the kidneys obtained from control (vehicle) and BK treated mice (three days).