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Fructose increases the activity of sodium hydrogen exchanger in renal proximal tubules that is dependent on ketohexokinase

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Abstract

High fructose intake has been known to induce metabolic syndrome in laboratory animals and humans. Although fructose intake enhances sodium reabsorption and elevates blood pressure, role of fructose metabolism in this process has not been studied. Here we show that by ketohexokinase — the primary enzyme of fructose — is involved in regulation of renal sodium reabsorption and blood pressure *via* activation of the sodium hydrogen exchanger in renal proximal tubular cells. First, wild-type and ketohexokinase knockout mice (Male, C57BL/6) were fed fructose water or tap water with or without a high salt diet. Only wild type mice fed the combination of fructose water and high salt diet displayed increased systolic blood pressure and decreased urinary sodium excretion. In contrast, ketohexokinase knockout mice were protected. Second, urinary sodium excretion after intraperitoneal saline administration was reduced with the decreased phosphorylation of sodium hydrogen exchanger 3 in fructose-fed WT; these changes were not observed in the ketohexokinase knockout mice, however. Third, knockdown of ketohexokinase attenuated fructose-mediated increases of NHE activity with decreased cAMP levels in porcine renal proximal tubular cells (LLC-PK1). In conclusion, fructose metabolism by ketohexokinase increases sodium hydrogen exchanger activity in renal proximal tubular cells *via* decreased intracellular cAMP level, resulting in increased renal sodium reabsorption and blood pressure in mice.

Keywords: Fructose; Ketohexokinase; NHE3; Blood pressure; Sodium reabsorption; Kidney

1. Introduction

Hypertension is caused by various factors, and it is considered that one of the major factors of blood pressure (BP) regulation is renal salt homeostasis [1,2]. The mechanism for salt sensitive hypertension includes increased arterial pressure following an expansion of extracellular fluid volume and increased cardiac output, designated by increased salt intake with dysfunction of renal excretion and total peripheral vessel resistance [3].

Along with the dramatic increase of sugar consumption in the 1900s, fructose consumption also showed a greater increase [4]. Fructose is a simple sugar, a monosaccharide, present primarily in added dietary sugars, honey, and fruit [5]. The introduction of an additional sweetener — high fructose corn syrup (HFCS; free fructose and free glucose; usually in a 55/45 proportion) — in the 1970s resulted in a further increase in total fructose intake. This increase in fructose intake is associated with a remarkable increase in the rates of obesity, diabetes, and hypertension [6,7]. Fructose intake from simple sugars has been epidemiologically linked to high blood pressure [8], and acute fructose administration has also been reported to increase blood pressure in humans [9]. Overweight men given 200 g of fructose for 2 weeks developed increased ambulatory and clinic BP [10]. Additionally, a systematic review of prospective cohort studies indicated a modest risk of developing hypertension in association with the consumption of fructose containing sugar sweetened beverages [11].

Fructose can be metabolized by both hexokinase and ketohexokinase (KHK, also known as fructokinase). The latter enzyme is the

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primary enzyme that metabolizes fructose and exists in two isoforms: KHK-A and KHK-C [12]. KHK-C is a rapid metabolizer of fructose and is expressed in the liver, intestines, and kidneys, whereas KHK-A is a slow metabolizer of fructose and is expressed ubiquitously, including in the kidneys [13]. We previously reported that KHK-KO mice were protected from fructose-induced obesity, hyperinsulinemia, NAFLD, and chronic kidney disease [13–18], and that fructose metabolism by KHK-C might be involved in those deleterious effects [19,20]. In addition, long-term high fructose intake increased blood pressure [21,22]. Moreover, the elevation of systolic blood pressure (SBP) has been particularly reported using the combination of high fructose and high salt diet in which fructose may enhance salt-mediated hypertension by enhancing sodium absorption [23,24]. In kidneys, KHK is predominantly localized to the proximal tubules [25]. Glut5 (SLC2A5), which is a major fructose transporter, is also abundant in the S3 proximal tubule [26]. Sodium hydrogen exchanger 3 (NHE3, also known as Slc9a3) is one of the principal mechanisms of sodium reabsorption in the kidney, expressed on apical membranes of the renal proximal tubule [27,28]. Recently, it has been reported that fructose stimulates NHE3 activity in the kidney proximal tubule [29]. However, its mechanism and the role of the fructose metabolizing enzyme KHK have not been elucidated. Here, we examined the role of fructose metabolism in the regulation of BP, renal sodium reabsorption, and NHE3 activity in renal tubular cells using KHK-KO mice and the renal proximal epithelial cell line LLC-PK1.

2. Materials and methods

2.1. Animal studies

KHK-KO mice (C57BL/6 background) lacking both KHK-A and KHK-C generated as described previously were used [30]. KHK-KO mice and wild-type (WT) litter mates (male, 3 months of age) were used. They were maintained in temperature and humidity controlled specific pathogen free conditions on a 12-h dark/12-h light cycle. Control diet (CD, normal laboratory chow, CE-2, 0.31% sodium) and high salt diet containing 4% NaCl (HSD) were obtained from CLEA Japan. Animal care and experimental procedures were approved by the Animal Experimentation Committee of the Nagoya University Graduate School of Medicine and were conducted according to the Nagoya University Regulations for Experiments.

Animal study 1; the study design is shown in Fig. 1A. WT and KHK-KO mice were assigned to four groups respectively (n=6, 3 mice per cage), with mean body weight matched among groups (Table 1). Groups included those fed CD or HSD with free access to water containing 10% (w/v) fructose (Fr, Sigma Aldrich, St. Louis, MO) or tap water (Wa) for 6 weeks (CD+Wa, CD+Fr, HSD+Wa, HSD+Fr). BP was measured in restrained, conscious mice by the indirect tail cuff technique under unstressed conditions (BA-98A, Softron, Japan) weekly until week 5. Before measurement, the mice were warmed with a heating pad for a few minutes. A total of 10 readings were taken for each mouse at 1 to 2-min intervals. Three days after blood pressure measurement, overnight urine was collected using metabolic cages. Four days after urine collection, they were anesthetized by 2.0 to 3.0% isoflurane and sacrificed after 6 h of fasting, and then blood and kidney samples were obtained.

Animal study 2; the study design is shown in Fig. 3A. To investigate the urinary sodium excretion in response to acute salt loading depending on fructose administration period without the influence of intestinal sodium absorption, salt loading *via* intraperitoneal administration was performed as previously described [31,32]. WT and KHK-KO mice were assigned to four groups respectively, and fed an *ad libitum* control diet with free access to water containing 10% (w/v) fructose for 0, 2, 4 and 6 weeks (n=6, one mouse per cage). At 0, 1, 3 and 5 weeks, mice were given 1.5 mL of normal saline (Otsuka Pharma, Tokyo, Japan) intraperitoneally (i.p.) and then placed into metabolic cages. Subsequently, urine was collected over the next 6 h, urinary volume, urinary sodium, and creatinine concentration were measured. One week later (to provide time separation from the IP injection) the mice were anesthetized by 2.0 to 3.0% isoflurane and sacrificed and kidney tissues were harvested.

The kidney and serum were removed rapidly, snap-frozen in liquid nitrogen, and stored at−80 °C until examinations were performed. Tissue was processed for histology, protein extraction, and RNA extraction. Blood was drawn from the inferior vena cava into chilled tubes. Serum and urine were separated by centrifugation and stored at−80 °C until measurement of each parameter was performed.

2.2. Histopathology

Methacarn-fixed, paraffin-embedded renal histology was evaluated by periodic acid-Schiff (PAS) staining. Immunohistochemistry for mouse KHK was performed using the anti-mouse KHK antibody (1:200 dilution, Sigma) and NHE3 (sodium-hydrogen

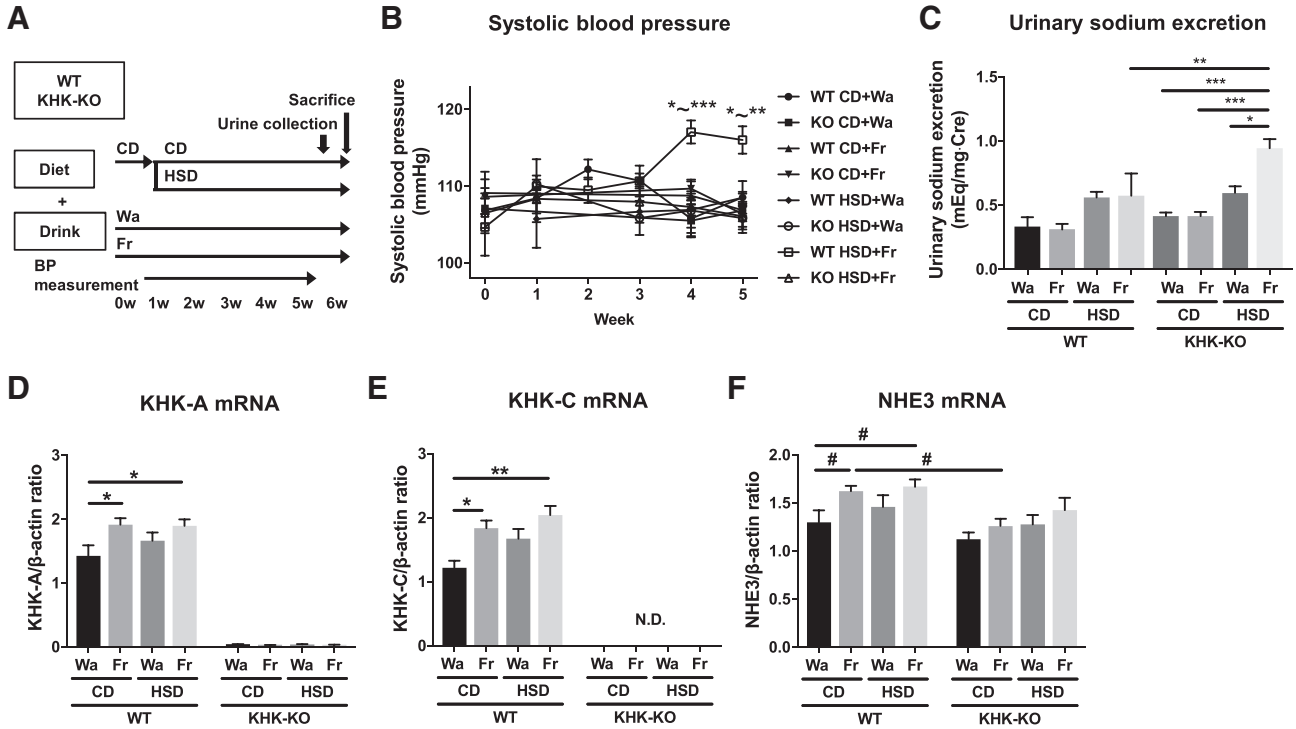

Fig. 1. WT and KHK-KO mice were given control diet (CD) or high salt diet (HSD) with tap water (Wa) or fructose water (Fr) for 6 weeks (n=6). A, Study design. B, SBP measured by tail cuff method. At 4 weeks; *P≪.05 vs. WT CD+Fr, **P≪.01 vs. WT HSD+Wa, KHK-KO HSD+Wa, KHK-KO HSD+Fr, ***P≪.001 vs. WT CD+Wa and KHK-KO CD+Wa. At 5 weeks; *P≪.05 vs. WT CD+Fr and KHK-KO CD+Wa, **P≪.01 vs. KHK-KO CD+Fr, KHK-KO HSD+Wa and KHK-KO HSD+Fr (two-way repeated ANOVA followed by Tukey's multiple comparisons tests). C, Urinary sodium excretion corrected by urine creatinine (n=6). D-F, Quantitative PCR for mouse KHK-A (D), KHK-C (E), and NHE3 (F) in kidney. β-actin was used as an internal control (n=5). Data represent means±S.E.M. *P≪.05, **P≪.01, ***P≪.001. #P≪.05 (t-test). N.D., not detected.

Table 1
Mice characteristics

	WT				KHK-KO			
	CD		HSD		CD		HSD	
	Wa	Fr	Wa	Fr	Wa	Fr	Wa	Fr
BW (g)	24.6±0.2	26.0±0.5	25.9±0.4	24.7±0.1	24.7±0.7	27.2±0.8	25.3±0.4	24.6±1.2
ΔBW (%)	5.1±0.9	2.9±1.0	5.2±0.9	6.9±1.1	3.0±0.4	5.0±1.4	1.9±0.7	2.6±2.4
Total energy	9.7±0.2	11.6±0.3[a]	12.1±0.1[*]	9.8±0.1[a]	10.7±0.3	14.8±0.1[*,#]	11.9±0.1	13.5±0.2[*]
Diet	9.7±0.2[§]	7.6±0.1[*, §, a]	12.1±0.1	5.1±0.1[#, a]	10.7±0.3	12.1±0.1[&]	11.9±0.1	9.7±0.1
Drinking	4.8±0.1[§]	9.9±0.4[*, a]	10.2±0.2	11.8±0.2	5.6±0.1	6.9±0.1[#]	10.5±0.2[*]	9.6±0.4[*]
Salt	26.1±0.4[§]	19.9±0.3[§, a]	144.9±1.3	63.9±0.1[*, a]	27.7±0.8	31.2±0.4[§]	142.5±0.7[*]	117±0.4[*,#]
UA	3.7±0.8	3.2±0.5	3.4±0.2	3.3±0.4	4.7±0.2	2.6±0.4[*]	3.9±0.4	2.5±0.5[*]

WT, wild type mice. KHK-KO, KHK knockout mice. CD, control diet. HSD, high salt diet. Wa, tap water. Fr, fructose water. BW, body weight (g) at week 0. delta BW, % increase of body weight for 5 weeks. Total energy, total energy intake (kcal/mouse/day). Diet, energy intake from chow (kcal/mouse/day). Drinking, drinking intake (ml/mouse/day). Salt, salt intake (mg/mouse/day). UA, serum uric acid (mg/dl). Data represent means±S.E.M. *P≪.05 vs. respective CD+Wa. #P≪.05 vs. respective HSD+Wa. &P≪.05 vs. respective HSD+Fr. §P≪.05 vs. respective HSD+Wa and HSD+Fr. a, P≪.05 vs. respective KHK-KO mice.

exchanger 3, anti-mouse NHE3 antibody, 1:200 dilution, Gene Tex, Irvine, CA, USA), as described previously [20]. Double immunofluorescent staining for mouse KHK and mouse NHE3 was also performed using Opal IHC kit (NEL840001KT, Perkin Elmer).

2.3. Biochemical analysis

Urine levels of creatinine and sodium, serum levels of uric acid were measured by LSI Medience (Japan). Serum insulin level was measured with the mouse insulin ELISA kit and ultrasensitive mouse insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

2.4. Cell culture

LLC-PK1 cells, a porcine (*Sus scrofa*) renal proximal epithelial cell line, was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). Serial culture was maintained in a 55 cm^2 tissue culture dish in medium composed of Dulbecco's modified eagle medium (DMEM, Sigma), 10% (vol/vol) heat inactivated fetal bovine serum (Sigma), 100 IU/mL penicillin, 100 μg/mL streptomycin (Life Technologies, Japan) with 0, 1 and 5 mmol/L fructose. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere in a CO_2 incubator (CO_2 incubator, Panasonic). To measure intracellular pH, cells were harvested with trypsin–EDTA (0.02%), seeded in medium without antibiotics on sterile glass coverslips (D11131H, Matsunami Glass IND. LTD, Osaka, Japan), and incubated for 48 h in the same medium to ensure confluence.

2.5. siRNA transfection

The siRNA targeted against the porcine *KHK* gene was constructed, using Dharmacon's siRNA Design Center algorithm, and synthesized by Dharmacon (Lafayette, CO). The 21 bp sequences of KHK siRNA which targeted all porcine KHK variants were as described below: sense: 5′-GCGGAUAGAGCAACACAAUUU-3′, antisense: 5′-AUUGUGUUGCUCUAUCCGCUU-3′. The lyophilized siRNAs were dissolved in RNase free water to a final concentration of 20 μM. The LLC-PK1 cells were seeded in DMEM with fructose (0, 1, or 5 mmol/L), without antibiotics, and transfected with 50 nM KHK siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as the manufacturer's instructions. Cells were incubated for 48 h before additional experiments were performed.

2.6. Measurement of intracellular pH by fluorescence microscopy

Intracellular pH (pHi) was measured in LLC-PK1 cells on glass coverslips using BCECF, the NH_4Cl pulse technique, and inverted confocal laser microscope (TiEA1R, Nikon Instech Co., Tokyo, Japan), as previously described [29,33]. Briefly, cells on glass coverslips in the control solution (Solution 1, Table 2) were loaded with 20 μmol/L BCECF-AM (B8806, Sigma) for 20 min. The acetoxymethyl ester from BCECF enters cells and is rapidly converted to the anionic free acid form by intracellular esterases. After the loading period, the glass coverslips were rinsed with control solution to remove the BCECF. Then they were placed in a thermo-regulated chamber mounted on an inverted epifluorescence microscope. Bathing solutions were rapidly exchanged without disturbing the position of the coverslips. Fluorescence was monitored using, alternately, 442 nm (pH insensitive) or 488 nm (pH sensitive) as the excitation wavelengths. pHi was calculated from the fluorescence emission ratio of the two excitation wavelengths using a standard calibration procedure based on the use of 10 μmol/L nigericin in high potassium ringer (solution 3, Table 2), at pH 5.8, 6.2, 6.6, 7, 7.4, 7.8, and 8.2. Following cell exposure to 20 nm NH_4Cl (Solution 2, Table 2) for 2 min, bathing solutions were rapidly replaced with Solution 1 in the presence of external 145 mmol/L Na^+, and cell pHi recovery was examined. In this study, we measured each five cells and calculated the initial rate of pHi recovery (delta pHi/delta time, pHi units/min) by linear regression analysis from the first 2 min after the start of the pHi recovery curve.

2.7. Quantitative RT-PCR

Total RNA from mouse whole kidney samples was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the RNA concentration was measured using a spectrophotometer (NanoDrop2000C, Thermo Fisher Scientific, Waltham, MA). According to the manufacturer's instructions, 1 μg of total RNA was reverse transcribed by using the cDNA synthesis kit (Qiagen). Quantitative PCR (qPCR) was performed with ABI Step One Plus Real Time PCR (Thermo Fisher Scientific) using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for mouse NHE3, ENaC-β, ENaC-γ and β-actin, pig (*Sus scrofa*) NHE3, and pig β-actin. qPCR for mouse KHK-A, KHK-C, and pig KHK was performed using Power SYBR Green PCR master Mix (Applied Biosystems). Primer pairs for mouse KHK-A and KHK-C were selected as previously described [30]. Primer pairs for pig KHK, which were designed to amplify regardless of variants, were as follows; forward 5'-CAACTCCTGCACGGTTCTCT-3′ and reverse 5'-CAGAGGTCCACGGAATAGCG-3′ (Invitrogen). All data were normalized based on β-actin expressions.

2.8. Protein extraction and western blot analysis

Proteins were extracted from renal cortex tissues and LLC-PK1 cells using radioimmunoprecipitation lysis buffer (Santa Cruz Biotechnology, Dallas, TX). Samples were derived at the same time and processed in parallel. Then the sample was centrifuged. Protein concentration was determined with Pierce BCA Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Western blot analysis was performed as described previously [13]. All samples were mixed and boiled with Laemmli sample buffer (BIO RAD, Hercules, CA) and subjected to SDS/PAGE analysis, then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with specific primary antibodies to rabbit anti-NHE3 (dilution, 1:1000; Gene Tex), mouse anti-pNHE3 (phosphorylated at Serine 552, dilution, 1:1000; Novus, Littleton, CO), rabbit anti-SGK1 (dilution, 1:500, Abcam, Cambridge, UK), rabbit anti-pSGK1 (phosphorylated at Serine 422, dilution, 1:500, Abcam), and mouse anti-β-actin (dilution, 1:1000, Sigma-Aldrich) overnight at 4 °C. This was followed by incubation with the secondary antibodies horseradish peroxidase conjugated rabbit IgG (dilution,

Table 2
Composition of solutions

	Solution 1 (Control)	Solution 2 (NH_4Cl)	Solution 3 (high K^+)
NaCl	141	121	20
KCl	5.4	5.4	130
$CaCl_2$	1	1	1
KH_2PO_4	0.4	0.4	0
$MgCl_2$	0.5	0.5	1
$MgSO_4$	0.4	0.4	0
Na_2HPO_4	0.3	0.3	0
HEPES	10	10	5
Glucose	0.6	0.6	0
NH_4Cl	0	20	0
Nigericin	0	0	0.01
pH	7.4	7.4	See method

All values are in mmol/l except for pH. HCL or NaOH was used to titrate to the appropriate pH.

1:2000; Cell Signaling Technology, Danvers, MA) or mouse IgG (dilution, 1:5000; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. Proteins were visualized on an Amersham Imager 600 and 680 with an enhanced chemiluminescence detection system (GE Healthcare UK Ltd), and the optical densities of protein bands were measured with the Amersham Imager 600 and 680 analysis software.

2.9. Metabolome analysis

Cells were washed twice with 10 mL of 5% (w/v) mannitol and lysed in 2 mL methanol containing 2.5 μM D-camphor-10-sulfonic acid (CSA) as internal standard. Metabolites were extracted from 400 μl lysate with 200 μl water and 400 μl chloroform, passing 400 μl of the aqueous phase through a 5 kDa-cutoff centrifugal filter tube (Human Metabolome Technologies, Tsuruoka, Japan). The filtrate was dried using a vacuum centrifuge and resuspended in 125 μl water prior to analysis by MS.

Fructose-1-phosphate (F1P), ATP and cAMP levels in LLC-PK1 cells were analyzed by capillary ion chromatography-mass spectrometry (capIC-MS) as described previously [34]. Fructose was analyzed by liquid chromatography- tandem mass spectrometry (LC–MS/MS). Data acquisition was performed using an Agilent 1290 Infinity liquid chromatography system coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with a JetStream interface (Agilent Technologies, Santa Clara, CA). The chromatographic separation was achieved on a Unizon UK-Amino 250×2.0 mm i.d. column (Imtakt Corporation, Kyoto, Japan) and maintained at 60 °C in the column oven. Mobile phase was water (eluent A) and acetonitrile (eluent B). The gradient profile of eluent B is 91% for 0 to 7 min, 87% at 15 min, 50% at 20 min, 10% for 20.1 to 25 min, and then 91% from 25 to 33 min. The mobile phase flow rate was 0.25 ml/min and the injection volume was 1 μl.

Tandem mass spectrometer was operated in the negative ion mode. The ion source parameters were as follow: gas temperature at 250 °C, gas flow at 20 l/min, nebulizer at 50 psi, sheath gas temperature at 350 °C, sheath gas flow at 10 l/min, capillary voltage at 3.5 kV, nozzle voltage at 2 kV, high pressure RF at 90 V, low pressure RF at 60 V, fragmentor voltage at 380 V and cell accelerator voltage at 7 V. The optimized multiple reaction monitoring transition and collision energy of fructose was *m/z* 179.1≫89 and 0 V respectively, and of CSA was *m/z* 231.1≫80 and 36 V. The dwell time was 200 ms.

2.10. Statistical analyses

Results were expressed as the mean±S.E.M. Data without indications were assessed by one-way ANOVA followed by Tukey's multiple comparisons tests using the GraphPad Prism software (GraphPad Software, San Diego, CA). Longitudinal data, such as BP, were analyzed with repeated measures two-way ANOVA. *P* values≪.05 were considered statistically significant.

3. Results

3.1. Combination of fructose and salt intake increased SBP

Utilizing mice that lack both isoforms of KHK, we examined the effects of fructose metabolism on SBP in WT and KHK-KO mice given CD or HSD with or without Fr for 6 weeks (Fig. 1A). Although energy intake was higher in the KHK-KO groups given fructose (both Fr and HSD+Fr) than it was in all other groups, WT and KHK-KO mice showed no significant difference in growth rate between control group and each treatment group for 5 weeks (Table 1). Likely this was because WT mice metabolize fructose easily, resulting in less chow intake in WT groups given fructose (both Fr and HSD+Fr) as well as lower salt intake in the WT HSD+Fr group compared to the other groups given HSD (Table 1). KHK KO mice possess little capacity to metabolize fructose *via* hexokinase, but they also lose fructose *via* the urine [13].

Despite lower salt intake, the WT mice fed fructose and salt exhibited significantly higher SBP after 4-weeks when compared to all other groups, and an increase in SBP was not observed in KHK-KO HSD +Fr mice (SBP at 5 weeks; 116±1.7 vs. 106.1±1.4 mmHg, *P*≪.05) (Fig. 1B). Urinary sodium excretion was significantly elevated in KHK-KO HSD+Fr mice compared to that in WT HSD+Fr mice consistent with increased sodium intake (Fig. 1C). PAS stained kidney sections showed no apparent renal injury in glomeruli and tubules in any of the groups (Supplementary Fig. S1). qPCR demonstrated that renal mRNA expressions of both KHK-A and KHK-C in WT mice given fructose (WT CD+Fr and WT HSD+Fr) were significantly increased compared to that of WT CD+Wa mice, and as expected, no KHK expression was observed in the KHK-KO mice (Fig. 1, D and E). In WT mice, mRNA expression of renal NHE3 was significantly increased by fructose intake regardless of CD or HSD, while no significant change was observed in KHK-KO mice (Fig. 1F).

3.2. Both KHK and NHE3 were expressed in renal proximal tubules

The distribution of NHE3 and KHK in the kidneys was evaluated by immunohistochemical staining and immunofluorescent staining (Fig. 2, A-D). NHE3 expression was confirmed in renal proximal tubular cells among all groups (Fig. 2A). Renal KHK expression was absent (minimal) in KHK-KO groups (Fig. 2B). The cytoplasm of the renal proximal tubules was stained with KHK, and serial sections stained with NHE3 and immunofluorescent double staining for KHK and NHE3 revealed that NHE3 was expressed in the brush border of the KHK-positive renal tubules, indicating both KHK and NHE3 were located in the proximal renal tubules (Fig. 2, C and D). These results suggest that the combination of increased fructose metabolism by KHK, which co-localized with NHE3 in renal proximal tubules, and high salt intake led to increased murine SBP possibly due to lowering urinary sodium excretion.

3.3. Fructose metabolism by KHK enhanced renal sodium reabsorption

The ileal neutral NaCl absorptive process is the major mechanism by which Na^+ is absorbed during the period between meals in the mammalian small intestine [35]. Further, urinary sodium excretion tends to reflect daily sodium intake. Hence, to evaluate the time dependent effect of fructose metabolism by KHK in renal sodium reabsorption in the absence of influences of intestinal sodium absorption and differences in daily salt intake, we examined urinary sodium excretion after the same dosage of i.p. saline administration in WT and KHK-KO mice after 0, 1, 3, and 5 weeks of fructose consumption with normal chow (Fig. 3A).

Fructose intake induced a significant decrease in urinary sodium excretion after i.p. saline administration in WT mice at 3 and 5 weeks compared to that observed at 0 week, but not in KHK-KO mice (Fig. 3B). qPCR revealed that renal mRNA expressions of KHK-A and KHK-C in WT mice were significantly increased at 4 weeks compared to that in WT mice at 0 week (Fig. 3, C and D). Western blot analysis revealed that fructose intake induced a slight but insignificant increase of renal NHE3 protein levels in WT mice (Fig. 3E). It has been reported that fructose stimulates NHE3 activity by decreasing the ratio of phosphorylation of NHE3 at serine-552 (pNHE3) to total NHE3 [29]. Therefore, we examined the phosphorylation of NHE3 in the kidney. In WT mice, the phosphorylation of NHE3 was decreased by fructose intake, and was significantly lower than that observed in KHK-KO mice at 4 weeks (Fig. 3E). These results suggested that the long-term fructose intake enhanced renal sodium reabsorption with the decreased NHE3 phosphorylation, and that is dependent on ketohexokinase.

3.4. KHK dependent activation of NHE in renal proximal tubules

Next, we examined the effect of fructose metabolism by KHK on NHE activity by measuring the rate of pHi change using the NH_4Cl pulse technique in LLC-PK1 cells under fructose stimulation. The delta pHi of LLC-PK1 cells was significantly increased, representing the increase of NHE activity by fructose stimulation in a dose-dependent manner (Fig. 4A). Then, we used synthesized siRNA targeting porcine KHK to suppress KHK mRNA expression in LLC-PK1 cells (Fig. 4B). To evaluate the efficacy of KHK siRNA on the suppression of fructose metabolism, metabolomic analysis was performed. Metabolomic analysis revealed that the intracellular content of both fructose and F1P — the fructose metabolite produced by the action of KHK — were

significantly increased by fructose stimulation (Fig. 4, C and D). Moreover, intracellular fructose content was further increased by KHK siRNA, compared to levels observed under mock transfection (Fig. 4C), while intracellular F1P content was significantly inhibited by KHK siRNA, indicating that KHK siRNA efficiently suppressed fructose metabolism (Fig. 4D). KHK knockdown significantly suppressed the fructose-mediated NHE activity increase (Fig. 5, A and B).

Phosphorylation of NHE3 is dependent on the cyclic adenosine monophosphate (cAMP) concentration [36], and fructose has been reported to decrease cAMP concentration and the ratio of pNHE3 to total NHE3, resulting in increased NHE3 activity in LLC-PK1 cells [29]. Consistent with these observations, we found that fructose stimulation decreased intracellular adenosine triphosphate (ATP), cAMP levels and the ratio of pNHE3 to total NHE3 in LLC-PK1 cells, and this was significantly abrogated by the suppression of KHK expression (Fig. 5, C-E). No difference was observed in the intracellular total NHE3 protein levels among experimental groups (Fig. 5E). These results suggested that fructose metabolism by KHK increased NHE activity, not NHE protein expression, through decreased cAMP in LLC-PK1 cells.

4. Discussion

High fructose intake has been implicated in the increased prevalence of obesity, diabetic mellitus, and hypertension in human and animals [4,6,7,13,21,22,30,37,38]. Recently, an association has been reported between fructose and sodium absorption and salt-sensitive hypertension [21–24]. Although fructose is primarily metabolized by KHK, the role of fructose metabolism by KHK in sodium absorption and salt-sensitive hypertension has not been examined. Here, we show that fructose metabolism by KHK is involved in the regulation of renal sodium reabsorption and blood pressure by activating the sodium hydrogen exchanger *via* cAMP reduction in the renal proximal tubular cells.

In the present study, both WT and KHK-KO mice given either fructose water or a high salt diet alone for 5 weeks showed no significant change in blood pressure, whereas the combination of fructose water and a high salt diet induced a significant increase in blood pressure in WT mice. Growth rate did not differ among all groups of WT mice, and no apparent renal injury was observed in all groups of WT and KHK-KO mice. Salt intake was even lower in WT mice given fructose water and high salt diet than in WT mice given high salt diet without fructose water. These data indicate that the combination of fructose intake with a high salt diet, not just the amount of digested salt, contributed to the regulation of blood pressure. In contrast, in KHK-KO mice, those increases in blood pressure were not observed in all groups, even though both WT and KHK-KO mice fed fructose and high salt diet consumed a similar amount of fructose, and KHK-KO mice ingested more salt than WT when they were fed the combination of fructose water and a high salt diet.

Fructose intake also stimulates salt absorption in the small intestine [22]. Additionally, gastrointestinal-renal regulation involving gut derived hormones secreted in response to salt sensors to modulate renal sodium excretion has been reported [39]. Hence, to evaluate the renal sodium excretion without the influences of intestinal sodium absorption, the urinary sodium excretion after intraperitoneal, not enteral, acute salt administration (same dosage for each mouse) in mice chronically given fructose water was

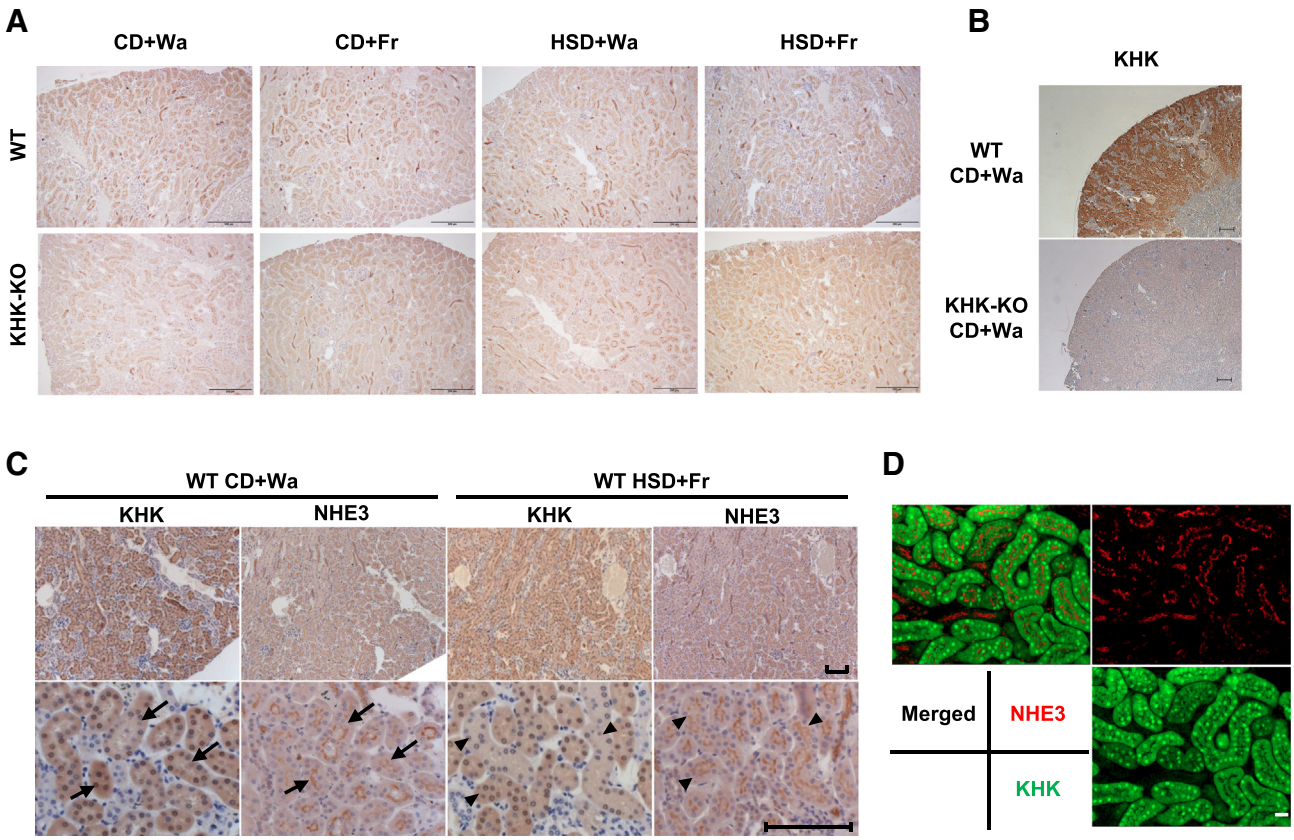

Fig. 2. WT and KHK-KO mice were given control diet (CD) or high salt diet (HSD) with tap water (Wa) or fructose water (Fr) for 6 weeks (n=6). A, Representative images of NHE3 immunostaining of kidney. Scale bar=200 μm. B, Immunohistochemistry in kidney for mouse KHK. Scale bar=200 μm. C, Immunohistochemistry in serial sections of kidney for mouse KHK and NHE3. Scale bar=100 μm. NHE3-positive staining in the brush border of KHK-positive renal tubules (arrow and arrowheads). D, Immunofluorescent double staining of kidney for mouse KHK (green) and NHE3 (red). Scale bar=20 μm.

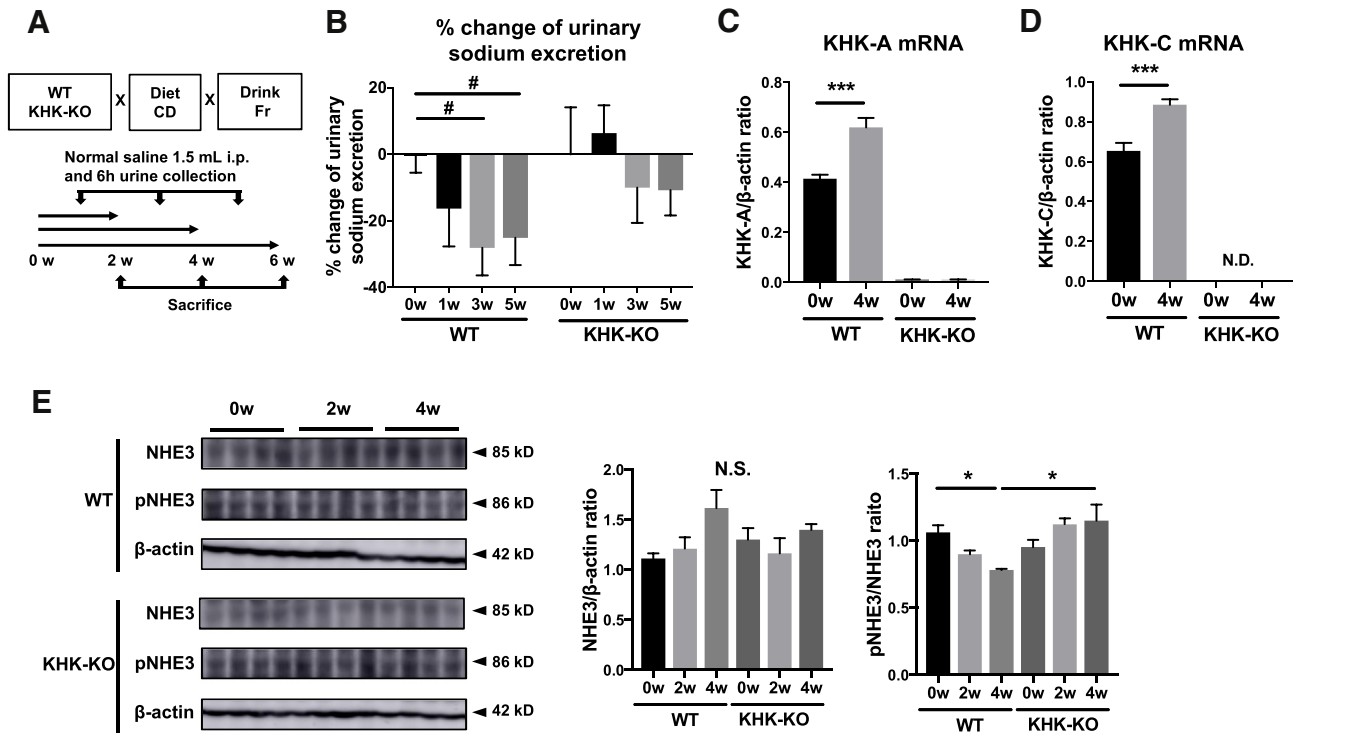

Fig. 3. WT and KHK-KO mice were given control diet with fructose water for 0, 2, 4 and 6 weeks, and saline was injected intraperitoneally at 0, 1, 3, and 5 weeks (n=6). A, Study design. B, Percent changes of urinary sodium excretion corrected by urinary creatinine at 0, 1, 3, and 5 weeks from 0 week (n=6). C and D, Quantitative PCR for mouse KHK-A (C) and KHK-C (D) in kidney. β-actin was used as an internal control (n=6). E, Western blot analysis for total NHE3 and the ratio of renal phosphorylated NHE3 (pNHE3) to total NHE3 in WT and KHK-KO at 0, 2, and 4 weeks (n=4). β-actin was used as an internal control. Data represent means±S.E.M. *P≪.05, **P≪.01, ***P≪.001. #P≪.05 (t-test). N.D., not detected.

analyzed. Fructose-fed WT mice exhibited reduced urinary sodium excretion after intraperitoneal salt administration, but not in KHK-KO mice. These results indicate that fructose metabolism is involved in the regulation of renal sodium excretion and blood pressure in a KHK dependent manner.

Renal proximal tubules are responsible for the reabsorption of 60–70% of the filtered load of salt and water, and the exchange of sodium and hydrogen ions across the cell membrane is regulated by NHE3 expressed in the apical membrane of proximal tubules [28,40]. In this study, we used serial kidney sections to confirm that NHE3 was co-localized with KHK at the apical membrane and cytoplasm of the renal proximal tubules, respectively. Further, renal mRNA expressions of KHK and NHE3 were increased by fructose intake in WT mice. While some effect of fructose in regard to increased NHE3 protein was observed, the primary mechanism of KHK action appeared to involve altering the activity of NHE3 through alteration in intracellular cAMP levels. Specifically, NHE3 activity in renal proximal tubular cells is mediated by the decrease of the ratio of phosphorylated NHE3 to total NHE3 and this was not observed in KHK-KO mice administered fructose. Further, we evaluated the fructose metabolism by KHK in the context of sodium hydrogen exchanger activity using LLC-PK1 cells which express both NHE1 and NHE3 [41,42]. While NHE3 is in the apical membrane of proximal tubule cells, NHE1 is in the basolateral membrane of multiple nephron segments [27,43]. NHE1 plays an important physiological role in maintaining cell volume and acidity, while NHE3 mediates sodium reabsorption [44,45]. Fructose is known to stimulate NHE3 activity, not NHE1, in LLC-PK1 cells by decreasing the ratio of pNHE3 to total NHE3 *via* PKA pathway downregulation through cAMP reduction [29]. cAMP is synthesized from ATP by adenylyl cyclase in the cytoplasm [46]; however, fructose metabolism

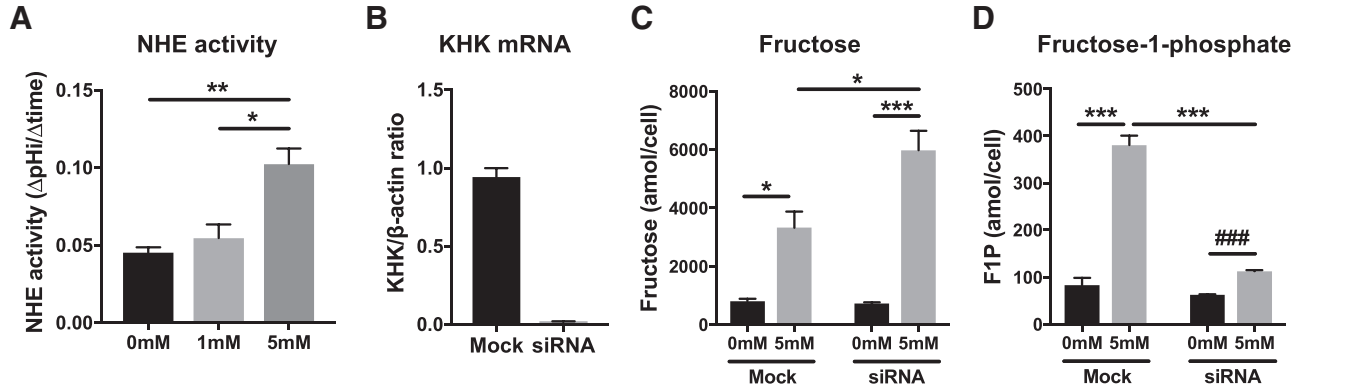

Fig. 4. Sodium hydrogen exchanger (NHE) activity and siRNA targeted against the porcine KHK in LLC-PK1 cells. A, NHE activity measured by delta pHi per minute by using the NH_4Cl pulse technique in LLC-PK1 cells stimulated with fructose. (n=3~4). B, Quantitative PCR for porcine KHK in mock or KHK siRNA transfected LLC-PK1 cells (n=3). β-actin was used as an internal control. C and D, Intracellular concentrations of fructose and fructose-1-phosphate (F1P) (n=3). Data represent means±S.E.M. *P≪.05, ***P≪.001. ###P≪.001 (t-test).

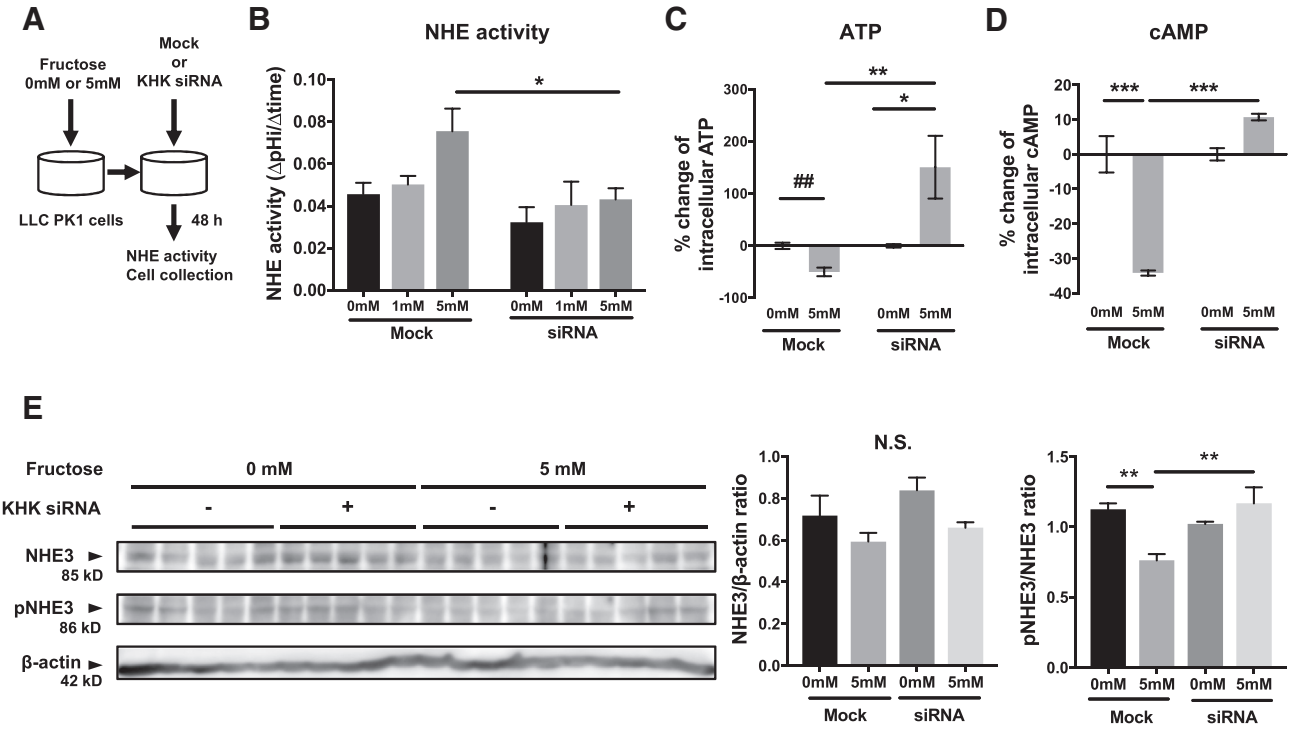

Fig. 5. Sodium hydrogen exchanger (NHE) activity, intracellular delta ATP, delta cAMP levels and phosphorylation of NHE in mock or KHK siRNA transfected LLC-PK1 cells stimulated with fructose. A, Study design. (n=3–6). B, NHE activity of LLC-PK1 cells. C and D, Percent changes of intracellular ATP (C) and cAMP (D) levels in mock or KHK siRNA transfected LLC-PK1 between 0 and 5 mmol/l fructose stimulation (n=3). E, Western blot analysis for total NHE3 and the ratio of renal phosphorylated NHE3 (pNHE3) to total NHE3 in mock or KHK siRNA transfected LLC-PK1 cells with 0 or 5 mmol/l fructose (n=5). β-actin was used as an internal control. Data represent means±S.E.M. *P≪.05, **P≪.01, ***P≪.001. ##P≪.01 (t-test). N.S., not significant.

by KHK is known to result in intracellular ATP depletion and hence might modify cAMP levels [5,13,47]. Indeed, intracellular ATP, cAMP levels and the phosphorylation of NHE3 displayed significant decreases in response to fructose, and those were reversed when KHK expression was knocked down. This suggests that fructose phosphorylation by KHK consumed intracellular ATP, subsequently decreasing intracellular cAMP and blunting NHE phosphorylation, ultimately resulting in increased NHE activity (Fig. 6).

Fructose overload positively correlated with the development of hypertension with the impairment of the renal dopaminergic system [48]. Further, dopamine inhibits NHE activity by stimulating adenylyl cyclase [49], suggesting the possibility that the regulation of renal NHE3 activity by KHK dependent fructose metabolism might involve the renal dopaminergic system.

Insulin has stimulatory effects on serum and glucocorticoid induced kinase 1 (SGK1), which is expressed in the aldosterone-sensitive distal nephron, and is involved in sodium retention, a salt sensitive hypertensive effect observed during high fructose intake [50–53]. Targets of SGK1 include the epithelial Na^+ channel (ENaC) and aldosterone is the principal hormone that affects ENaC and stimulates Na^+ reabsorption by distal segments of the renal tubule [53–55]. SGK1 has also been shown to regulate NHE3 [56–58]. In our study, qPCR demonstrated that fructose intake did not increase renal mRNA expressions of ENaC-β and ENaC-γ in both WT and KHK KO mice (Supplementary Fig. S2). In addition, no significant difference was observed in the serum insulin (data not shown) and intrarenal phosphorylation of SGK1 among the groups (Supplementary Fig. S2), implying that these mechanisms were not directly involved in our animal study. Furthermore, hyperuricemia as a result of a long-term high fructose diet participates in the pathogenesis of fructose-induced hypertension particularly in humans and in rats [6]. However, in our study, elevated serum uric acid levels were not observed, consistent with previous reports using mice (Table 1) [13].

A limitation of this study is that the role of intestinal sodium absorption in the context of SBP increase was not evaluated. As both KHK and NHE are known to be expressed in the intestine, further

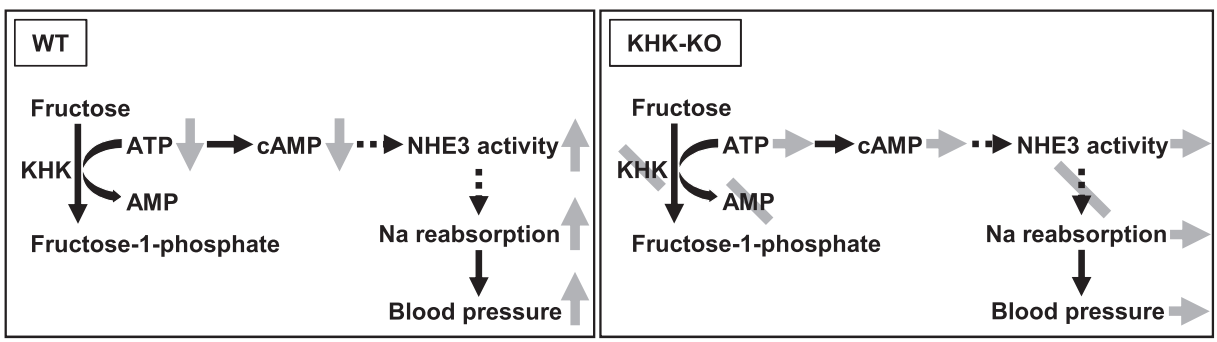

Fig. 6. Possible mechanism. Enhanced fructose metabolism by KHK induces ATP depletion and subsequent decrease of cAMP leading to the increase of NHE3 activity in renal proximal tubular cells, resulting in increases of renal sodium reabsorption and blood pressure.

investigation of the roles of intestinal KHK and NHE would also be valuable.

In conclusion, the combination of high fructose and high salt intake increased renal sodium reabsorption and blood pressure, and these effects were prevented in mice lacking KHK. Fructose metabolism by KHK increased sodium-hydrogen exchanger activity in renal proximal tubular cells, likely mediated by the decrease of cAMP. These results suggest that fructose metabolism by KHK might play an important role in sodium homeostasis and salt sensitivity of blood pressure.

Disclosure

RJJ is an inventor on patents related to lowering uric acid as it relates to blood pressure, insulin resistance, and diabetic renal disease. He also has equity in XORT therapeutics, Inc. and Colorado Research Partners LLC, both which are startup companies interested in developing novel xanthine oxidase inhibitors and fructokinase inhibitors, respectively. RJJ has also received honoraria from Danone, Astra Zeneca, and Horizon Pharmaceuticals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2019.05.017.

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