Aging-associated renal disease in mice is fructokinase dependent

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1Division of Renal Diseases and Hypertension, University of Colorado, Aurora, Colorado; 2Department of Nephrology, Nagoya University Graduate School of Medicine, Nagoya, Japan; 3TMK Project, Medical Innovation Center, Kyoto University, Kyoto, Japan; 4Developmental Biology and Cancer Programme, UCL Institute of Child Health, London, United Kingdom; 5Laboratory of Renal Physiopathology and Department of Nephrology, Instituto Nacional de Cardiología I.J.Ch., Mexico City, Mexico; 6Department of Internal Medicine, Ewha Womans University School of Medicine, Ewha Medical Research Center, Seoul, Republic of Korea; and 7Division of Nephrology, Eastern Colorado Health Care System, Department of Veteran Affairs, Denver, Colorado

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Roncal-Jimenez CA, Ishimoto T, Lanaspá MA, Milagres T, Hernando AA, Jensen T, Miyazaki M, Doke T, Hayasaki T, Nakagawa T, Marumaya S, Long DA, García GE, Kuwabara M, Sánchez-Lozada LG, Kang DH, Johnson RJ. Aging-associated renal disease in mice is fructokinase dependent. Am Physiol Renal Physiol 311: F722–F730, 2016. First published July 27, 2016; doi:10.1152/ajprenal.00306.2016.—Aging-associated kidney disease is usually considered a degenerative process associated with aging. Recently, it has been shown that animals can produce fructose endogenously, and that this can be a mechanism for causing kidney damage in diabetic nephropathy and in association with recurrent dehydration. We therefore hypothesized that low-level metabolism of endogenous fructose might play a role in aging-associated kidney disease. Wild-type and fructokinase knockout mice were fed a normal diet for 2 yr that had minimal (<5%) fructose content. At the end of 2 yr, wild-type mice showed elevations in systolic blood pressure, mild albuminuria, and glomerular changes with mesangial matrix expansion, variable mesangiolysis, and segmental thrombi. The renal injury was amplified by provision of high-salt diet for 3 wk, as noted by the presence of glomerular hypertrophy, mesangial matrix expansion, and alpha smooth muscle actin expression, and with segmental thrombi. Fructokinase knockout mice were protected from renal injury both at baseline and after high salt intake (3 wk) compared with wild-type mice. This was associated with higher levels of active (phosphorylated serine 1177) endothelial nitric oxide synthase in the proximal tubule by fructokinase, and this results in transient ATP depletion with the generation of oxidative stress and inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) (5). The administration of fructose to rats results in modest proximal tubular injury, and has also been shown to accelerate preexistent kidney disease (9, 26). Fructose metabolism also results in the generation of uric acid, and this is associated with the development of afferent arteriolar disease with loss of autoregulation, resulting in glomerular hypertension (29, 30). While most studies have focused on dietary fructose, fructose can also be generated in the kidney and liver by the aldose reductase-sorbitol dehydrogenase polyol pathway, and modest fructose levels can be detected even in fasting animals (13, 21). Indeed, fructose can be generated in the kidney in diabetes or with dehydration, and in both situations may lead to local renal damage (20, 28).

We hypothesized that some of the renal damage associated with aging could be due to fructose-dependent renal injury, even in the absence of dietary fructose. To investigate this hypothesis, we studied aging wild-type mice and aging mice that could not metabolize fructose via the fructokinase-dependent pathway [fructokinase knockout, also known as ketohexokinase knockout (KHK-A/C KO mice)]. KHK-A/C KO mice have a normal phenotype when young (6), but have not been examined in the aging state.

MATERIALS AND METHODS

Experimental protocol and animals. Ketohexokinase-A and -C knockout (KHK-A/C KO) mice of C57BL/6 background and lacking both ketohexokinase-A and ketohexokinase-C, were originally provided by David Bonthron at Leeds University (6). KHK-A/C knockout homozygous mice and wild-type (WT) littermates (male, 24–25 mo old) were used. They were maintained in temperature- and humidity-controlled specific pathogen-free conditions on a 14-hour light/10-hour dark cycle. Both WT and KHK-A/C KO mice were fed regular diet ad libitum [Harlan Teklad; no. 2918, containing 58 percent carbohydrate, 24 percent protein, and 18 percent fat and containing minimal (<5%) of fructose or sugar], with free access to tap water.

AGING is associated with the development of glomerulosclerosis and tubulointerstitial disease in humans and rodents (12, 23, 35). Interestingly, aging-associated renal injury can vary greatly, and some individuals may show minimal reduction in kidney function and relatively preserved kidney histology with age. This raises the possibility that some of the “normal” deterioration in renal function during the aging process observed in Western cultures may be subtle renal injury driven by diet or other mechanisms.

The ingestion of sugar has been associated with albuminuria in humans (3, 4, 31). Sugar contains fructose and glucose, and evidence suggests that the fructose component may be responsible for the renal injury. Specifically, fructose is metabolized in the proximal tubule by fructokinase, and this results in transient ATP depletion with the generation of oxidative stress and inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) (5). The administration of fructose to rats results in modest proximal tubular injury, and has also been shown to accelerate preexistent kidney disease (9, 26). Fructose metabolism also results in the generation of uric acid, and this is associated with the development of afferent arteriolar disease with loss of autoregulation, resulting in glomerular hypertension (29, 30). While most studies have focused on dietary fructose, fructose can also be generated in the kidney and liver by the aldose reductase-sorbitol dehydrogenase polyol pathway, and modest fructose levels can be detected even in fasting animals (13, 21). Indeed, fructose can be generated in the kidney in diabetes or with dehydration, and in both situations may lead to local renal damage (20, 28).

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Table 1. General characteristics of aging WT and KHK-A/C KO mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KHK-A/C KO</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>36.9 ± 1.7</td>
<td>37.1 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.40 ± 0.09</td>
<td>1.52 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Epididymal fat weight, g</td>
<td>1.12 ± 0.23</td>
<td>1.41 ± 0.33</td>
<td>NS</td>
</tr>
<tr>
<td>AST, IU/l</td>
<td>28.6 ± 4.6</td>
<td>25.0 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Serum uric acid, mg/dl</td>
<td>2.6 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Glomerular size</td>
<td>no different</td>
<td>no different</td>
<td></td>
</tr>
<tr>
<td>Glomerular thrombi</td>
<td>6 of 7</td>
<td>involved 20% of the glomeruli</td>
<td></td>
</tr>
<tr>
<td>Serum fructose, pg/ml</td>
<td>1404/1104</td>
<td>193/194</td>
<td>NS</td>
</tr>
<tr>
<td>Serum glucose, mg/dl</td>
<td>191.6</td>
<td>191.6</td>
<td>NS</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>19.1</td>
<td>19.1</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>106.6</td>
<td>106.6</td>
<td>NS</td>
</tr>
<tr>
<td>Serum uric acid, mg/dl</td>
<td>2.6</td>
<td>2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Serum fructose, µmol/l</td>
<td>335.1 ± 19.3</td>
<td>403.9 ± 22.4</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE. WT, wild-type; KHK-A/C, ketohexokinase-A and -C knockout.

Two experimental studies were performed. In the first set of experiments, WT and KHK-A/C KO mice (n = 7 per group) underwent urine collection using a metabolic chamber (Techniplast, Philadelphia, PA) at 24 mo of age, and were euthanized at 25 mo with collection of kidney tissues and serum. A second set of studies were done in which 24 mo old WT and KHK A/C KO mice (n = 5–6 per group) were challenged for 3 wk with a high salt load (1% NaCl in water with 0.04% sucralse). Systolic and diastolic blood pressure was assessed weekly during the period of high salt intake by tail cuff sphygomanometry (Visitech BP2000; Visitech Systems, Apex, NC); mice underwent conditioning prior to any measurements being taken. Urine was collected from metabolic cages at 18–20 mo of age, and again both before and after high salt intake. Mice were euthanized at 25 mo of age by anesthesia and cardiac exsanguination, with serum and kidney tissues collected for analyses.

All experiments were conducted with adherence to the NIH “Guide for the Care and Use of Laboratory Animals.” The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado.

Biochemical analysis. Biochemical analysis for serum alanine aminotransferase, aspartate aminotransferase, total cholesterol, triglycerides, glucose, and urinary creatinine were done with an automated chemistry analyzer (VetACE Clinical Chemistry System, Alfa Wasserman Diagnostic Technologies). Urinary albumin concentration was determined by Albwell M (Exocell, Philadelphia, PA) and urine neutrophil gelatinase-associated lipocalin (NGAL) was measured using the Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). Serum creatinine concentration was analyzed by the high-performance liquid chromatography-tandem mass spectrometry method (33). Urinary nitrates and nitrates were measured using a Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI). Serum fructose was measured using the EnzyChrom Fructose Assay Kit (Bioassay Systems, Hayward, CA) and serum uric acid was measured using QuantChrom Uric Acid assay kit (BioAssay Systems). Kidney tissue samples were homogenized in a buffer containing 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, and 0.5% (vol/vol) Triton X-100. Homogenates were centrifuged at 13,000 rpm for 10 min (4°C) and protein in the collected supernatant quantified. Intrarenal fructose and uric acid levels were assessed by utilizing the Bioassay Systems kits (see above); values were normalized to protein concentration in the lysate determined by the BCA assay (Pierce). Histology. Tissues were fixed in 10% formalin or methyl Carnoy’s and embedded in paraffin. Three-micrometer sections were stained with periodic acid-Schiff reagent (PAS). On coronal sections of each kidney, the area of 50–100 individual glomeruli was determined by

![Figure 1](http://ajprenal.physiology.org/DownloadedFrom/10.1152/ajprenal.00306.2016)

Fig. 1. Focal glomerular thrombi in aging wild-type (WT) mice but not ketohexokinase-A and -C knockout (KHK-A/C KO) mice. Shown are representative glomeruli from WT mice (A) and KHK-A/C KO mice (B). WT mice showed focal glomerular thrombi (A, arrows) whereas thrombi are absent in KHK-A/C KO mice. Glomerular thrombi were present in 6 of 7 aging WT mice and involved 20 percent of the glomeruli (C). Glomerular size was no different between groups (D). Mild mesangial matrix expansion (based on type IV collagen staining) was present in WT aging mice (E) compared with KHK-A/C KO mice and was significantly different (*P < 0.05) when quantified (F). Sample size: n = 7 in WT and n = 6 in KHK A/C KO mice. A, B, E: 400X. ND, not detected.
outlining the glomerular tuft using Aperio software (Aperio Technologies, Vista, CA). Mesangial matrix expansion was determined by measuring the glomerular area containing type IV collagen on tissue sections stained with rabbit anti-type IV collagen antibody (Chemicon International, Temecula, CA) as described elsewhere (18, 34). Specifically, the relative mesangial area (proportion of type IV collagen positive area per glomerular tuft area) was calculated using Aperio Software. Mesangial cell activation (15) was measured using a rabbit anti-smooth muscle actin antibody (RB-9010-P, Thermo Fisher Scientific, Fremont, CA) and determining the ratio of actin positive staining to overall glomerular tuft area in all glomeruli in the tissue section.

Western blotting. Kidney lysates from WT and KHK-A/C KO mice were homogenized in mitogen-activated protein kinase lysis buffer as previously described (19). Briefly, tissues (~50 mg) were homogenized in 500 µl of buffer containing 0.5% Triton X-100, 2 mM MgCl₂, 1 mM EGTA and 1 mM dithiothreitol supplemented with protease and phosphatase inhibitors (Roche); samples were then incubated on ice for 30 min with occasional vortex and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatant was collected and protein content determined by the BCA assay (Pierce). Fifty micrograms of total protein was loaded per lane for SDS-PAGE (10% wt/vol) analysis and then transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies [all at 1:1,000 dilution; peNOS (S1177) (Cell Signaling, 9571S); eNOS (Cell Signaling, 9572S); α-actin (Cell Signaling, 4967S); KHK (Sigma, HPA007040)] followed by appropriate horseradish peroxidase secondary antibodies (1:2,000). Blots were visualized using the HRP.

Fig. 2. Renal functional injury in WT mice compared with KHK A/C KO mice. We observed no differences in serum creatinine (A) or urinary NGAL excretion (C) between 2-yr-old WT and KHK A/C KO mice. However, urinary albumin/creatinine ratios were higher in 2-yr-old WT mice compared with KHK A/C KO mice (B).

Fig. 3. Baseline studies prior to salt loading in aged mice. Baseline weights were slightly higher in WT compared with KHK A/C KO mice (A). Similarly systolic blood pressure (BP) and pulse rate were also higher in WT mice (B–D). In contrast, in this set of animals no difference in urine albumin/creatinine excretion was observed (E). During the subsequent 3 wk of salt loading, the daily intake of salt (1%) was similar between both groups (F, P = NS).
Supersignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). Chemiluminescence was recorded with an Image Station 440CF and results were analyzed with the 1D Image Software (Kodak Digital Science, Rochester, NY).

Statistical analysis. All data are presented as means ± SE. Data graphics and statistical analysis were performed using Prism 5 (GraphPad). Data was analyzed by t-test, or Mann-Whitney U-test when normality could not be assumed. Two-way ANOVA with Bonferonni was used to compare urinary nitrite excretion pre and post salt challenge. P < 0.05 was regarded as statistically significant.

RESULTS

General characteristics of aging (2-yr-old) mice. Both KHK A/C KO and WT littermate mice showed normal behavior at 24 mo with similar levels of activity. There were no differences in body weight or amount of epididymal fat. Similarly, no differences were noted in serum lipids (cholesterol, triglycerides), liver function tests (aspartate and alanine aminotransferase), or serum glucose or insulin in blood samples obtained after 6 h of fasting (Table 1).

C57BL6 mice are known to develop some aging-associated kidney damage, with mesangial expansion, low-grade interstitial fibrosis, and albuminuria (22). We confirmed that aging WT mice showed mild mesangial cell proliferation and matrix expansion (Fig. 1). Interestingly, low-grade mesangiolitic injury was also present, in association with focal glomerular thrombi in 6 of 7 WT mice. In contrast, KHK A/C KO mice showed no histologic abnormalities in their kidneys. Quantification revealed the presence of thrombi in nearly 20% of glomeruli of WT mice compared with <1% of glomeruli in KHK A/C KO mice (Fig. 1). Mesangial matrix expansion, determined by measuring glomerular type IV collagen, was significantly higher in WT mice compared with KHK A/C KO mice, and glomeruli also tended to be larger in the WT mice compared with the KHK A/C KO mice although this was not significant (Fig. 1). KHK A/C KO mice also showed significantly less albuminuria that WT mice. However, serum creatinine (measured by HPLC) and urinary NGAL levels were not different (Fig. 2). Furthermore, no tubulointerstitial disease was noted in either group.

Effect of high-salt diet on aging mice. Aging-associated renal disease is known to be associated with decreased functional reserve and increase susceptibility to salt-sensitive hypertension. We therefore performed a second set of studies to determine if aging mice lacking fructokinase might be protected from high salt intake. In these studies 2-yr-old aging WT and KHK A/C KO mice were administered a high-salt diet (1 percent NaCl with 0.04% sucralose to stimulate drinking) for 3 wk. Baseline systolic blood pressure and pulse prior to salt loading were lower in the KHK A/C KO mice (Fig. 3). During the 3 wk of high salt intake, the mean intake of salt water was equivalent between two groups (Fig. 3). At the end of the 3 wk, the animals were euthanized and assessed for blood pressure, renal function, and injury. Renal function (as noted by HPLC-determined serum creatinine) were not different between WT and KHK A/C KO mice. However, albuminuria was markedly higher with salt loading in both WT mice and KHK A/C KO mice compared with baseline levels, with WT mice showing more than twice the level of proteinuria as KHK A/C KO, although this was not significant due to the wide range of values in the WT mice (Fig. 4). In addition, there remained a
difference in systolic BP (Fig. 4D), although both groups showed an increase in blood pressure at a similar degree over the 3-wk period (Fig. 4).

Despite no differences in measured renal function, marked differences in renal injury were present, with 5 of 5 WT mice showing focal glomerular thrombi with fibrin deposits whereas only rare thrombi were present in the KHK A/C KO mice (Fig. 5). In addition, glomeruli in WT mice showed evidence of glomerular hypertrophy and increased mesangial matrix expansion with hypercellularity, whereas this was not noted in KHK A/C KO mice (Fig. 5). Quantification of type IV collagen documented increased mesangial matrix in the WT mice compared with KHK A/C KO mice (Fig. 5). Similarly, alpha smooth muscle actin, which is known to reflect activation of mesangial cells (15), was also increased in the WT mice compared with the KHK-A/C KO mice (Fig. 5).

Endothelial nitric oxide synthase expression. Aging kidneys show evidence for endothelial dysfunction and impaired angiogenesis (16, 24). Urinary nitrates/nitrates, which are a general reflection of both endothelial and non-endothelial nitric oxide were significantly lower in WT mice compared with KHK A/C KO mice both before and after saline challenge (Fig. 6). Western blotting of KHK A/C KO mice performed after salt loading showed significantly higher levels of activated endothelial nitric oxide synthase (phosphorylated at the serine 1177 site) compared with WT mice, especially when factored for total eNOS expression (Fig. 6). These studies suggest that the KHK A/C KO mice had preserved endothelial function.

Fructose and uric acid levels. We also measured both serum and renal fructose and uric acid levels in the first set of aging mice. As shown in Fig. 7, fructokinase knockout mice had higher serum fructose levels consistent with their reduced ability to metabolize fructose (13). However, there was no difference in renal fructose or serum or renal uric acid levels.

DISCUSSION

Aging is associated with the development of kidney disease in mice, rats, and humans (17, 22). While several mediator systems are involved in aging-associated renal disease, including the renin-angiotensin system, endothelial nitric oxide, and oxidative stress (1, 7, 8), the role of fructose metabolism is not known. Dietary fructose is known to cause renal injury in rats, even with as little as 20 percent of the diet as fructose (9, 10, 26), so it would not be particularly insightful to evaluate the role of high fructose diet on aging-associated renal disease. However, stealth amounts of fructose are generated daily from glucose via the endogenous aldose reductase-sorbitol dehydro-
mice had mild mesangial expansion (noted by type IV collagen staining), mild glomerular hypertrophy, and focal thrombi observed in the majority (85%) of mice. In contrast, the fructokinase knockout mice showed less glomerular matrix expansion and almost no thrombi that were statistically significant. Indeed, glomeruli generally appeared normal in the fructokinase knockout mice.

We also performed a second study in which aging mice were challenged for 3 wk with a high-salt diet. High salt intake is known to increase glomerular filtration rate, hypertension, and proteinuria in subjects, especially those who are salt-sensitive including the elderly (2). Perhaps not surprisingly, we found that high-salt diet dramatically increased albuminuria in wild-type mice, and this was associated with an amplification of renal injury, with marked glomerular hypertrophy, mesangial matrix expansion, alpha smooth muscle actin expression in the mesangium (which marks mesangial activation), and segmental glomerular thrombi. In contrast, fructokinase knockout mice showed significantly less glomerular hypertrophy, mesangial actin and collagen expression, and glomerular thrombi. Interestingly, the fructokinase KO mice stilled showed some evidence for salt-mediated effects, as the level of albuminuria and glomerular size were higher than that observed in fructokinase knockout mice on a normal diet, consistent with better endothelial function in mice lacking fructokinase.

The primary finding from our study was that mice lacking fructokinase were relatively protected from developing aging-associated kidney damage. Aging wild-type littermates developed slightly elevated systolic blood pressure, a higher pulse, and variable albuminuria that were significantly greater than that observed in the fructokinase knockout mice. While we could not document differences in renal function, histologically there were substantial differences. First, the wild-type mice had mild mesangial expansion (noted by type IV collagen staining), mild glomerular hypertrophy, and focal thrombi observed in the majority (85%) of mice. In contrast, the fructokinase knockout mice showed less glomerular matrix expansion and almost no thrombi that were statistically significant. Indeed, glomeruli generally appeared normal in the fructokinase knockout mice.

We further investigated possible mechanisms underlying the renal protection in aging fructokinase KO mice. Both mice and rats are known to have impairment in endothelial function with age, with reduced renal levels of nitric oxide, altered eNOS expression, and with some impairment in expression of vascu-
lar endothelial growth factor-A and endothelial hyperpolarizing factor (11, 16, 24, 27, 35). Fructose is also known to mediate endothelial dysfunction, reduce endothelial nitric oxide levels, transiently reduce eNOS protein, and block acetylcholine-induced dilation of aortic rings (10, 25). It was thus of interest that the fructokinase KO mice showed higher expression of phosphorylated eNOS with higher urinary nitrate/nitrite excretion. That preservation of eNOS may account for protection is supported by a study in eNOS knockout mice which also develop glomerular injury and thromboses at age 13 mo (younger than wild-type mice) (27).

A limitation of the study is that we could not specifically show evidence for fructose metabolism in the aging mice. Specifically, we found similar levels of fructose and uric acid in the kidneys of aging WT and KHK A/C KO mice. However, it is likely that the blockade of fructokinase acted by preventing fructose metabolism, as fructose is the only common sugar metabolized through the fructokinase pathway. A second limitation of the study is that it was only performed in male animals (1), which are known to be more susceptible to kidney damage, and whether similar protection would be observed in female mice is not known.

In summary, these studies raise the possibility that some aging-associated renal changes may not represent the consequences of age-related degeneration, but rather may involve active metabolic processes that can be potentially interrupted.

Second, these studies alert one to consider that one might not simply consider dietary fructose as a potential nephrotoxin, but rather that generation of endogenous fructose may have a stealth role in driving kidney disease. Indeed, endogenous fructose has already been implicated in both diabetic nephropathy and in dehydration-mediated chronic kidney disease (20, 28). Finally, these studies emphasize a linkage between endothelial dysfunction, thrombosis, and fructose metabolism that warrant further study. It has been reported that overexpression of eNOS can prevent fructose-induced metabolic syndrome in rats (36). Thus studies to improve endothelial function might be an approach for preventing aging associated renal disease that could have a significant impact on human health and aging.

**GRANTS**

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**DISCLOSURES**

R. J. Johnson and M. A. Lanaspa have a patent application with the University of Colorado to block fructose metabolism as a means for blocking sugar craving and acute kidney injury. R. J. Johnson, M. A. Lanaspa, C. A.
Roncal-Jimenez, and L. G. Sánchez-Lozada are members of Colorado Research Partners, LLC, that is trying to develop an inhibitor of fructose metabolism. R. J. Johnson is also on the Scientific Board for Amway, and Amway also has interest in developing nutraceuticals to block fructose metabolism.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


