Aging-associated Renal Disease in Mice is Fructokinase Dependent

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Abstract

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 years that had minimal (<5%) fructose content. At the end of two years, 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 weeks, 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 week) compared with wild-type mice. This was associated with higher levels of active (phosphorylated serine 1177) endothelial nitric oxide synthase in their kidneys. These studies suggest that aging-associated renal disease might be due to activation of specific metabolic pathways that could theoretically be targeted therapeutically, and raise the hypothesis that aging-associated renal injury may represent a disease process as opposed to normal age-related degeneration.

Key words: Chronic Kidney Disease; Aging; Fructose
Introduction

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 pre-existent 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) litter mates (male, 24 to 25 month old) were used. They were maintained in temperature- and humidity-controlled specific pathogen-free conditions on a 14-hour dark/10-hour light 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.

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) at 24 months of age, and were sacrificed at 25 months with collection of kidney tissues and serum. A second set of studies were done in which 24 month old WT and KHK A/C KO mice (*n* = 5-6 per group) were challenged for 3 weeks with a high salt load (1% NaCl in water with 0.04% sucralose). 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 to 20 months of age, and again both before and after high salt intake. Mice were sacrificed at 25 months 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 Albuwell M (Exocell, Philadelphia, PA) and urine NGAL was measured using the Mouse
Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN). Serum creatinine concentration was analyzed by the high-performance liquid chromatography–tandem mass spectrometry method (33). Urinary nitrites and nitrates were measured using a Colorimetric Assay Kit from Cayman Chemical Company (Ann Arbor, Michigan). Serum fructose was measured using the EnzyChrom Fructose Assay Kit (Bioassay Systems, Hayward, CA) and serum uric acid was measured using QuantiChrom 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% (v/v) Triton X-100. Homogenates were centrifuged at 13,000 rpm for 10 minutes (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 μm 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 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 wild type 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 r.p.m. 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% w/v) analysis and then transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies (all at a 1:1,000 dilution; peNOS (S1177), (Cell Signaling, 9571S); eNOS, (Cell Signaling, 9572S); β-Actin, (Cell Signaling, 4967S); KHK, (Sigma, HPA007040) followed by appropriate horseradish peroxidase secondary antibodies (1:2,000). Blots were visualized using the HRP Supersignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). Chemiluminescence was recorded with an Image Station 440CF and results were analysed with the 1D Image Software (Kodak Digital Science, Rochester, NY).

**Statistical analysis.** All data are presented as the mean ± s.e.m. 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. 2-way ANOVA with Bonferroni 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 (Two year old) Mice. Both KHK A/C KO and WT littermate mice showed normal behavior at 24 months 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 hours 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 (Figure 1). Interestingly, low grade mesangiolytic 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 percent of glomeruli of WT mice compared to <1% of glomeruli in KHK A/C KO mice (Figure 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 (Figure 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 (Figure 2). Furthermore, no tubulolinterstitial 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 year 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 weeks. Baseline systolic blood pressure and pulse prior to salt loading were lower in the KHK A/C KO mice (Figure 3). During the three weeks of high salt intake, the mean intake of salt water was equivalent between two groups (Figure 3). At the end of the 3 weeks, the animals were sacrificed 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 (Figure 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 three week period (Figure 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 (Figure 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
(Figure 5). Quantification of type IV collagen documented increased mesangial matrix in the
WT mice compared with KHK A/C KO mice (Figure 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 (Figure 5).

Endothelial Nitric Oxide Synthase Expression. Aging kidneys show evidence for
endothelial dysfunction and impaired angiogenesis (16, 24). Urinary nitrites/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 (Figure 6).
Western blotting of KHK A/C KO mice performed after salt loading showed significant 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 (Figure 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 Figure 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 dehydrogenase pathway (13), and this pathway can be enhanced if aldose reductase is activated by glucose, salt, or dehydration (20, 21, 28). Hence, we tested the hypothesis that blocking fructose metabolism might reduce aging associated kidney disease even when the diet is very low in fructose.

The first observation was that fructokinase knockout mice appeared healthy and there were no apparent toxicity from lacking fructokinase observed. The observation that the fructokinase knockout mice are healthy are consistent with the clinical literature, in which humans lacking fructokinase (a condition known as essential fructosuria) are clinically healthy throughout their lives (32). Importantly, we observed no benefit in mice lacking fructokinase as evaluated by a large number of metabolic tests (liver function, lipid profile and glucose-insulin level). However, these mice were on a standard mouse diet and not one high in sugar and fat where a lack of fructokinase has been shown to have a benefit on fatty liver and metabolic syndrome (14). We did observe a slightly lower body weight in the second set of aging fructokinase knockout animals compared to wild type littermates, but since this difference was not observed in the first set of mice, it remains unclear if a lack of fructokinase might be associated with slightly lower body mass with aging.

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 was 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 three weeks
with a high salt diet. High salt intake is known to increase glomerular filtration rate,
hypertrophy, 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 still 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 the concept that the high salt diet might still be inducing mild
glomerular hyperfiltration and hypertension in these 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 vascular 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 knock-out mice who also develop glomerular injury and thromboses at age 13 months
(approximately a year 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 was 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.
<table>
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<th>WT</th>
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<th>p value</th>
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<td><strong>Liver weight (g)</strong></td>
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<td><strong>Epididymal fat weight (g)</strong></td>
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<td><strong>AST (IU/l)</strong></td>
<td>28.6 ± 4.6</td>
<td>25.0 ± 1.7</td>
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<tr>
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<td>117.0 ± 16.0</td>
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<td><strong>Triglyceride (mg/dl)</strong></td>
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<td><strong>Blood urea nitrogen (mg/dl)</strong></td>
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<td>NS</td>
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<td><strong>Serum glucose (mg/dl)</strong></td>
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<td><strong>Insulin (pg/ml)</strong></td>
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Acknowledgments

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Financial Disclosure

RJJ and MAL have a patent application with the University of Colorado to block fructose metabolism as a means for blocking sugar craving and acute kidney injury. RJJ, MAL, CR and LGL are members of Colorado Research Partners, LLC, that is trying to develop an inhibitor of fructose metabolism. RJJ is also on the Scientific Board for Amway and Amway also has interest in developing nutraceuticals to block fructose metabolism.


29. **Sanchez-Lozada LG, Tapia E, Bautista-Garcia P, Soto V, Avila-Casado C, Vega-Campos IP, Nakagawa T, Zhao L, Franco M, and Johnson RJ.** Effects of febuxostat on metabolic and renal alterations...


Figure Legends

**Figure 1. Focal glomerular thrombi in Aging WT Mice but not 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 (Fig 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 to KHK-A/C KO mice and was significantly different when quantified (F). Sample size: (n=7 in WT and n = 6 in KHK A/C KO mice) (A-C, E; 400x). N.D., not detected.

**Figure 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 year old WT and KHK A/C KO mice. However, urinary albumin/creatinine ratios were higher in 2 year old WT mice compared with KHK A/C KO mice (B).

**Figure 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 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. During the subsequent three weeks of salt loading, the daily intake of salt (1%) water were similar between both groups (F, p=NS).

**Figure 4. Effect of High Salt Loading on Renal Function.** At the end of three weeks of high salt loading, no differences were observed in either serum creatinine or urine NGAL, but urinary albumin/creatinine ratio tended to be higher in WT mice compared with KHK A/C KO mice (Fig A-C). However, after three weeks of salt treatment there remained significant differences in systolic BP (Fig D) and pulse rate (Fig E).

**Figure 5. Renal Histology following High Salt loading.** WT mice showed significant renal injury, with segmental thrombosis present in 5 of 5 WT mice (Fig A, B, PAS stain), involving approximately 10 percent of glomeruli (Fig D, PAS stain), whereas thrombi were minimally present in the KHK A/C KO mice (Fig C, PAS). Wild-type mice showed greater mesangial hypercentrularity (Fig B), mesangial matrix expansion (as noted by type IV collagen immunostaining, Fig D), and mesangial alpha smooth muscle actin expression (Figure I) than KHK A/C KO mice (Fig F and J, respectively). Quantitation of the histologic changes confirmed increased glomerular tuft area (Fig G), mesangial type IV collagen deposition (Fig H), and expression of alpha smooth muscle actin in the mesangium in wild-type mice on salt compared with KHK-A/C knockout mice. Magnification 400x.

**Figure 6. Effect of High Salt Diet on Endothelial Function in Aging Mice.** Urinary nitrites/nitrates were significantly higher in KHK A/C KO mice compared with WT Mice at 18-20 months of age (Figure A, p<0.05) and at 24 months following salt loading (Figure B). Renal tissue obtained after salt loading also showed a significantly higher level of p-eNOS in renal tissue by Western blotting although total eNOS protein was lower in KHK A/C KO mice compared with WT mice (Figure C). Quantification of p-eNOS/total eNOS by densitometry showed a significantly higher ratio in KHK A/C KO mice compared with WT mice, consistent with better endothelial function in mice lacking fructokinase.
Figure 7 Serum and Renal Fructose and Uric acid Levels. Serum fructose (Figure A), renal fructose (Figure B), serum uric acid (Figure C) and renal uric acid (Figure D) were measured in wild-type and fructokinase knockout mice at 2 years. Serum fructose levels were higher in the fructokinase knockout mice (KHK KO). Otherwise no differences were observed between these two groups of mice. *, P < 0.05. N.S., not statistically significant.
Figure 1

A

B

C

D

E

F

% of glomeruli with hyaline thrombosis

WT

KHK-A/C

Globular size

WT

KHK-A/C

Globular CV (% of

WT

KHK-A/C
A. Serum Creatinine

B. Urine albumin

C. Urine NGAL
A  Serum Creatinine

B  Urine Albumin

C  Urine NGAL

D  Systolic BP

E  Pulse Rate
A. Urine NOx (18-20 months age)

B. Urine NOx (24 months age)
C. Renal eNOS and p-eNOS

D. Renal p-eNOS / eNOS ratio

WT KHK-A/C

p=0.029
A. Serum Fructose

B. Renal Fructose

C. Serum Uric acid

D. Renal Uric acid

Fructose (μmol/l)

Uric acid (mg/dl)