Ketohexokinase-Dependent Metabolism of Fructose Induces Proinflammatory Mediators in Proximal Tubular Cells

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ABSTRACT
Increased consumption of fructose may play an important role in the epidemic of metabolic syndrome and may presage the development of diabetes, cardiovascular disease, and chronic kidney disease. Once in the cell, fructose is phosphorylated by ketohexokinase (KHK), leading to consumption of ATP, formation of AMP, and generation of uric acid through xanthine oxidoreductase (XOR). This study aimed to examine the direct effects of fructose in human kidney proximal tubular cells (HK-2) and whether they are mediated by the fructose metabolism via KHK. At a similar concentration to that observed in peripheral blood after a meal, fructose induced production of monocyte chemotactic protein 1 (MCP-1) and reactive oxygen species in HK-2 cells. Knockdown of KHK by stable transfection with small hairpin RNA demonstrated that these processes were KHK dependent. Several antioxidants, including specific inhibitors of NADPH oxidase and XOR, prevented MCP-1 secretion. We detected XOR mRNA in HK-2 cells and confirmed its activity by identifying uric acid by mass spectrometry. Fructose increased intracellular uric acid, and uric acid induced production of MCP-1 as well. In summary, postprandial concentrations of fructose stimulate redox- and urate-dependent inflammatory mediators in proximal tubular cells.


Metabolic syndrome, characterized by visceral obesity, insulin resistance, elevated BP, hypertriglyceridemia, and microalbuminuria,1–2 is strongly associated with the development of type 2 diabetes, cardiovascular disease, and all-cause mortality.3–6 The prevalence of metabolic syndrome has been increasing worldwide during the past decades.7,8 Several epidemiologic9–11 and experimental studies12–14 reported that increased consumption of fructose, which is ubiquitous in modern Western diets, may play an important role in the development of the current epidemic of metabolic syndrome in addition to lack of exercise, overnutrition, and genetic predisposition. Fructose is a monosaccharide normally present in fruit and vegetables and in table sugar (sucrose); however, the majority of dietary fructose intake in the United States comes from high-fructose corn syrup, a widely used sweetener. Clinical studies showed that fructose intake can induce hypertriglyceridemia, weight gain, increased BP, and hyperuricemia.15–17

In humans, fructose is absorbed unchanged in the small intestine following transport to the
liver, which metabolizes ≥50% of the total absorbed fructose with the remainder taken up primarily by the kidney and adipose tissue. Fructose enters cells predominantly through the transporters GLUT5 and/or GLUT2. Both transporters are expressed in the kidney, mostly in the proximal tubule. In the liver, as well as in the kidney, fructose is phosphorylated by fructokinase (ketohexokinase [KHK]) to yield fructose-1-phosphate followed by cleavage by aldolase B to dihydroxyacetone phosphate and glyceraldehyde. Fructose can also be phosphorylated by hexokinase, but the Km for fructose is much higher than for glucose, which minimizes fructose phosphorylation through this pathway.

Unlike hexokinase, the KHK pathway of fructose metabolism bypasses tightly regulated glycolytic checkpoints, especially phosphofructokinase. Because KHK has no negative feedback, all fructose entering the cell is rapidly phosphorylated, which can result in ATP depletion, which has been well documented in vitro and in vivo in animal models and humans. ATP depletion activates enzymes of purine metabolism, which degrade adenine nucleotides to uric acid via xanthine oxidoreductase (XOR) with the development of hyperuricemia. KHK is expressed predominantly in the liver, small intestine, and kidney. The highest KHK activity along the nephron is present in the proximal tubule.

Our group showed recently that fructose consumption can accelerate the progression of chronic kidney disease (CKD) in the rodent remnant kidney model. A fructose-rich diet enhanced glomerulosclerosis, tubular atrophy, tubulointerstitial inflammation, interstitial collagen deposition, and peritubular monocyte-macrophage infiltration. Control rats fed equal amounts of a starch (glucose)-based diet showed significantly less progression. Although the effects of fructose may be mediated by some components of the metabolic syndrome such as dyslipidemia and hyperinsulinemia, tubular epithelial cells express fructose transporters and KHK, which may target these cells for direct effects of fructose. We therefore performed an in vitro study using human epithelial tubular cells (HK-2) to examine the effects of fructose in these cells and determine potential mechanisms by which fructose may induce tubular damage and whether they involve KHK.

RESULTS

KHK-Mediated Monocyte Chemotactic Protein 1 Production Induced by Fructose

Our group reported previously that a high-fructose diet increases the renal expression of monocyte chemotactic protein 1 (MCP-1) in the remnant kidney model of rats and confirmed this effect in vitro in HK-2 cells. MCP-1 is a potent chemoattractant of monocytes/macrophages and plays a key role in the proinflammatory response, and its action is predominantly confined to the renal tubule, which is also a major site of production. MCP-1 has been shown to play a crucial role in the inflammatory-mediated renal damage. To explore this effect further in renal epithelial cells and to determine whether it occurs with physiologic concentrations of fructose, we stimulated HK-2 cells with various concentrations of fructose for 72 h and found that 1 mM fructose (equal to the physiologic postprandial concentration in the peripheral blood) induced more than a two-fold increase in MCP-1 production (Figure 1A). Further increasing the fructose concentration to 5 and 10 mM plateaued with an additional 30% increment, statistically not different from the MCP-1 level at 1 mM. Interestingly, glucose did not stimulate MCP-1 (Figure 1B) and even attenuated the effect of fructose at the concentration 25 mM (Figure 1C). D-Mannitol did not produce a significant effect on MCP-1 production, excluding the osmotic mechanism. Similarity of the effects of fructose and tagatose on MCP-1 production. In all experiments, cells were treated with fructose, glucose, tagatose, and mannitol for 72 h. MCP-1 content in the medium was assayed by ELISA and is expressed as pg/mg of cellular protein content. Data are means ± SEM *P < 0.05 versus untreated cells or corresponding control, nonparametric U test and, in A, one-way ANOVA; #P = 0.06 versus 5 mM fructose + 5 mM glucose; n = 3, each experiment performed in triplicate.
Stimulation of mannitol also did not induce an elevation of MCP-1 level excluding an osmotic mechanism (Figure 1D). When we compared the effects of fructose and tagatose, which is also metabolized by KHK, we found that both sugars, at 5 mM, induced a significant increase in MCP-1 production (Figure 1E), suggesting that MCP-1 release depends largely on the first step of fructose metabolism because tagatose, after phosphorylation by KHK, is metabolized much slower than fructose-1-phosphate.

Because KHK expression in the kidney and, specifically, in the proximal tubule is very high, we next sought whether metabolism by KHK is required for the proinflammatory effect of fructose to induce MCP-1 production. First, we detected expression of KHK in HK-2 cells and found that mRNA for KHK in these cells is expressed at a level comparable to the liver (Figure 2A). Next, we transfected HK-2 with four different small hairpin RNA (shRNA) constructs for human KHK containing a gene coding resistance to puromycin and used this latter property to select stably transfected clones in puromycin-containing medium. Among the resistant clones, two showed an almost absent expression of the mRNA for KHK (Figure 2B). Western blot analysis confirmed that >75% of the KHK expression was suppressed at the protein level (Figure 2C). Stable transfection of HK-2 cells with nonrelevant shRNA did not affect KHK expression showing the absence of nonspecific effects on transfection and expression of small interfering RNA duplexes in the cells (Figure 2, B and C). We also confirmed a reduction in the functional activity of KHK in the silenced clones as reflected by the absence of ATP depletion in response to fructose (Figure 2D) because ATP depletion by acute fructose loading is strictly KHK dependent. That an increase in ATP was seen after fructose loading in these clones (Figure 2D) is because, in the absence of KHK, fructose enters the glycolytic cascade via hexokinase, resulting in an increase in net ATP production. Next, we incubated nontransfected HK-2 cells, KHK-silenced HK-2 clone, and cells stably expressing a nonrelevant shRNA with fructose. To obtain a robust response, we used a concentration of fructose 5 mM. In nontransfected cells and pRS-GFP shRNA clone, fructose induced MCP-1 production, whereas stable silencing of KHK abolished the effect of fructose even at 5 mM, indicating a specific requirement for KHK (Figure 2E).

**Involvement of Reactive Oxygen Species in the KHK-Mediated MCP-1 Production Induced by Fructose**

MCP-1 production can be blocked by antioxidants in proximal tubular cells and other cells. We therefore examined fructose-induced MCP-1 production in the presence of antioxidants N-acetylcysteine, cell-permeable superoxide scavenger MnTMPyP, and inhibitors of NADPH oxidase (apocynin) and xanthine oxidoreductase (allopurinol). All tested antioxidants completely abrogated the effect of fructose, suggesting that fructose-induced MCP-1 stimulation in HK-2 cells is redox dependent and mediated by superoxide production (Figure 3A). Apocynin suppressed both basal and stimulated reactive oxygen species (ROS) production (Figure 3A).

These results prompted us to examine the effect of fructose on ROS production in HK-2 using live cell imaging with the ROS-specific fluorescence probe 5-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate-acetyl ester (CM-H₂DCFDA). As shown in Figure 3B, fructose induced an increase in the probe fluorescence, which was ROS specific because the antioxidant tempol prevented this increase (data not shown). At concentrations of 0.5 to 5.0 mM, fructose-
induced ROS production was dose dependent. An increase in oxidants was observed with concentrations as low as 1 mM (physiologic) and reached a plateau between 5 and 10 mM (Figure 3C). In the presence of D-mannitol, ROS production was not affected (Figure 3C), excluding an osmotic mechanism in the effect of fructose. Time-course experiments showed that the level of ROS increased as early as 15 min and remained at the same level for approximately 4 h with return to basal levels after 16 h, when, most likely, all of the added fructose was metabolized (Figure 3D).

To test whether fructose-induced ROS production was KHK dependent, we incubated nontransfected cells, KHK-silenced HK-2 cells, and cells transfected with nonrelevant shRNA in the presence of a high level of fructose (5 mM) for 2 h. Fructose produced a significant increase in ROS production in normal HK-2 and in pRS-GFP(−) clone cells, whereas no effect was observed in cells with silenced KHK expression (Figure 3E). Finally, to test whether fructose-induced ROS production is dependent on xanthine oxidoreductase (XOR) and urate production as suggested by results in Figure 3A, we tested the effect of fructose in the presence of oxypurinol (0.1 mM), a XOR inhibitor. Oxypurinol did not modify the level of ROS in untreated cells but partially reduced ROS accumulation in cells treated with 5 mM fructose, suggesting involvement of XOR in this pathway (Figure 3F).

**XOR Expression and Uric Acid Production in Response to Fructose in Tubular Epithelial Cells**

Fructose metabolism via KHK results in the stimulation of AMP-deaminase followed by a XOR-mediated increase in uric acid formation. This process has been usually studied in the liver; however, KHK is highly expressed in HK-2 cells and fructose-induced MCP-1 and ROS production might be suppressed by XOR inhibition. We therefore examined the expression of XOR in HK-2 cells and measured uric acid, the XOR reaction product. Reverse transcriptase–PCR (RT-PCR) analysis confirmed modest expression of XOR (Figure 4A). To identify uric acid, we used mass spectrometry. Uric acid was identified in the extract of HK-2 cells as fragments at m/z 124 and 96 by mass spectrometry (MS)/MS analysis as described in...
the Concise Methods section (Figure 4B), confirming that XOR is active. Next we tested whether fructose increases uric acid production in these conditions and whether uric acid is responsible for the increase in MCP-1 expression. Uric acid was measured in fructose-treated cells by both enzymatic assay with uricase and by MS. Figure 4, C and D, shows that fructose treatment induced an increase in intracellular level of uric acid in HK-2 cells that was completely prevented by allopurinol (Figure 4D). To determine whether fructose can induce local accumulation of uric acid in the kidney, we measured its content in the crude kidney homogenates from rats pair fed with the high-fructose diet or standard rat diet for 16 wk; however, in this case, we observed only a tendency to an increase in the uric acid content from 0.122 ± 0.025 to 0.145 ± 0.031 nmol/mg protein ($P < 0.1; n = 6$).

### DISCUSSION

This study demonstrates that fructose exerts direct and potentially detrimental effects on cultured human proximal tubular epithelial cells. These cells, along with hepatocytes, adipocytes, and possibly endothelial cells, metabolize fructose via KHK, bypassing common checkpoints for carbohydrate metabolism.$^{18-21}$ Fructose, at physiologic concentrations, induced proinflammatory mechanisms (MCP-1 synthesis) and oxidative stress in the proximal tubular cells. We further demonstrated that KHK, the first enzyme of the main pathway of fructose metabolism, is required for this redox-dependent proinflammatory effect.

The level of fructose in the peripheral blood$^{38}$ and in the renal tubule$^{27}$ in fasting conditions is usually very low (10 to 60 μM). Postprandial levels of fructose in the portal blood rise to 2.2 mM, and because the liver takes up 50 to 75% of absorbed fructose, postprandial levels in the peripheral blood are approximately 1 mM$^{21}$ and depend on the amount and speed by which fructose and sugar are ingested.$^{39}$ Upon fructose loading, its concentrations along the nephron may reach levels equaling or exceeding the concentration in peripheral blood.$^{27}$ Thus, it is likely that the effects we observed in HK-2 cells could be physiologically relevant. It is noteworthy that levels as low as 1 mM were able to stimulate MCP-1, ROS, and uric acid production in our cellular system. Although we used higher concentrations (5 to 10 mM) in some experiments to get robust responses to help discern pathways, the response of HK-2 cells to supraphysiologic concentrations of fructose (5 to 10 mM) versus physiologic 1 mM was similar in character and magnitude.

We did observe that marked increases in glucose concentration (to 25 mM) could dampen the effect of fructose to induce MCP-1 production. Nevertheless, postprandial elevations in glu-
The metabolism pathways in renal epithelial cells by inducing MCP-1 production may, indeed, play a key role in fructose-induced renal damage. Previous demonstrated ability of fructose to accelerate the progression of CKD in the remnant kidney model, stimulated MCP-1 production. Because MCP-1 production was effectively inhibited by allopurinol and antioxidants, the proinflammatory activity of oxidative stress and/or uric acid play an important role downstream from HK. Interestingly, experiments using the superoxide scavenger MnTMPyP revealed that fructose-induced ROS are mediated by generation of superoxide. The experiments with other antioxidants showed that HKH-dependent ROS production might involve two major sources: NADPH oxidase and XOR. The role of ROS and uric acid in mediating MCP-1 production is well established in several cell types. We also previously reported that both fructose and uric acid increase MCP-1 in the kidneys of rats. Our results suggesting an involvement of NADPH oxidase are also in agreement with an in vivo study demonstrating that ROS production by vascular and blood cells in fructose-fed rats increased simultaneously with expression of the NADPH oxidase subunit p22phox.

It has been known since 1968 that phosphorylation of fructose by HKH may rapidly deplete ATP in the liver and kidney, with the most dramatic drop in the renal cortex. These processes are associated with an increase in the degradation of AMP to hypoxanthine, followed by conversion to uric acid by XOR. Although intracellular urate production, which was confirmed in our experiments by MS, is not unexpected, it was not, to the best of our knowledge, previously reported. Moreover, fructose-induced increase in uric acid production confirms activity of XOR. Consistent with these findings, we observed that HKH-mediated MCP-1 and ROS production after fructose stimulation was dependent on XOR. Because oxypurinol blocks uric acid synthesis and ROS formation by xanthine oxidase, the ROS production in response to fructose might be partially mediated either by XOR-dependent ROS or by uric acid. Despite being an antioxidant in the serum, uric acid can engage redox-dependent signaling in adipocytes with the production of oxidants by NADPH oxidase. Conversely, fructose feeding induced only a mild and NS increase in urate content in the kidney homogenates, suggesting that uric acid could be increased only in proximal tubular cells, where it is produced via a HKH-dependent mechanism, but not in other cell types, which do not express HKH.

In conclusion, our studies suggest that the intake of foods high in fructose content can induce proinflammatory changes in the proximal tubule through a direct HKH-dependent mechanism, with stimulation of MCP-1 and the induction of oxidative stress. These studies could provide a causal link between obesity and metabolic syndrome and the development of renal disease.

CONCISE METHODS

Cell Culture

HK-2, an immortalized cell line from normal adult human kidney that maintains phenotype of proximal tubular cells and represents the best in vitro model of human proximal tubule cells, were obtained.
from American Type Culture Collection (Rockville, MD). Cells were grown to confluence in DMEM/F-12 (30:50) with l-glutamine and HEPES buffer (Mediatech, Herndon, VA) supplemented with 10% FBS; 100 U/ml penicillin; 100 g/ml streptomycin; and 2 mM l-glutamine and insulin (10 mg/L), transferrin (5.5 mg/L), and sodium selenite (6.7 μg/L). Cells were cultured at 37°C in 95% air and 5% carbon dioxide (CO₂) to 70 to 75% of confluence.

Animal Protocol
All animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee. Twenty-four 150-g male Sprague-Dawley rats (Charles Rivers Laboratories, Wilmington, MA) were divided into four groups of six rats each and received a diet of either 60% fructose (F60) or standard rat food (control), which contains complex carbohydrates, instead of purified sugars (Harlan-Teklad, Madison, WI). The animals were pair fed to ensure equal food intake. Rats were maintained in temperature- and humidity-controlled specific pathogen–free conditions on a 12:12-h light-dark cycle. After 16 wk, the rats were anesthetized with isoflurane and killed. Samples of the kidney tissues were stored at −80°C until analysis.

Stable Transfection of HK-2 Cells and KHK Silencing with shRNA
For the silencing of KHK in HK-2 cells, we used HuSH shRNA KHK-specific constructs in the pRS backbone under control of U6 promoter (Origene, Rockville, MD). To achieve long-term silencing, we used stable transfection. Cells were transfected with plasmids for four different KHK shRNA and shRNA for nonrelevant gene (GFP; pRS-GFP) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the protocol of the manufacturer followed by the selection of the transfectants resistant to puromycin in the selective medium containing 1 μg/ml puromycin for 8 to 10 wk. The best HK-silenced clones were selected and validated on the basis of the lowest KHK mRNA expression and the absence of the functional KHK activity (see Figure 2). KHK-silenced HK-2 clone selected for experiment (pRS-KHK) and the clone stably expressing nonrelevant shRNA were maintained in the medium with 0.5 μg/ml puromycin, which was removed before experiments.

RNA Isolation and RT-PCR
Total RNA extraction was performed with TRIZOL (Invitrogen). The RNA purity and concentration were evaluated by spectrophotometric measurements. HK2 was detected in normal HK-2 and in clones stably expressing nonrelevant shRNA were maintained in the medium with 0.5 μg/ml puromycin which was removed before experiments.

Measurement of Intracellular ATP Content
Cellular ATP was measured by using an ATP bioluminescence assay (Roche assay kit CLS II; Roche, Mannheim, Germany), following the manufacturer’s instructions. After the experimental treatments, cells were washed with ice-cold PBS and detached from the culture plates with 0.05% trypsin/0.02% EDTA, and then we added to the cell suspension boiling 200 mM Tris/8 mM EDTA (pH 7.75) solution and incubated for 2 min at 98°C. Cell debris was precipitated at 10,000 × g for 2 min. Then ATP was determined in the supernatant by adding luciferin/luciferase by automated injection in a luminometer (Opto-comp II; MGM Instruments, Hamden, CT) with integration of the light signal for 2 s. The ATP concentration was calculated by interpolation from a standard curve. Cellular protein content was determined by using the BCA protein assay. The ATP content was normalized to protein concentration and expressed as nanomoles per milligram of protein.

Uric Acid Assay
To assess intracellular uric acid content, we rinsed cells with ice-cold PBS and harvested them in buffer containing 25 mM HEPES (pH 7.1), 100 mM KCl, 1 mM dithiothreitol, and 0.1 mM EDTA. The cell extracts were homogenized by 100 strokes of the pestle and thawed in liquid nitrogen three to four times. The intracellular level of uric acid was measured using Amplex Red UA/Uricase Assay Kit from Invitrogen-Molecular Probes (Eugene, OR). The fluorescence was measured using excitation in the range of 530 to 560 nm and emission detection at approximately 590 nm. Uric acid concentration in the samples was determined from the standard curve. The uric acid levels were normalized to protein concentration and expressed as nanomoles per milligram of protein.

Detection of Uric Acid by MS
All samples were stored at −80°C before analysis. Before the analysis, they were thawed in a cold water bath and filtrated through a 0.2-μm Micro Centrifuge Nylon filter (Millipore, Billerica, MA) by centrifugation for 10 min at 14,000 rpm. The filtrates were sealed in amber glass vials using Teflon-lined caps for liquid chromatography (LC)-MS analysis. The calibrators were 0.050, 0.125, 0.250, 0.500, 1.000, and 5.000 μM uric acid in aqueous solution.

The LC-MS analyses were carried out with a ThermoFinnigan Surveyor liquid chromatography system (ThermoFinnigan, San Jose, CA) and a TSQ Quantum triple quadruple mass spectrometer (ThermoFinnigan) equipped with ESI interface operated in negative-ion mode. LC analyses were performed in a gradient elution mode using Phenomenex Luna 5 μm C18 (2) 100A (150 mm × 4.6 mm) column (Phenomenex, Torrance, CA) coupled with a Phe-
nomenex Luna C18,(2.5)-µm particle size guard column. The mobile phase used included 5 mM ammonium acetate/0.1% acetic acid (A) and methanol (B). The mobile phase flow was 600 µl/min, and the sample injection volume was 20 µl. The gradient began at 95% A. The composition was linearly ramped to 25% B over the next 4.5 min, and it was maintained constant for 1.5 min. Then, it was reversed to the original composition of 95% A over 0.5 min, after which time it was kept constant for 0.5 min to reequilibrate the column. In the TSQ Quantum, nitrogen was used for both the sheath (60 psi) and auxiliary (20 units) gases. The heated capillary temperature was maintained at 350°C. The collision pressure was 1.5 U, and collision energy was 25 V. The single-reaction monitoring method was applied for the analysis. The ions selected for single-reaction monitoring were m/z 124.1 and 96.1. The operation of the LC-MS and data analysis were performed using ThermoFinnigan Xcalibur 1.4 software. The calibration was linear with the R² value of 0.998.

ROS Detection
We assessed intracellular ROS with the fluorescence probe CM-
H₂DCFDA (Molecular Probes) as described previously in detail.⁴⁹ Briefly, at the end of treatments, cells were washed with HBSS followed by incubation in the presence of CM-H₂DCFDA (5 µM) for 30 min in HBSS. Then cells were transferred to HBSS without probe, and green fluorescence was measured using an inverted microscope Axiovert 200 (Carl Zeiss Microimaging, Thornwood, NY) equipped with CCD camera and image acquisition/analysis software AxioVision 4.5 (Carl Zeiss Microimaging, Thornwood, NY).

ELISA for MCP-1
At the end of the treatments, media supernatants were collected, quantified, and centrifuged at 1000 × g for 10 min to remove dead and nonadherent cells, and the remnant cell monolayer was lysed as described for protein determination. MCP-1 content in the supernatant was assayed by human MCP-1/CCL2 immunoassay Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. Experiments were performed in triplicate and confirmed on three independent occasions. The MCP-1 content was normalized to protein content and was indicated as picograms per milligram of protein.

Statistical Analysis
At least three independent experiments were performed in triplicate each. Data were analyzed by one-way ANOVA followed by Fisher least significant test, unpaired t test, or Mann-Whitney U test, with P < 0.05 considered significant. Comparison between two values was performed by t test or U test. ANOVA was used to test differences among several means.

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DISCLOSURES
R.J.J. and Y.S. are listed as inventors on patent applications by the University of Florida related to the role of fructose in hypertension and metabolic syndrome. R.J.J. has also written a book on fructose for the lay public (Rodale Press, 2008).

REFERENCES


