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Miguel A. Lanaspa, ... , Richard J. Johnson, Dean R. Tolan

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Ketohexokinase C Blockade Ameliorates Fructose-Induced Metabolic Dysfunction in Fructose-Sensitive Mice

Miguel A Lanaspa^{1#}, Ana Andres-Hernando^{1#}, David J. Orlicky¹, Christina Cicerchi¹, Cholsoon Jang², Nanxing Li¹, Tamara Milagres¹, Masanari Kuwabara¹, Michael F. Wempe³, Joshua D. Rabinowitz², Richard J Johnson,¹ and Dean R Tolan^{4,*}

¹Division of Renal Diseases and Hypertension, University of Colorado, Aurora, CO, USA,

²Department of Chemistry and Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ, USA and ³Department of Pharmacology, University of Colorado, Aurora, CO, USA. ⁴Department of Biology, Boston University, Boston; MA, USA

*Correspondence: Dean R Tolan, Department of Biology, Boston University, 5 Cummington Mall, Boston, USA. E-mail: tolan@bu.edu Phone: 617-353-5310

#equally contributed

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Abstract

Increasing evidence suggests a role for excessive intake of fructose in the Western diet as a contributor to the current epidemics of metabolic syndrome and obesity. Hereditary fructose intolerance (HFI) is a difficult and potentially lethal orphan disease associated with impaired fructose metabolism. In HFI, the deficiency of a particular aldolase, aldolase B, results in the accumulation of intracellular phosphorylated fructose thus leading to phosphate sequestration and depletion, increased ATP turnover and a plethora of conditions leading to clinical manifestations including fatty liver, hyperuricemia, Fanconi syndrome and severe hypoglycemia. Unfortunately, to date, there is no treatment for HFI and avoiding sugar and fructose in our society has become quite challenging. In this report, through use of genetically modified mice and pharmacological inhibitors, we demonstrate that the absence or inhibition of ketohexokinase (Khk), an enzyme upstream of aldolase B, is sufficient to prevent hypoglycemia and liver and intestinal injury associated with HFI using aldolase B knockout mice. We thus provide evidence for the first time of a potential therapeutic approach for this condition. Mechanistically, our studies suggest that it is the inhibition of the Khk C isoform, not the A isoform, that protects animals from HFI.

Introduction

Fructose is a major monosaccharide present in sugars whose dietary intake has increased over 40-fold since 1700 (1, 2) especially from the 1970s with the introduction of high fructose corn syrup (HFCS). Added sugars, especially as HFCS, are now found in a wide variety of products including infant formulas and foods aimed for children (3). Reducing sugar intake is now recommended by multiple agencies including the American Heart Association (AHA) and World Health Organization (WHO),(4, 5) since it increases the risk for dental caries and metabolic syndrome(4). However, it is very difficult to avoid exposure to HFCS and sucrose in today's culture since it is commonly added to a great variety of foods.

One patient group that suffers dire consequences from the widespread use of added sugars are individuals with Hereditary Fructose Intolerance (HFI), an autosomal recessive disease with an incidence near 1:30,000, although the frequency may be higher due the difficulty of its diagnosis (6, 7). The disease arises from a deficiency in aldolase-B activity in the liver, kidney, and small intestine. Subjects with HFI develop severe reactions following fructose ingestion, with abdominal pain, vomiting, diarrhea, symptomatic hypoglycemia, hyperuricemia, and other pathologies, including death. One of the greatest risk periods is for the newborn infant or the baby being weaned from breast milk since the autosomal recessive nature of the disease may result in parental ignorance of the disorder(8). In this setting, acute mortality from fructose exposure may result after hypoglycemia, seizures, lactic acidosis, and coma.

Fructose metabolism in liver, kidney, and intestine requires the coordinated action of two enzymes, ketohexokinase (KHK), which phosphorylates fructose to fructose 1-phosphate (Fru 1-P) and aldolase B, which splits Fru 1-P into dihydroxyacetone phosphate and glyceraldehyde. In HFI, mutations in the aldolase B gene (*aldob*) lead to deficiency in aldolase-B activity and the accumulation of Fru 1-P. This results in a marked phosphate and ATP depletion, and subsequent uric acid generation, following fructose ingestion, which is much greater than that observed in normal individuals (9). The depletion of phosphate, sequestered in Fru 1-P, leads to glycogen accumulation (10) due to lack of phosphate, as well as competitive inhibition of glycogen phosphorylase b and phosphoglucomutase by Fru 1-P(11). The sum of these alterations is thought to account for the severe acute hypoglycemia and steatohepatitis that develop in HFI subjects (12-14). Furthermore, these findings have led to the hypothesis that the disease manifestations of HFI might be prevented by blocking the accumulation of Fru 1-P by blocking upstream

Khk activity (15). In this regard, subjects with essential fructosuria who have a Khk deficiency, show no symptoms from ingesting fructose, and have a normal lifespan (16-18).

Recently an aldolase B knockout (AldoB-KO) mouse was shown to have symptoms similar to HFI (19). In this report, the hypothesis that blocking Khk activity will prevent HFI in mice is tested employing Khk-knockout mice. If correct, this hypothesis would open the door for novel therapeutic agents for this disease, for which the only treatment to date (avoidance of fructose) has become almost impossible in our society.

Results

Lack of aldolase B is associated with ATP depletion and over-activation of hepatic ketohexokinase induced by fructose.

The first two enzymes in fructose metabolism are Khk and aldolase B. Importantly, unlike other sugar kinases, Khk favors the metabolism of fructose over any other sugars and this is associated with reductions in ATP upon fructose exposure in both human cultured cells and in vivo (20, 21). As a consequence of Fru-1-P buildup and reduced ATP levels, inorganic-phosphate levels decrease and AMP levels increase, respectively, which activates AMP deaminase-2(22). In turn, excess AMP enters into the purine degradation pathway resulting in increased uric acid (Fig. 1a). After an acute exposure of mice to fructose (1 g/kg for 90 min), the livers were dissected and extracts made. Consistent with HFI, AldoB-KO mice showed exacerbated intrahepatic ATP depletion (presumably due to blocked regeneration of ATP from Fru-1-P, Fig. 1b) and decreased phosphate levels (Fig. 1c). Importantly, the overall Khk protein level did not differ significantly between wild type, aldolase-B heterozygous, and AldoB-KO mice (Fig. 1d), however; hepatic fructose-dependent ATP depletion (as a marker for Khk activity), was significantly increased in AldoB-KO mice compared to the other groups (Fig. 1e). Moreover, when a similar fructose challenge was given to ketohexokinase knockout (Khk-KO) mice there was a lack of significant changes in hepatic ATP, phosphate, or uric acid levels suggesting that these changes were part of a mechanism dependent on Khk (Fig. 1b-d,f).

Ketohexokinase deficiency protects against HFI pathology in AldoB-KO mice.

Previously, our group demonstrated that mice lacking aldolase B are phenotypically identical to human HFI and are characterized by severe lethality, inflammation, fatty liver, and growth retardation after

exposure to fructose (19). Since Khk is located upstream to aldolase B in the metabolism of fructose, and Khk is necessary for fructose-dependent ATP depletion and the generation of Fru 1-P, the hypothesis that blockade of Khk activity would protect from the sequela of HFI was tested. Accordingly, we crossed a Khk-KO mouse with an AldoB-KO mouse to generate an AldoB-KO mouse lacking both isoforms of Khk (A and C)(23, 24). Protein expression in liver extracts from progeny mice confirmed our expectations (Fig. 2a). Interestingly, aldolase-B heterozygous breeding pairs fed fructose (5%) demonstrated significantly reduced percentage of homozygous *Aldob*^{-/-} pups in the offspring at birth, which was abrogated when ketohexokinase gene (*khk*) was deleted (Fig. 2b).

At weaning, pups bred in the presence of fructose had significantly lower body weight, which indicated a failure to thrive seen in HFI (Fig. 2c)(25). Similarly, after weaning, AldoB-KO mice demonstrated significant weight loss after exposure to various fructose-containing sugars in the drinking water (fructose, sucrose, or high fructose corn syrup (HFCS) 5% w/v), which was not observed in the *Khk*^{-/-}*Aldob*^{-/-} double knockout (Khk/AldoB DKO) mice (Fig. 2d). The drop in body weight loss after exposure of AldoB-KO mice to fructose-containing sugars was paralleled by significant reduced adipose and muscle weight suggestive of increased catabolic rates in these tissues. In this regard, epididymal fat as well as the weight of several muscle groups including tibialis anterior, gastrocnemius and soleus are significantly lower in AldoB-KO but not Khk/AldoB DKO mice. Consistently, serum levels of creatinine kinase (CPK) a marker of muscle breakdown was significantly elevated in AldoB KO mice after exposure to fructose (Fig. 2e-g). This observation suggests that Fru-1-P accumulation in the liver and other Khk-C expressing tissues trigger the mobilization of energy stores in distant organs, particularly skeletal muscle. Consistent with published data (26) and as a consequence of the absence of Khk activity, the Khk-KO mice had a significantly higher excretion of urinary fructose both at baseline and after orally exposed to fructose (Fig. 2h). The level of urinary fructose in the Khk/AldoB DKO mice was 10-fold higher than in AldoB-KO mice.

Since the majority of dietary sugars, including fructose, are metabolized by the liver and intestines, which coincides with the main sites of expression of *Aldob* and *Khk* (24, 27), hepatic and intestinal metabolic effects induced by fructose in AldoB-KO and Khk/AldoB DKO mice exposed to fructose were analyzed. As shown in Fig. 3a (left panel), after chronic exposure to small amounts of fructose in the chow (0.3%), histological examination of livers of AldoB-KO mice as well as liver injury scoring analysis (Supplementary table 1) demonstrated extensive inflammation characterized by the presence of significant numbers of both apoptotic and necrotic cells, pigmented macrophages, and overall “ductal response” with lots of diffuse macrophage infiltration in the parenchyma. Ductal response refers to the reactive processes in

disease and injury occurring at the interface of the portal and parenchymal compartments as described by Gouw et al (28) and others (Fig. 3a, left panel, colored arrows). Consistent with a greater inflammatory phenotype, mRNA levels of both pro-inflammatory cytokines (*Il6* and *Tnfa*) and pro-inflammatory chemokines (*Cxcl1*) were significantly up-regulated in AldoB-KO mice compared to the other groups (Fig. 3b). Of interest, minimal or no inflammation was observed in AldoB-KO mice when *khk* expression was deleted (Fig. 3a, right panel). Furthermore, consistent with a pro-inflammatory phenotype in AldoB-KO mice chronically exposed to fructose, these mice but not Khk/AldoB DKO animals developed severe hepatic periportal (zone 1) fibrosis demonstrated with picro-sirius red staining under polarized light (Fig. 3c). The increase fibrosis was paralleled by significantly elevated levels of hepatic hydroxyproline (Fig. 3d) and by increased expression levels of fibrotic markers including *alpha smooth muscle actin (Asma)* and *timp1* (figure 3e). Lastly, AldoB-KO mice treated with fructose demonstrated elevated serum levels of AST and ALT (transaminitis), markers of liver inflammation and injury compared to Khk/AldoB DKO animals (Fig. 3f). It appears that the observed Khk-dependent leukocyte infiltration in AldoB KO is in response to substantial hepatocyte death. Depletion of intracellular phosphate secondary to its sequestration as Fru 1-P and concomitant reduction in ATP through phosphorylation of fructose by Khk results in reduced ATP availability for other cellular processes. These events are further exacerbated in AldoB KO mice by the absence of proper glycolysis for oxidative phosphorylation and ATP production accompanied by a significant glycogen store disorder. Together, these events substantially predispose AldoB KO mice hepatocytes to death thus triggering an inflammatory response characterized by significant leukocyte infiltration.

To better understand the mechanism whereby hepatic triglyceride accumulation occurs in aldolase B deficient mice chronically exposed to fructose, we analyzed the expression of enzymes involved in *de novo* lipogenesis (fatty acid synthase (FAS), acetyl-coa carboxylase (ACC) and ATP-citrate lyase (ACL)) as well as in fatty acid oxidation (carnitine-palmitoyl transferase 1 (CPT1) and enoyl-CoA hydratase (ECH1)). As shown in Fig. 3g and supplemental Fig. 1, while the baseline expression of CPT1 and ECH1 does not significantly differ between groups fed fructose, aldolase B knockout mice have significantly higher levels of FAS, ACC and ACL suggestive of increased ability for *de novo* lipogenesis. Of interest, of these lipogenic enzymes, only the expression of ACL is significantly down-regulated when Khk expression is deleted in aldolase B knockout mice suggesting that the significant increased expression of lipogenic enzymes in aldolase B knockout mice is independent of fructose metabolism. However, Khk/AldoB DKO mice do not develop fatty liver (see hepatic TG data in table 1), indicating that despite elevated expression of FAS and ACC in these mice, no increased *de novo* lipogenesis occurred. This effect could be mediated by increased

AMPK-dependent inhibition of ACC in Khk/AldoB DKO mice as demonstrated by a greater pACC/ACC ratio (supplemental Fig. 1).

One of the major risks associated with fructose consumption in HFI subjects is the development of a hypoglycemic shock (29). Consistent with this shock in HFI, in AldoB-KO mice, acute oral exposure to fructose resulted in a consistent and severe hypoglycemia in a dose-dependent manner (0.75 to 2.25 g/kg) (Fig. 4a). Importantly, Khk/AldoB DKO mice administered fructose maintained serum glucose even at the high dose of 1.75 g/kg dose of fructose (Fig. 4b). This result provided strong evidence that the observed hypoglycemic effect in AldoB-KO animals depends directly on active Khk. The mechanism whereby Khk-dependent hypoglycemia occurs in hereditary fructose intolerance is likely multiple and complex. Based on published data, it would involve a series of molecular events that include an impaired glycogenolysis and glycogen accumulation disorders, increased glucokinase-mediated glucose uptake and reduced de novo glucose production. In this regard, the observation that deletion of Khk prevents the hypoglycemic response to fructose suggests a critical role for Fru 1-P in the mechanism.

To examine gluconeogenesis, we evaluated the expression levels of PEPCK and G6Pase, which are known as good indicators of gluconeogenesis (30). However, and as opposed to our initial hypothesis, hepatic levels of PEPCK and G6Pase are not down-regulated in Aldo-B KO mice but in contrast, are higher than those observed in wild type mice (Fig. 4c and supplemental Fig. 1). Of interest- the up-regulation of these key gluconeogenic enzymes is not prevented although it is substantially ameliorated in Khk/AldoB DKO. It is possible that the elevation of PEPCK and G6Pase in Aldo-B KO mice do not necessarily mean increased gluconeogenesis in these animals and could be the consequence of a compensatory mechanism secondary to reduced flux. Therefore, to further evaluate whether increased PEPCK and G6Pase expression in aldolase B knockout mice was really associated with enhanced ability for gluconeogenesis, we performed a pyruvate tolerance test. As shown in Fig. 4d, and consistent with previous reports in HFI subjects (29, 31), Aldo-B KO mice are able to produce glucose from pyruvate. However, their ability to produce glucose endogenously is however substantially blunted compared to wild type mice. Furthermore, an intermediate effect in glucose production was observed in Khk/AldoB DKO mice. This benefit observed in Khk/AldoB DKO mice could be due to not only improved gluconeogenic rate compared to aldolase B knockout mice but also to improved glycogenolysis. Despite some gluconeogenic ability in AldoB KO mice, 18-hour fasting serum glucose levels were significantly lower compared to wild type and Khk/Aldob DKO mice, although relatively normal suggestive of some ability for de novo glucose production in these animals (119 mg/dl WT vs 96 mg/dl KO vs 112.3 mg/dl DKO).

In subjects with HFI hepatomegaly is believed to be a consequence of increased glycogen accumulation, and results in significantly impaired glucose availability and homeostasis. Production and degradation of glycogen is tightly regulated by controlling the activity of glycogen synthase (GS) for production- and glycogen phosphorylase (GYPL), for degradation-. Activities of these enzymes are maintained by phosphorylation. AMP kinase (AMPK) phosphorylates and inhibits GS while protein kinase A (PKA) phosphorylates and activates GYPL. Glycogen levels as well as GS activity are significantly elevated in AldoB KO mice compared with wild type animals (Figure 4e, supplemental Fig. 1 and table 1). It is important to note that both glycogen accumulation as well as the continuous activation of GS is prevented in Khk/AldoB DKO mice suggesting that unlike AldoB KO mice, wild type and DKO animals could potentially maintain better glucose homeostasis by mobilizing their glycogen reservoir in the liver.

Glucose uptake is stimulated upon energy demand by cells. In fed states, insulin promotes glucose uptake by insulin-sensitive tissues. In the liver, besides insulin action, the release of glucokinase from the nucleus to the cytosol is an important step to stimulate glucose uptake for glycolysis. Thus, one possibility is that HFI is associated with an exacerbated glucokinase-mediated response leading to severe hypoglycemia following a fructose challenge. In this regard, our data show that fructose exposure is not associated with greater insulin release over time (table 1) which is consistent with previous observations including the lack of an active fructose transporter (GLUT5) in pancreatic islets and the failure of fructose to stimulate the release of insulin by pancreatic β -cells (32). In contrast to the minimal effect of fructose on the release of insulin, hepatic glucokinase expression and location significantly differ between AldoB KO mice and the rest of the groups. Our data in figure 4f demonstrate that AldoB KO mice have significantly greater baseline expression of glucokinase as well as fructose-dependent cytosolic release from the nucleus than wild type or Khk/AldoB DKO mice. At 90 min post-fructose load, cytosolic glucokinase levels are 2.6 and 1.7-fold greater in AldoB KO mice than wild type or DKO animals, respectively. Since Fru 1-P has been shown to disrupt the bond between glucokinase and its regulatory protein in the nucleus thus allowing for its cytosolic translocation(33, 34), our metabolomics data demonstrate that AldoB KO mice have significantly greater accumulation of Fru 1-P than wild type and DKO mice (Figure 4g). It appears that glucose taken up by AldoB-KO mice is directed towards glycogen accumulation. As described above, glycogen synthase activity is not inhibited in AldoB KO mice and consistently, glycogen levels are significantly higher in these mice after a fructose challenge compared to the rest of groups (table 1). Furthermore, consistent with preferential shifting of glucose towards glycogen accumulation, analysis of alternative pathways to glycolysis like the pentose phosphate pathway indicate that there is a significantly reduced levels of metabolites from this pathway in AldoB KO mice, identified by substantially lower levels

of 6-phosphogluconate and sedoheptulose-7-phosphate. Similarly, other parameters besides hypoglycemia commonly observed in HFI include hypermagnesemia, hyperuricemia, hypophosphatemia and transaminitis (25). Serum and liver parameters were analyzed after a 90 min, 1.75 g/kg dose challenge of fructose for wild type, AldoB-KO, Khk-KO, and Khk/AldoB DKO mice. Overall, there was protection from acute changes in the Khk/AldoB DKO mice, except for elevated fructosemia, which was observed in all mice after fructose exposure (Table 1). Lastly, an inflammatory component was observed in the duodenum and jejunum of fructose exposed AldoB-KO but not Khk/AldoB DKO mice. This response was characterized by the presence of apoptotic cells in the intestinal lumen and derangement of apical villi (Fig. 4h), particularly in the duodenum and jejunum corresponding to the intestinal segments that highly express both Khk and aldolase B (35, 36).

Ketohexokinase-A depletion exacerbates pathophysiology of HFI in AldoB-KO mice

Two alternative splice isoforms of the Khk gene coexist in most mammals including humans, namely Khk-A and Khk-C(23, 24). Of these isoforms, Khk-C has higher affinity for fructose and thus, causes greater ATP reduction in response to a fructose challenge compared to Khk-A(26). The specific knockout of Khk-A (*Khk^{3a/3a}*) is associated with an exacerbated metabolic phenotype in mice in response to long-term exposure to fructose (26). Consistent with a role in buffering the overall fructose metabolism in other tissues, the Khk-A/AldoB-KO double knockout mice (*Khk^{3a/3a}Aldob^{-/-}*) are not protected against the pathology of fructose exposure and had an exacerbated hypoglycemic response in response to fructose compared to AldoB-KO mice (Fig. 5a-b). In parallel, there was a slight increase in liver-Khk activity as determined by percent ATP depletion (Fig. 5c). Perhaps as a result of this increased Khk activity, serum levels of liver transaminases (AST and ALT), as well as uric acid, were significantly higher in *Khk^{3a/3a}Aldob^{-/-}* compared to AldoB-KO animals (Fig. 5d-f and table 1). Furthermore, when chronically exposed to low amounts of fructose, Khka deficiency was associated with significantly greater lipogenic, pro-fibrotic and pro-inflammatory hepatic profile compared to regular AldoB KO mice (Figure 5g-j). This study provides evidence supporting the hypothesis that the inhibition of Khk activities in tissues expressing the Khkc isoform like liver confer protection against HFI in mice.

Effective pharmacological inhibition of Khk for the prevention and the treatment of HFI in mice.

Osthole, a coumarinic derivative obtained from plants of the Angelica family, has efficient inhibitory activity against Khk (37). To test whether osthole could be used for inhibition of Khk activity in the liver

and thus confer protection against HFI in mice, AldoB-KO mice were exposed to osthole (25 mg/kg, Santa Cruz biotechnologies SC-205780) in the drinking water for 7 days before an acute fructose challenge of (1.75 g/kg). AldoB-KO mice pre-treated with osthole demonstrated significantly reduced Khk activity and Fru-1-P accumulation in the livers compared to vehicle-exposed mice and demonstrated less ATP decrease (Fig. 6a-b). Consistently, exposure of mice to osthole resulted in significantly increased urinary fructose excretion compared to vehicle treated animals consistent with blocking fructose metabolism (as in essential fructosuria) (Fig. 6c). More importantly, monitoring blood glucose for 90 min following an acute fructose challenge showed that osthole-treated AldoB-KO mice had significantly improved glycemia (Fig. 6d). Consistent with improved glycaemia and reduced Fru-1-P levels, the release of GCK to the cytosol from the nucleus was significantly reduced in osthole-treated mice compared to vehicle (Fig. 6e). Furthermore, monitoring other serum metabolites 90 min after acute fructose challenge showed that osthole-treated mice had reduced transaminitis, hyperuricemia, and hypermagnesemia (Fig. 6f-i, respectively). However, it is important to note that osthole is not a pure specific fructokinase inhibitor and as such, the beneficial effects of fructokinase may be also related to multiple off-target effects(38). Nevertheless, our study suggests that the prophylactic pharmacological inhibition of Khk is therapeutically effective in prevention of acute pathology in this animal model for HFI.

Discussion

For the first time, evidence is reported herein that the blockade of Khk could be an excellent therapeutic approach for the prevention and treatment of HFI. The need for new therapeutic approaches for HFI lies in the increased challenges in excluding fructose and added sugars in the diet due to their ubiquitous use in food preparations (3, 39). This issue alone severely affects the quality of life for HFI subjects, with constant concern about both acute and chronic exposure that leads to severe hypoglycemia and liver disease, respectively. In this regard, by using both the newly created AldoB-KO mice¹⁶ and a newly discovered Khk inhibitor, osthole,²⁴ the multiple deleterious effects that fructose poses for HFI subjects, as well as for this animal model, were blocked including acute hypoglycemic shock, liver inflammation, and intestinal damage.

Moreover, ample recent evidence strongly indicates that the increased consumption of fructose and sugar in recent decades has significantly contributed to the current epidemics of obesity and metabolic syndrome (40-43). It is commonly thought that this association between sugar consumption and metabolic syndrome is merely due to the hypercaloric effect of fructose and sugar as a consequence of excessive intake (44, 45). However, there are isocaloric data to the contrary that highlight the peculiarity

of fructose and its metabolism(46, 47). Importantly, by using AldoB-KO mice, here we show that even when fructose cannot be directly metabolized into triglycerides, glucose, or glycogen, it can induce specific metabolic harmful effects in the organism, including fatty liver and liver inflammation, which would underline the importance of the particular metabolism of fructose in disease.

Despite complete avoidance of fructose-containing foods, subjects with HFI often develop episodes of hypoglycemia, show signs of general ill health and overall symptoms of chronic fructose intoxication (8). As there is little to no ingestion of fructose, these symptoms must be related to the metabolic and not the caloric properties of fructose. The multi-variant symptoms of chronic fructose intoxication could be from inadvertent ingestion of foods that contain fructose or fructose-containing sugars (sucrose and high fructose corn syrup), which are heavily added to foods in order to make them tastier and more appealing to consumers. These foods are not necessarily sweeter, which is a powerful aversion in those with HFI (48). In addition, it has become increasingly apparent that fructose can be produced endogenously in the body by the activation of aldose reductase and the polyol pathway (49, 50). These reports demonstrate that the endogenous fructose production and its metabolism through Khk is an important deleterious step in the pathogenesis of fatty liver and steatohepatitis. Consistently, HFI subjects do not tolerate sorbitol-containing foods, an intermediate product of the polyol pathway (51) leading to fructose production. Therefore, given the prevention of pathology by limiting the metabolism of fructose through Khk shown here, further studies employing aldose reductase knockout mice or aldose reductase inhibitors, which are already commercially available (unlike drugs targeting Khk), are warranted to prevent the endogenous production and metabolism of fructose.

The mechanism whereby HFI pathology progresses so rapidly after acute fructose exposure remains unclear. What is clear is that this mechanism likely depends on the phosphate depletion that occurs in its sequestration as Fru 1-P. However, substantial ATP depletion and uric acid generation also occur and is exacerbated by the over-activation of Khk (Fig. 1). Therefore, this substantial ATP depletion and uric acid production and its hepatic accumulation may be a yet unknown underlying factor important in the pathogenesis of HFI. Consistent with this hypothesis, uric acid is an important pro-oxidant molecule that can cause intracellular oxidative stress, inflammation, and mitochondrial dysfunction (52-55). In this study, inhibiting Khk activity did not reveal the details of the molecular mechanisms linked with the disease, but did reveal the intracellular hallmarks (i.e. phosphate depletion, ATP depletion, Fru-1-P generation, etc) that are part of the pathogenic pathway. Given the model organism and the effective “cure” described here, deciphering this pathway is within reach.

Methods:

Animal study. Aldolase B knockout (AldoB-KO) and Khk knockout (Khk-KO) mice, in the C57BL/6 background were generated as described previously(19, 24, 56). Mice were maintained in temperature- and humidity-controlled specific pathogen-free condition on a 14-h dark/10-h light cycle, and allowed *ad libitum* access to normal laboratory chow (Harlan Teklad, #2920X) or fructose-free diet (Bioserv F6700) for AldoB-KO mice. Aldolase B/Khk (lacking both A and C isoforms of Khk) knockout mice and aldolase B/Khk-A knockout mice were developed by crossing F2 heterozygous mice ($khk^{+/-} aldob^{+/-}$ or $(khk^{+/3a} aldob^{+/-})$) and maintained on a fructose-free diet. 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. In all studies, 8 week old male mice were employed.

Oral fructose tolerance test was performed on 8-wk old fasting (6 hour) male mice by gavage of a fixed amount of fructose as indicated for each experiment. Baseline, 15, 30, 45 60 and 90 min glucose levels were determined with a glucometer from tail snips. Area under the curve (AUC) for glucose was calculated by arbitrarily providing 1 cm per 50% glucose levels and per 30 min of time. Therefore, the disappearance of 50% of baseline blood glucose in 30 min would result in half a cm square loss in the AUC. Similarly, oral pyruvate tolerance test was performed in 6-hour fasting mice exposed to 1.5 g/kg pyruvate and serum glucose determined over a 120-min period.

Biochemical analysis. Blood was collected in microtainer tubes (BD) from cardiac puncture of mice under isoflurane, and serum was obtained after centrifugation at 13000 rpm for 2 min at room temperature. Biochemical analysis for alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, and uric acid was performed on serum and liver lysates with an automated chemistry analyzer (VetACE Clinical Chemistry System, Alfa Wassermann Diagnostic Technologies). Serum or liver fructose, phosphate, and magnesium determination was performed biochemically following manufacturer's instruction (Fructose: bioassay systems EFRU250, phosphate and magnesium: Biovision, K410 and K385). Urinary fructose levels were normalized to units of creatinine (determined biochemically on liver extracts with a Creatinine Reagent Kit, Pointe Scientific, INC). Liver hydroxyproline was determined biochemically following manufacturer's protocol (Biovision, K555). To extract metabolites from liver samples, frozen liver samples were ground at liquid nitrogen temperature with a Cryomill (Retsch, Newtown, PA). The resulting tissue powder was weighed (~20 mg). The extraction was then done by adding -20°C extraction solvent to the powder and incubated in -20°C overnight, followed by vortexing and centrifugation at 16,000 x g for 10

min at 4°C. The volume of the extraction solution (μl) was 40 x the weight of tissue (mg) to make an extract of 25 mg tissue per ml solvent. Dried extracts were then re-dissolved in LC-MS grade water. Metabolites were analyzed via reverse-phase ion-pairing chromatography coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The mass spectrometer was operated in negative ion mode with resolving power of 100,000 at m/z 200 and scan range of m/z 75-1000. The LC method used an Atlantis T3 column (150 mm × 2.1 mm, 3 μm particle size, 100 Å pore size, Waters, Cat#186003719), with a gradient of solvent A (97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid) and solvent B (methanol). The LC gradient was 0 min, 0% B, 200 μl/min; 2 min, 0% B, 200 μl/min; 4 min, 20% B, 200 μl/min; 13 min, 80% B, 200 μl/min; 17 min, 100% B, 200 μl/min; 17.5 min, 100% B, 300 μl/min; 20 min, 100% B, 300 μl/min; 20.5 min, 0% B, 300 μl/min; 24 min, 0% B, 300 μl/min; 25 min, 0% B, 200 μl/min. Other LC parameters were, column temperature 25°C, autosampler temperature 5°C, injection volume 10 μL.

Histopathology. Formalin-fixed paraffin-embedded liver and jejunal sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Histological examination was performed as described previously by Orlicky et al (57). For the liver injury scoring, the entire cross section of liver including all zones were analyzed from each mouse. Images were captured on an Olympus BX51 microscope equipped with a four megapixel Macrofire digital camera (Optronics; Goleta, CA) using the PictureFrame Application 2.3 (Optronics). Composite images were assembled with the use of Adobe Photoshop. All images in each composite were handled identically. To quantitate the fibrosis, ten polarized images were made in a “tiling” fashion across each PSR stained slide, then quantitated and averaged using the 3I Slidebook program (3I, Denver, Colorado) to arrive at the PSR stained pixels per 100x field for that slide/animal. The criteria used in the liver injury scoring are modified from the criteria used by Brunt (Brunt reference included), and as previously modified for mouse liver (45). and include: 1) liver cell injury (ballooning, acidophil bodies, necrotic cells, pigmented macrophages, megamitochondria, etc), 2) inflammation (lobular inflammation, foci of inflammatory cells, lipogranulomas, portal inflammation, Langhans giant cells, etc), 3) Steatosis (macro and microvesicular steatosis), 4) fibrosis (perisinusoidal, periportal, bridging fibrosis, cirrhosis, etc) and 5) other features: and other features like glycogenated nuclei, mitotic figures, etc. To score all animals and as described above, we take 10 (ten) images in a “tiling” fashion across a section of tissue per animal to obtain an average per animal, then we average the averages of each animal in the various groups and supply a mean +/- std dev. For intestinal analysis, several sections of each mice (3-5 sections each) were analyzed and scored including duodenal,

jejunal, ileal and cecal sections. Score points include loss of papillae tip, luminal presence of dead and apoptotic cells and leukocyte infiltration.

Western blotting. Protein lysates were prepared from mouse tissue employing MAP Kinase lysis buffer as previously described(58). Protein content was determined by the BCA protein assay (Pierce). Total protein (50 µg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% w/v), and transferred to PVDF membranes (BioRad). Membranes were first blocked for 1 h at 25 °C in 4% (w/v) instant milk dissolved in 0.1 % Tween-20 Tris-Buffered Saline (TTBS), incubated with primary rabbit or mouse-raised antibodies (1:1000 dilution in TTBS) (Aldolase B (H00000229-A01), GCK (H000026465-B02P) and PEPCCK (H00005105-B01P) Novus biotechnologies; G6Pase Abcam (ab96142) , Khk, Sigma (HPA007040); Actin (8432), FAS, ACC (3676),p-ACC (Ser79, 11818) AMPK (2603), pAMPK (Thr172, 2535), ACL (4332), CPT1 (12252), GS (3886), pGS (Ser641 3981) Cell Signaling, and PYGL (15851-1-AP) and ECH1 (11385-1-AP) from Proteintech,, and visualized using an anti-rabbit (7074) or anti-mouse IgG (7076) horseradish-peroxidase conjugated secondary antibody (1:2000, Cell Signaling) using the HRP Immunstar® detection kit (Bio-Rad, Hercules, CA). Chemiluminescence was recorded with an Image Station 440CF and results analyzed with the 1D Image Software (Kodak Digital Science, Rochester, NY). For real time PCR, RNA was extracted with the RNeasy kit (QIAGEN) and converted to cDNA with the iScript kit (Bio-rad). Renal levels of il6 and ccl2 were analyzed with specific primers (59).

Ketohexokinase activity. Khk activity was determined as previously described with slight modifications (49). Briefly, liver samples were first homogenized in 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, and 1 mM DTT using a polytron homogenizer, and centrifuged for 10 min at 13000 rpm at 4 °C. The protein content of the supernatant fraction was quantified with the protein BCA assay (Pierce), and Khk activity was measured on 50 µg lysate protein after addition of a buffer to 5 mM fructose in 50 mM imidazole, 1 M potassium acetate, pH 5.2, and 1 mM ATP. ATP was measured both before and after 2 h incubation at 37 °C using the ATP determination kit (K354-100, Biovision) as per manufacturer's instructions. Khk activity was calculated as the ratio between ATP levels at 2 h versus baseline for each sample at zero time.

Statistical analysis. All numerical data are presented as the mean ± s.e.m. Independent replicates for each data point (n) are identified in figure legends. Data graphics and statistical analysis were performed using Prism 5 (GraphPad). Data without indications were analyzed by one-way ANOVA, Tukey post hoc

test. A value of $p < 0.05$ was regarded as statistically significant. For figure 3b and assuming not normal distributions of genotypes, data was analyzed by two-tail chi-square.

Study Approval. All animal experiments were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals (60). The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado (Aurora, CO).

Author Contributions

MAL, MFW, RJJ and DRT helped designing research studies, MAL and AAH conducted the experiments, MAL, AAH, CC, CJ, NL, TM and MK acquired the data, MAL, CJ, JDR, RJJ, DJO and DRT analyzed the data, and MAL, RJJ and DRT wrote the manuscript.

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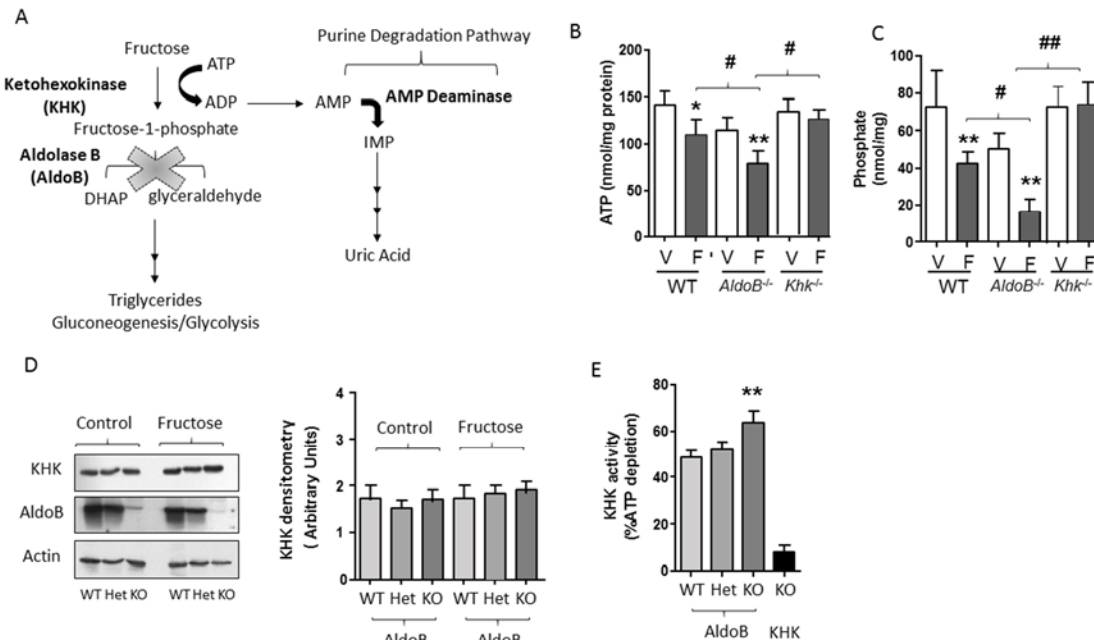


Figure 1: Lack of aldolase B is associated with increased ATP depletion and ketohexokinase activation in response to fructose.

a) Schematic describing fructose metabolism and its association with the purine degradation pathway leading to uric-acid generation. The loss of the aldolase B gene, indicate by the X is associated with Fru-1-P accumulation, phosphate and ATP depletion, and increased nucleotide turnover resulting in uric acid accumulation. **b,c)** Intrahepatic ATP and phosphate levels in wild type (WT), AldoB-KO (*aldob*^{-/-}), and Khk-KO (*khk*^{-/-}) mice acutely exposed to water vehicle (V) or fructose (F) (1 g/kg for 90 min). **e)** Representative western blot for aldolase B (AldoB) and ketohexokinase (Khk) in liver extracts from wild type (WT), Heterozygous (Het), and AldoB-KO (KO) mice ± fructose exposure (1 g/kg for 90 min). Right, densitometry values from n=15 animals per group **f)** Ketohexokinase activity in liver extracts from wild type (WT AldoB), heterozygous for AldoB-KO (Het AldoB), AldoB-KO (KO AldoB) mice, and Khk-KO (KO Khk) mice. Values were evaluated for statistically significant differences (One Way ANOVA-Tukey post hoc analysis, n=7

animals per group, * $p < 0.05$, ** $p < 0.01$ versus respective vehicle controls or # $p < 0.05$, ## $p < 0.01$ versus respective genotypes).

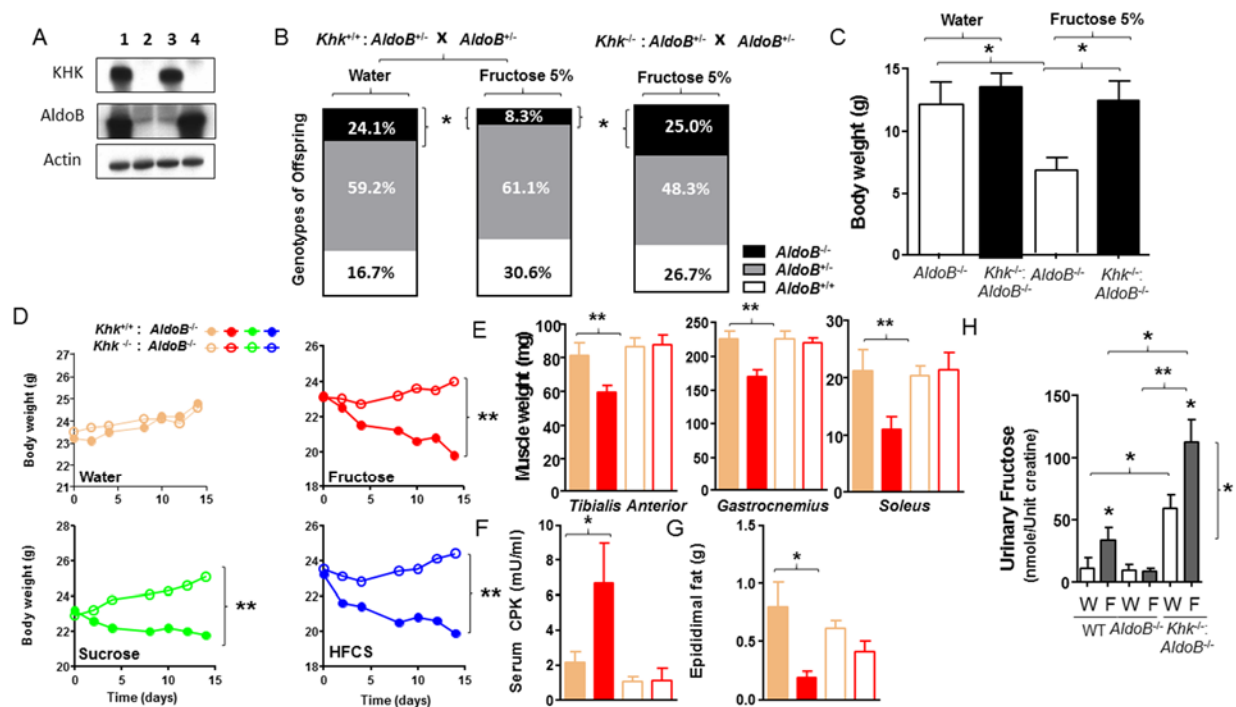


Figure 2: Metabolic responses associated with ketohexokinase deletion in AldoB-KO mice

a) Representative western blot for aldolase B (AldoB) and ketohexokinase (Khk) in wild type (lane 1), *Khk*/*Aldob*-double KO (lane 2), *Aldob*-KO (lane 3) and *Khk*-KO (lane 4) mice. **b)** Distribution of different genotypes in offspring from breeding *aldob* heterozygous (*Aldob*^{+/-}) pairs exposed to water control or 5% fructose in the drinking water with either wild-type (*Khk*^{+/+}) (left and center) or *Khk*-KO background (*Khk*^{-/-}) (right) (n≥15 pups analyzed from ≥3 offspring per breeding pair). Total pups born in each group: Left graph: wild type (13/54), heterozygous (32/54) and knockouts (9/54). Center graph: wild type (11/36), heterozygous (22/36) and knockouts (3/36). Right graph: wild type (15/60), heterozygous (29/60) and knockouts (16/60). **c)** Average body weight (g) at weaning of *Aldob*-KO mice (white) or *Khk*/*Aldob*-doubleKO mice (*Khk*^{-/-} *Aldob*^{-/-}) (black) from heterozygous breeding pairs. **d)** Changes in body weight (g) of 8-wk old *Aldob*-KO (*Khk*^{+/+};*Aldob*^{-/-}) (solid) or *Khk*/*Aldob*-doubleKO (*Khk*^{-/-};*Aldob*^{-/-}) (open) mice exposed to the indicated fructose-containing sugars (5 % w/v) for 14 d. **e-g)** Muscle weight, serum CPK and

epididimal fat weight at day 15 after water (brown bars) or fructose (red bars) exposure in AldoB-KO – solid- and AldoB/Khk DKO mice –clear bars. **h)** Urinary fructose excretion, normalized to units of creatinine, in wild type (WT), AldoB KO (*Aldob*^{-/-}), or Khk/AldoB-doubleKO (Khk AldoB-DKO) mice exposed to water (W) (white) or 5% fructose w/v (F) (gray) for 24 h. Statistical significance between values is indicated by braces with p-levels as follows: For b: two-tail chi-square. Total n number described in b section. For c,e and h: One Way ANOVA-Tukey post hoc analysis For d: T-test 2 tail. **p*<0.05, ***p*<0.01 *n*=7 animals per group.

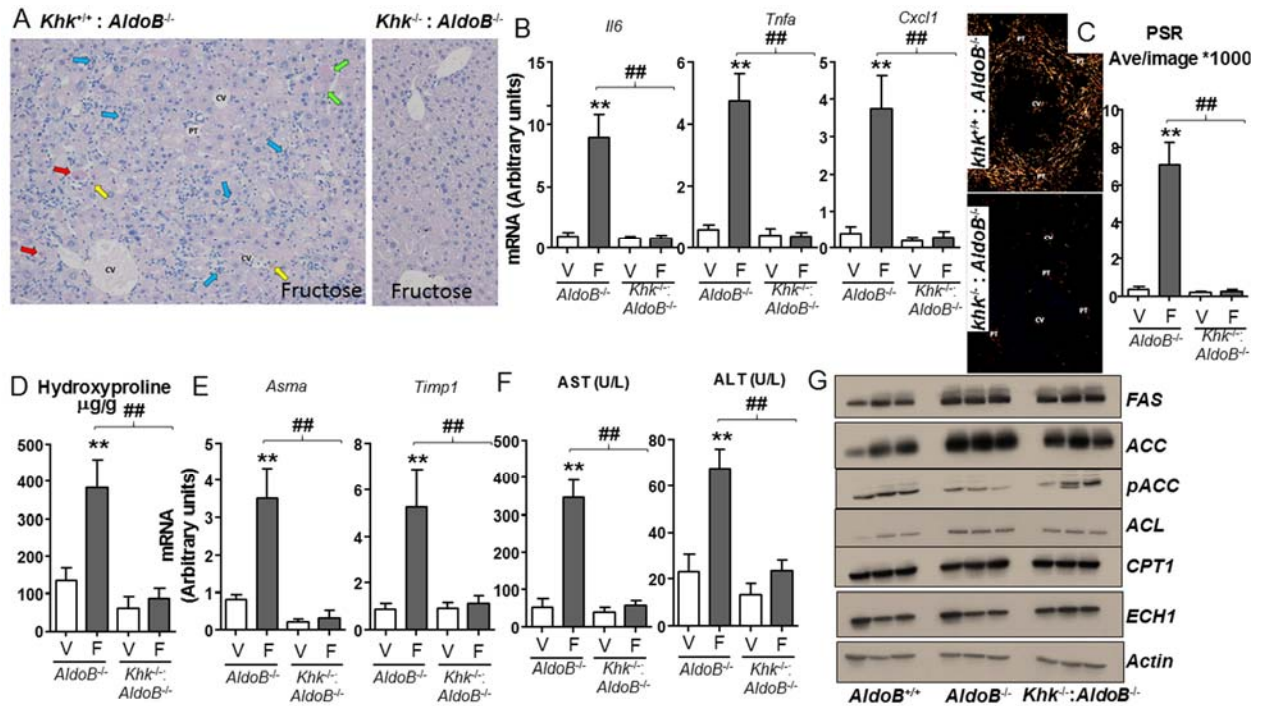


Figure 3: Reduced hepatic inflammation and fibrosis in Khk/AldoB-DKO mice compared to AldoB-KO mice exposed to fructose.

a) Representative H&E images from livers of AldoB-KO (*khk*^{+/+}:*aldob*^{-/-}) exposed to 0.3% fructose in the chow (left), or Khk/AldoB-DKO (*khk*^{-/-}:*aldob*^{-/-}) mice identically exposed to the same chow. Red Arrows- Apoptotic cells, Green Arrows-Necrotic cells, Yellow Arrows-pigmented macrophages, Blue Arrows- "ductal response", and lots of diffuse macrophage inflammation. CV indicates central vein and PT indicates portal triad. **b)** mRNA levels of pro-inflammatory cytokines (*Il6* and *Tnfa*) and chemokines (*Cxcl1*) in mice exposed to sucrose-free (V) or fructose (F) containing chow. **c)** Liver Picro-Sirius red (PSR) staining pictured under polarized light and positive pixel signal denoting increased fibrosis in AldoB-KO mice compared to Khk/AldoB-DKO mice exposed to fructose. CV and PT are indicated as in A). **d-f)** Liver hydroxyproline,

mRNA levels of fibrotic markers *asma* and *timp1*, serum AST, and serum ALT levels in AldoB-KO mice or Khk/AldoB-DKO as denoted in a) exposed to sucrose-free chow (V) or fructose (F). **g)** Representative western blot for lipogenic (Fas, Acc (and Ser69 phosphorylated ACC) and Acl) and fat oxidation related enzymes (Cpt1 and Ech1) in low fructose (0.3%) fed mice. Values were evaluated for statistically significant differences (n=7 animals per group, One Way ANOVA-Tukey post hoc analysis * $p<0.05$, ** $p<0.01$ versus respective vehicle controls or # $p<0.05$, ## $p<0.01$ versus respective genotypes).

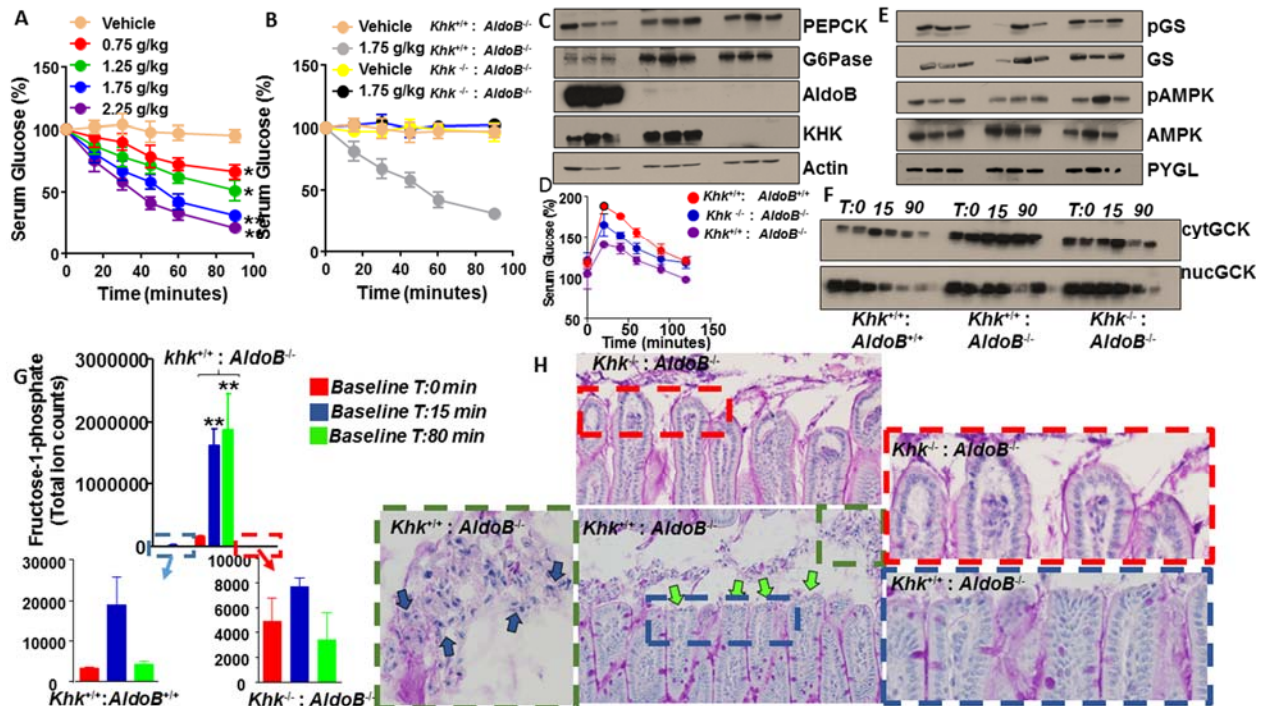


Figure 4: Fructokinase deficiency protects against fructose-induced severe hypoglycemia and metabolic imbalances in AldoB KO mice.

a) Time-course of serum glucose levels in AldoB-KO mice acutely exposed to increasing levels of oral fructose. **b)** Time-course of serum glucose levels in AldoB-KO (*Aldob*^{-/-}) and Khk/AldoB-DKO (*Khk*^{-/-}; *Aldob*^{-/-}) mice acutely exposed to fructose (1.75 g/kg) or water (Vehicle) control. **c)** Representative western blot for gluconeogenic enzymes PEPCCK and G6Pase in low fructose (0.3%) fed wild type (left), AldoB-KO (center) and Khk/AldoB-DKO (right) mice. **d)** Serum glucose levels after pyruvate tolerance test in the same groups as in c). **e)** Representative western blot for glycogen synthase (GS) total and inhibited (pGS) as well as total and active AMPK and total glycogen phosphorylase (PYGL) in the same groups as in c). **f)** Representative western blot for cytosolic and nuclear glucokinase expression at baseline and 15 and 90

min post fructose challenge in the same groups as in c. **g**) Intrahepatic Fru-1-P levels at baseline and 15 and 90 min post fructose challenge in the same groups as in c. **h**) Representative PAS images from the small intestine of AldoB-KO mice (*Khk*^{+/+}:*Aldob*^{-/-}) (left and center) or *Khk*/AldoB-DKO mice (*Khk*^{-/-}:*Aldob*^{-/-}) (right) collected 90 min after exposure to oral fructose (1.75 g/kg). The destruction in the tip of the papilla (green arrows) and luminal apoptotic cells (blue arrows) in the jejunum area are indicated. Values were evaluated for statistically significant differences (n=7 animals per group, One Way ANOVA-Tukey post hoc analysis **p*<0.05, ***p*<0.01 versus respective vehicle controls

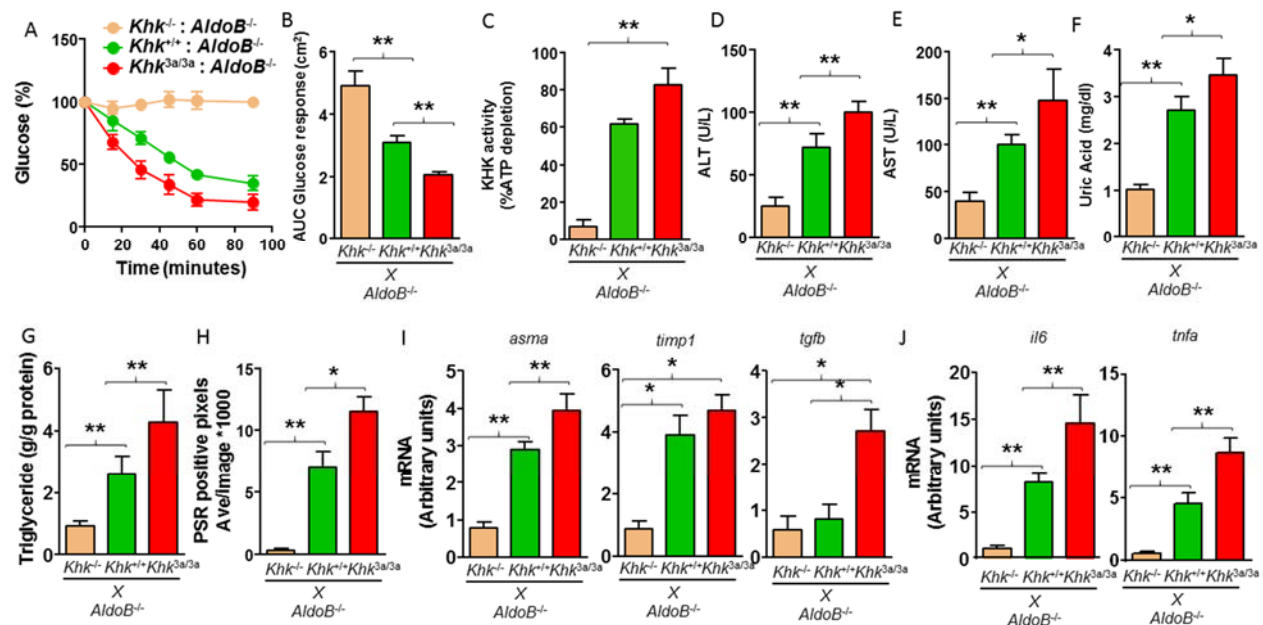


Figure 5: Isoform-specific effects of Khk Knockout in the protection of AldoB KO mice exposed to fructose.

a) Time-course of serum glucose levels in AldoB-KO mice (*Khk*^{+/+}:*Aldob*^{-/-}), *Khk*/AldoB-DKO (*Khk*^{-/-}:*Aldob*^{-/-}), or AldoB-KO plus *Khk*-A only KO (*Khk*^{3a/3a}:*Aldob*^{-/-}) mice acutely exposed to oral fructose (1.75 g/kg). **b**) Area under the curve calculate for the first 40 min of serum glucose levels after acute exposure to fructose (1.75 g/kg) for the same animal groups as in A. **c**) *Khk* activity in liver extracts attained 2 h after fructose exposure for the same animal groups as in A). ATP depletion was calculated versus the baseline for each sample at zero time. **d-f**) AST, ALT, and uric acid levels in the serum of the same animal groups as in A. **g**)

Intrahepatic triglycerides in chronically fed fructose in the chow (0.3%). **h-i)** Liver fibrosis determined as positive pixel area of picro sirius red (PSR) staining and mRNA levels of profibrotic genes *asma*, *timp1* and *tgfb*. **j)** mRNA levels of pro-inflammatory genes *Il6* and *Tnfa*. Pairwise statistical significance was calculated using One Way ANOVA-Tukey post hoc analysis, and significant differences denoted by * $p < 0.05$ and ** $p < 0.01$ (n=75 animals per group).

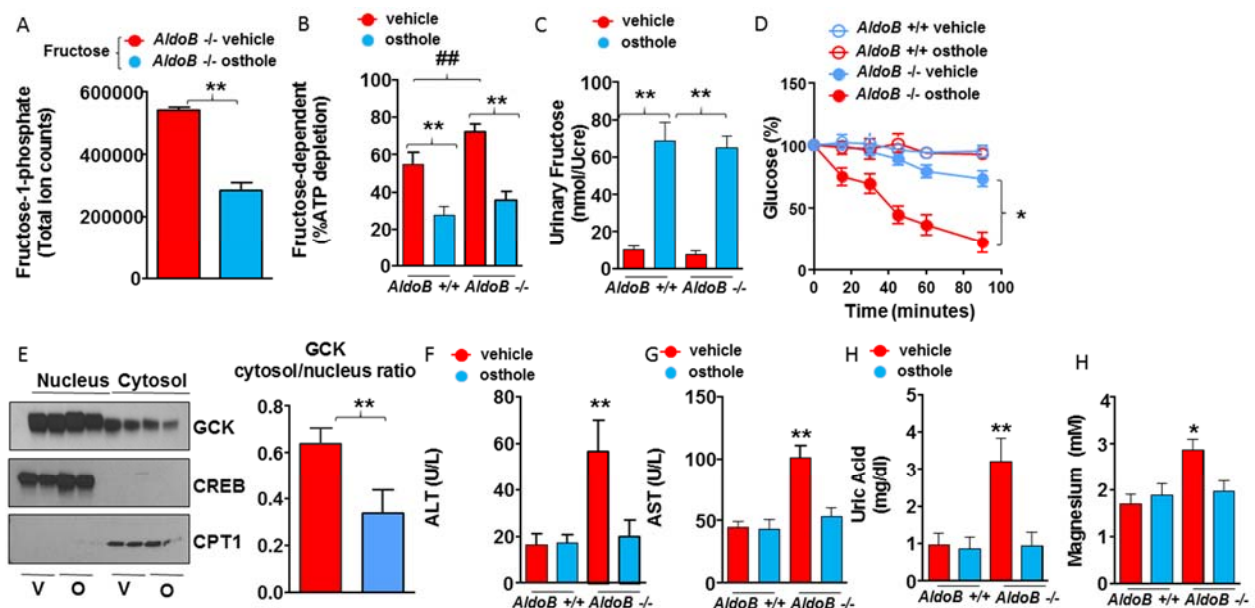


Figure 6: Prophylactic pharmacological inhibition of Khk protects AldoB-KO mice against the deleterious effects of fructose

AldoB-KO mice (*Aldob*^{-/-}) or wild type littermates (*Aldob*^{+/+}) were treated with either vehicle (red) or osthole (blue) (25 mg/kg) for 7 d in the drinking water (water was changed twice daily). **a)** Fru-1-P levels in liver at 90 min post fructose challenge **b)** Fructose-dependent ATP depletion attained 2 h after an acute oral dose of fructose (1.75g/kg). **c)** Urinary fructose excretion in the same groups as in **b)** **d)** Time-course of

serum glucose levels in these AldoB-KO mice after acute oral dose of fructose (1.75 g/kg). *e*) Representative western blot for glucokinase (GCK) expression in nucleus and cytosol of AldoB KO mice exposed to fructose for 90 minutes and treated with either vehicle (v) or osthole (o). CREB and CPT1 are nuclear and cytosolic markers of the nuclear and cytosolic fractions. *f-i*) Serum from AldoB-KO mice attained after acute oral dose of fructose (1.75 g/kg) was analyzed for AST, ALT, uric acid, and magnesium, respectively. Pairwise statistical significance was calculated using One Way ANOVA-Tukey post hoc analysis, and significant differences denoted by * $p < 0.05$ and ** $p < 0.01$ ^{##} $p < 0.05$ (n=7 animals per group).

	Wild Type		AldoB KO		Khk/AldoB DKO		Khk-A/AldoB DKO		
Biochemical Blood Analysis	Water (N=5)	Fructose (n=5)	Water (N=5)	Fructose (n=5)	Water (N=5)	Fructose (n=5)	Water (N=5)	Fructose (n=5)	AldoB KO vs Khk/AldoB DKO Fructose (ANOVA)
Glucose (mg/dl)	124.2 ± 6.8	120 ± 10.4	109.4 ± 7.9	30.4 ± 7.8**	128 ± 9.5	124.4 ± 12.3	105.8 ± 6.4	27.6 ± 9.4**	P<0.01
Glucose (AUC over 90')	4.8 ± 0.3	4.6 ± 0.3	4.4 ± 0.3	3.0 ± 0.2**	4.8 ± 0.3	4.9 ± 0.3	4.5 ± 0.2	2.0 ± 0.7**	P<0.01
Insulin (ng/ml)	0.62 ± 0.1	0.46 ± 0.2	0.46 ± 0.2 [#]	0.55 ± 0.1	0.68 ± 0.1	0.68 ± 0.1	0.57 ± 0.1	0.66 ± 0.3	NS
Magnesium (mM)	2.0 ± 0.4	2.2 ± 0.3	2.2 ± 0.3	2.8 ± 0.2**	2.0 ± 0.3	2.0 ± 0.2	2.2 ± 0.1	2.6 ± 0.1**	P<0.01
Uric Acid (mg/dl)	1.2 ± 0.3	1.4 ± 0.2	1.5 ± 0.2	2.9 ± 0.3**	0.9 ± 0.3	1.2 ± 0.2	1.3 ± 0.2	3.4 ± 0.4**	P<0.01
Fructose (mM)	0.1 ± 0.1	0.7 ± 0.3*	0.3 ± 0.1	1.4 ± 0.2**	0.5 ± 0.2	1.7 ± 0.2**	0.2 ± 0.1	0.8 ± 0.3**	NS
AST (U/L)	36 ± 10	35 ± 9	48 ± 11 [#]	122 ± 16**	32 ± 6	30 ± 6	49 ± 9	140 ± 27**	P<0.01
ALT (U/L)	22 ± 8	25 ± 10	32 ± 6 [#]	68 ± 10**	21 ± 4	19 ± 4	30 ± 8	89 ± 18**	P<0.01
Biochemical Liver Analysis									
Phosphate (nmol/mg)	71.3 ± 19.6	40.8 ± 9.4**	53.8 ± 6.9 [#]	22.2 ± 2.8**	88.6 ± 7.3	85.2 ± 14.3	70.6 ± 2.3	38.1 ± 3.3**	P<0.01
Uric Acid (µg/mg)	11.8 ± 2.9	18.6 ± 5.2	16.6 ± 2.2	45.3 ± 14.3**	7.8 ± 1.1	6.2 ± 3.3	14.4 ± 4.4	60.8 ± 8.1**	P<0.01
FKA activity (% ATP depleted)	47.3 ± 4.6	53.3 ± 2.2	61.5 ± 4.4 [#]	64.9 ± 7.7	9.3 ± 3.1	9.0 ± 5.1	61.3 ± 5.1	70.8 ± 4.1**	P<0.01
Glycogen (mg/g)	2.1 ± 0.2	2.5 ± 0.5	10.3 ± 2.3 [#]	16.8 ± 1.5**	5.1 ± 2.1	3.5 ± 1.5	14.3 ± 3.2	20.2 ± 3.4**	P<0.01
Triglyceride (g/g)	0.6 ± 0.2	0.5 ± 0.2	2.3 ± 0.6 [#]	2.2 ± 0.3**	0.9 ± 0.2	0.7 ± 0.4	4.3 ± 0.6	4.1 ± 0.9**	P<0.01
6-PG (total ion count*1000)	11.5 ± 1.9	10.9 ± 3.3	3.7 ± 0.9 [#]	1.5 ± 0.8**	3.0 ± 0.2	5.1 ± 0.1**			P<0.01
S7P (total ion count*1000)	64.4 ± 22.3	80.0 ± 8.3	42.6 ± 4.5	8.8 ± 6.1**	39.8 ± 4.7	69.9 ± 11.9			P<0.01

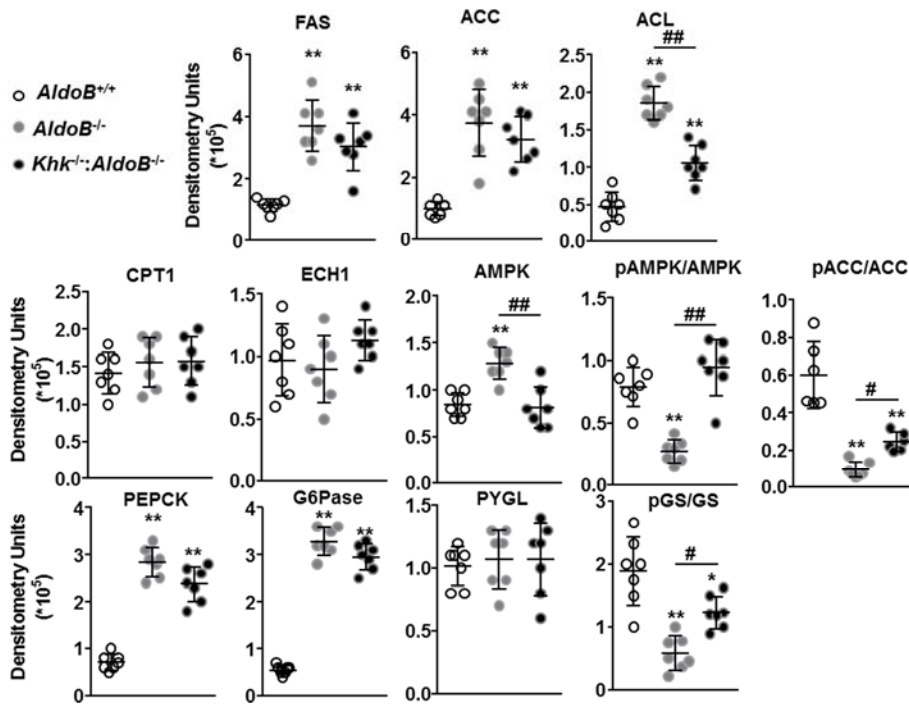
Table 1 Biochemical blood and liver parameters in wild type, AldoB KO, Khk/AldoB DKO and Khk-A/AldoB DKO mice exposed to water or fructose. Mice were given a dose of 1.75 g/kg of fructose and liver and serum were collected after 90 min. Liver parameters were normalized to the mg of liver protein. Statistical analysis was performed on all the genotypes dosed with fructose using ANOVA coupled with Tukey post hoc analysis with those parameters with significant differences noted in bold and the level indicated, * = $p < 0.05$, ** = $p < 0.01$. Bold values denote statistically significant versus water in each group. The last column indicate significance analysis between AldoB KO and khk/AldoB DKO on fructose

Footnotes: AST= Aspartate Transaminase, ALT= Alanine Transaminase, 6-PG= 6-phosphogluconate, S7P= Sedoheptulose-7-phosphate.

			Wild Type		AldoB KO		AldoB/FKA-A/C KO	
			Water (N=5)	Fructose (n=5)	Water (N=5)	Fructose (n=5)	Water (N=5)	Fructose (n=5)
Feature		Points						
Liver Cell Injury								
Ballooning	None	0	x	x		x	x	x
	Few	2			x			
	Many	4						
Acidophil Bodies	None	0	x	x	x		x	x
	Few	2						
	Many (>1/200x field)	4				x		
Necrotic Cells	None	0	x	x	x		x	x
	Present (>2/200x field)	2				x		
Pigmented Macrophages	None	0	x	x	x		x	x
	Many	1				x		
Megamitochondria	None	0	x	x	x	x	x	x
	Present	1						
Sinusoidal Dilation & Congestion	None	0	x	x	x	x	x	x
	Yes (>3 RBC diameter)	1						
True abscesses (>6 cell diameter)	No	0	x	x	x	x	x	
	Yes (>1/40x field)	1						x
Liver Cell Injury Subtotal			0	0	2	7	0	1
Inflammation								
Lobular Inflammation	<2/200x field	0	x	x			x	x
	2/200x field	1						
	2-4/200x field	2						
	>4/200x field	3			x	x		
Foci of inflammatory cells	Absent (<1/200x field)	0	x	x				x
	Present (>1/200x field)	1			x	x	x	
Lipogranulomas	Absent	0	x	x		x	x	x
	Present	1			x			
Portal Inflammation	None to minimal	0	x	x	x		x	x
	More than minimal	1				x		
Ceroid Laden Kupffer C (DAPI only stn)	No	0	?	?	?	?	?	?
	Yes (>3/200x field)	1						
Langhans Giant Cells	No	0	x	x	x	x	x	x
	Yes (>1/200x field)	1						
Foamy Macrophages (PAS stn)	No	0	x	x	x	x	x	x
	Yes (>1/200x field)	1						
Inflammation Subtotal			0	0	4	5	1	0
Other								
Mallory's Hyaline	None	0	x	x	x	x	x	x
	Many	1						
Glycogenated nuclei	<1/200x field	0	x	x	x		x	x
	1-3/200x field	1				x		
	>3/200x field	2						
Ductal Reaction	None	0	x	x	x		x	x
	Minor	1						
	Robust	2				x		
Mitotic Figures	<2/200x field	0	x	x	x		x	x
	>2/200x field	1				x		
Hyperpl nod regen morph	No	0	x	x	x	x	x	x
	Yes	1						
	None	0	x	x	x	x	x	

Hyalinized & Thickened PV&HA	Present	1						
GC Transformation	No	0	x	x	x	x	x	x
	Yes	1						
Other Subtotal			0	0	0	4	0	0
Steatosis								
Macrovesicular steatosis (vesicles larger than nuclei)	<10%	0	x	x			x	X
	10-33%	1				x		
	33-66%	2			x			
	>66%	3						
Microvesicular steatosis (filled with small vesicles, central nucleus)	Not present	0	x	x	x	x	x	x
	Present	1						
Steatosis Subtotal			0	0	2	1	0	0
Fibrosis								
Stage	None	0	x	x	x		x	x
	Perisinusoidal (PS)	1						
	Periportal(PP)/Centrilob (CL) only	1						
	Bridging fibrosis	2				x		
	Cirrhosis	3						
Fibrosis Subtotal			0	0	0	2	0	0
Scoring by Category			Wild Type		AldoB KO		AldoB/FKA-A/C KO	
			Water (N=5)	Fructose (n=5)	Water (N=5)	Fructose (n=5)	Water (N=5)	Fructose (n=5)
Injury			0	0	2	7	0	1
Inflammation			0	0	4	5	1	0
Other			0	0	0	3	0	0
Steatosis			0	0	2	1	0	0
Fibrosis			0	0	0	2	0	0
Total			0	0	8	18	1	1
Additional Comments								
1) Score macrosteatosis at 200x								
2) Macrovesicular CLD must be equal to or larger in size than hepato nuclei								
3) Microvesicular steatosis must completely fill the hepatocyte with small CLD and have a central nucleus								
4) Assess fibrosis with Trichrome stained slide								
5) Lobar inflammation refers to the sum of all types of inflammation								

Supplemental Table 1: Brunt Scoring on mice exposed to water or fructose.



Supplemental Figure 1: Western blot densitometry of lipogenic (top row), fat-oxidation related (middle row) and glucose metabolism-related proteins in wild type (*Aldob*^{+/+}), *AldoB*-KO mice (*Aldob*^{-/-}) or *AldoB*/*Khk* DKO (*Khk*^{-/-};*Aldob*^{-/-}) mice exposed to fructose. n=7 mice per group. Pairwise statistical significance was calculated using One Way ANOVA-Tukey post hoc analysis, and significant differences denoted by **p*< 0.05 and *p*<0.01 versus wild type. # *p*<0.05 ## *p*<0.01 (n=7 animals per group).**