Adiponectin resistance and pro-inflammatory changes in the visceral adipose tissue induced by fructose consumption via ketohexokinase-dependent pathway

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ABSTRACT

An epidemic of obesity and type 2 diabetes is linked with the increase in consumption of fructose-containing sugars, such as sucrose and high fructose corn syrup (HFCS). In mammalian cells, fructose is metabolized predominantly via phosphorylation to fructose-1 phosphate by ketohexokinase (KHK) or by alternative pathways. Here we demonstrate that KHK-dependent pathway mediates insulin resistance and inflammatory changes in the visceral fat in response to high fructose.

We used mice (males, C57BL/6 background) including littermate wild type control and mice lacking both isoforms of KHK (KHK-null).

Fructose diet induced metabolic syndrome including visceral obesity, insulin resistance, proinflammatory changes in the visceral fat (production of proinflammatory adipokines and macrophage infiltration), the endoplasmic reticulum (ER) stress signaling, and decrease of the high molecular weight (HMW) adiponectin followed by decrease in the downstream signaling. KHK-KO mice consuming the same high fructose diet remained lean, with normal insulin sensitivity and healthy visceral adipose tissue with normal adiponectin function not distinguishable from the control by any of the tested parameters.

This study demonstrates that blocking KHK and redirecting fructose metabolism to alternative pathways is an effective way to prevent visceral obesity and insulin resistance induced by high fructose, a widespread component of Western diets.
INTRODUCTION

Metabolic syndrome and type 2 diabetes have been linked with increased consumption of added sugars containing fructose, such as sucrose and high fructose corn syrup (1-3). Fructose is distinct from other sugars in its initial metabolism, particularly in its phosphorylation to fructose-1 phosphate by a fructose-specific enzyme ketohexokinase (KHK, fructokinase). Together with two downstream enzymes (aldolase and triokinase), KHK forms a fructose-specific metabolic pathway (4). There are two KHK isoforms produced by alternative splicing of the KHK pre-mRNA: KHK-C, which is highly active and mostly responsible for the fructose metabolism and expressed abundantly in fructose-metabolizing organs (liver, kidney, intestine), and KHK-A, an isoform with very low affinity to fructose, which is widely distributed in most tissues at the low level of expression and apparently not involved in the fructose metabolism (5; 6). KHK-C pathway bypasses tightly regulated glycolytic checkpoints, such as hexokinase and phosphofructokinase. Without negative feedback mechanisms, metabolic flow of fructose through this pathway remains largely unregulated and greatly exceeds energy demands (4; 7). This causes dramatic stimulation of lipogenesis and fat accumulation in the liver, which is commonly followed by hepatosteatosis (8; 9). Recently, Ishimoto et al. (10) demonstrated that fructose-induced, abnormal lipogenesis in the liver, hepatosteatosis, triglyceridemia, and visceral obesity were absent in mice lacking both isoforms of KHK. While consuming similar amounts of fructose, KHK-deficient mice were excreting substantial amounts of it with urine but still maintained positive energy balance but on the lower level than wild type mice fed with fructose (10). Liver retains from 50 to 75% of the total fructose absorbed in the intestine and metabolizes it via KHK-C while the remaining part is taken up mostly by the kidney and adipose tissue (4; 11). If the kidney metabolizes fructose via KHK-C, which is highly expressed in proximal tubular cells (5; 12), KHK expression in the adipose tissue is very low (5; 10), and represented predominantly by the A-isoform with no (5) or very low expression of KHK-C (10) while fructose is metabolized via alternative pathway, likely via hexokinase-dependent glycolytic pathway (11).

In the previous work (10), we demonstrated that fructose-induced lipogenesis in the liver, dyslipidemia, and hepatosteatosis are dependent on the metabolism of fructose in the liver via KHK. Even though hepatosteatosis can contribute to the development of the insulin resistance, in obesity it is predominantly mediated by the low-grade inflammation in the visceral adipose...
tissue and production of the proinflammatory cytokines (13-15), imbalance in production of beneficial adipokines such as loss of adiponectin in the adipose tissue (16; 17), as well as fat-induced endoplasmic reticulum (ER) stress and other stress mechanisms in adipocytes as an underlying molecular mechanism (18-20). Fructose consumption causes accumulation of visceral fat (10; 21; 22). This is why the goal of this study is to characterize the role of the adipose tissue in the development of insulin resistance in response to elevated consumption of fructose including i) production of the proinflammatory cytokines and macrophage infiltration in the adipose tissue as a crucial mechanism causing insulin resistance at the organism level, ii) production of adiponectin, an anti-inflammatory and insulin-sensitizing adipokine, and, specifically, production of the biologically active high molecular weight form of this adipokine followed by detecting adiponectin-dependent signaling in the targets at least partially responsible for the control of lipogenesis in the liver, and iii) to test if fructose causes ER stress signaling in the adipose tissue. All these effects were examined in the wild type and KHK-A/C-KO mice fed with normal or high-fructose diet to detect whether balance of pro-inflammatory and anti-inflammatory changes in the adipose tissue and the resulting insulin resistance can be improved in the absence of KHK.

RESEARCH DESIGN AND METHODS

Animal model. The Animal Care and Use Committee of the University of Florida has approved protocols for all animal experiments. To test effects of fructose on the visceral adipose tissue and the role of KHK, we used KhkΔ/Δ mice with the C57BL/6 background that lack KHK-A and KHK-C, as a result of deletion of exons 4-7 (6). We obtained breeders of these mice from Dr. D. Bonthron (University of Leeds, UK). Prior to our study, we backcrossed these KHK-A/C mice to C57BL/6 mice to obtain a congenic strain. 8-10 week old age-matched littermate male mice were used for the experiments. To assess effects of fructose, wild-type and KHK-A/C mice were divided in two groups (6-8 animals per group), and fructose was administered with water (30% in a drinking water, ad libitum) given to all animals in fructose-treated groups for twelve weeks. At the same time, all experimental groups had an access ad libitum to the standard rodent diet (Teklad LM-485 diet from Harlan Laboratories). The concentration of fructose in drinking water was selected based on our previous study (10), which showed that 30% fructose in drinking
water consumed *ad libitum* is needed for developing the robust signs of metabolic syndrome (BW increase, insulin resistance) within 10-12 weeks while with lower concentration of fructose the effect of the same magnitude would require substantially longer period of time. We also analyze the effect of the identical treatment with glucose to compare effects of these sugars. Body weight was measured weekly for each animal. Food and water intake measured two weeks before the end of experiments. One week before the end of the experiment, we performed the insulin tolerance test. Animals were fasted for four hours starting 6 am and then injected intraperitoneally with insulin (Humulin: R; Eli Lilly, Indianapolis, IN) at 1 units/kg body wt. Glucose in tail vein blood was measured immediately before injection (time 0) and at 15, 30, 60, 90, and 120 min after injection using One Touch Ultra glucometer (Lifescan, Milpitas, CA). At the end of the treatment period, animals were sacrificed by the intraperitoneal injection of 150 mg/kg sodium pentobarbital. Blood was collected via cardiac puncture, and serum was stored in aliquots at -80° C. Samples of visceral adipose tissue, liver and kidney were collected on ice and rinsed in ice-cold PBS. For RNA isolation, tissue samples were preserved in RNA Later solution (Applied Biosystems/ Ambion, Austin, TX) at -80° C.

**Real time qRT-PCR.** Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Trace DNA was removed using DNA-free kit (Applied Biosystems/Ambion, Austin, TX). 0.5µg of total RNA was converted to cDNA using iScript cDNA synthesis kit (BioRad, Hercules, CA). Quantitative real time RT-PCR was performed using SsoFast EvaGreen Supermix (BioRad, Hercules, CA) with primers spanning two or more exons and optimized for real-time PCR, which were designed using software GENEious Pro (v. 4.8; Biomatters Ltd. Auckland, New Zealand) and Beacon Designer v.7.70; Premier Biosoft International, Palo Alto, CA). Sequences for all used primers are shown in the Table 1. Real time PCR was performed using CFX96 real-time PCR detection system. Our detailed protocol for QPCR and assay and validation was described earlier (23). Relative gene expression was analyzed by ΔΔC(t) method using the CFX Manager software (v. 1.6; Bio-Rad, Hercules, CA).

**Western blotting.** Phosphorylated and total Acetyl-CoA carboxylase (ACC) were detected by Western blotting with phosphorylation state-specific rabbit monoclonal antibody for Ser79 and phosphorylation-independent ACC polyclonal antibody (Cell Signaling Technology (Beverly, MA)). Antibodies for AdipoR1 (C-14) and for AdipoR2 (N-16) were from Santa Cruz
Biotechnology (Santa Cruz, CA). GAPDH was detected with monoclonal antibodies from Chemicon (Temecula, CA). Control of protein loading was also confirmed in some experiments by staining gels/membranes with EZBlue reagent (Sigma, St. Louis, MO). Our protocol of Western blotting was described in details earlier (24). The images were digitalized using the Alpha Ease FluorChem digital imaging system (Alpha Innotech Corp., San Leandro, CA). Band densitometry was performed using NIH Image software.

**Immunohistochemistry.** To assess the macrophage infiltration in the adipose tissue, samples of the visceral adipose tissue were collected in the 10% buffered formalin, processed, embedded in paraffin, and sectioned. Macrophages were stained as described (25) with the F4/80 antibody from Invitrogen/Caltag Labs (Carlsbad, CA), secondary HRP-conjugated antibody and diaminobenzidine (DAB) chromogen and a hematoxylin counterstain. Positive cells were counted in blind fashion in three different fields, two slides per animal and expressed as percentage of F4/80-positive cells.

**Detection of XBP-1 activation by mRNA splicing.** Activation of transcription factor by mRNA splicing producing mRNA of its active form was analyzed by PCR analysis of spliced and unspliced forms of XBP-1 mRNA as described in (26).

**Assays for measurement of total and HMW adiponectin, and insulin.** Total adiponectin in the serum was measured either with the ELISA kit for mouse adiponectin from AdipoGen (Liestal, Switzerland) or ELISA kit from ALPCO Diagnostics (Salem, NH) for detection of total and high molecular weight adiponectin, which we also used to detect HMW adiponectin. Insulin was measured with the mouse ELISA assay from Chrystal Chem (Downers Grove, IL).

**Statistics.** At least three independent experiments were performed in the case of *in vitro* studies, and or 6-8 animals per group were used for *in vivo* studies performed in triplicate each. Data were analyzed by two-way ANOVA followed by Bonferroni’s multiple comparisons test, unpaired Student’s t-test, or Mann-Whitney U test, with a value of P<0.05 considered to represent a significant difference. Comparisons between two values were performed by t-test or U-test. ANOVA was used for two-factor analysis and to test differences among several means.

**RESULTS**

**Effect of fructose on energy intake in wild type and KHK-A/C-KO mice.** From the values of food and water intake per cage with 2-3 animals, we calculated average energy
consumption per animal from the rodent chow and fructose consumed with drinking water. The results clearly demonstrate (Fig. 1,A) that mice showed clear preference for sweetened water and decreased amount of energy consumed from chow by about 30% and replaced it with fructose. Total energy intake from chow and fructose combined was modestly higher than in control group that consumed energy only from chow. Similar pattern was also detected in KHK-A/C-KO mice but in this case mice consumed less fructose (31% vs. 41% in the WT group (Fig. 1,A). It is important to emphasize that our results on energy consumption in WT and KHK-A/C-KO treated or not with fructose are highly similar with the results of our previous analysis of the same model (10), which demonstrated in greater details energy expenditure by indirect calorimetry as well as analysis of the fructose metabolism and excretion. The finding crucial for this study was that fructose consumption caused cumulative energy increase in WT and, to a smaller extent, in KHK-A/C-KO mice with the positive energy balance not only in WT but also in KHK-A/C-fed mice. KHK-A/C-KO fructose fed mice showed dramatic loss of fructose with urine and some increase of fructose level in the blood (10). It is safe to assume that in the absence of KHK-C fructose was rerouted away from lipogenesis and the subsequent transport to the adipose tissue. Next, we analyzed changes in the energy consumption for each of three macronutrients (Fig. 1,B). In the fructose-treated WT mice, energy from carbohydrates increases from 58% WT control to 75% in fructose-treated group and to 71% in KHK-A/C-KO mice. Energy consumed from proteins and fat is decreased correspondingly. Thus, absence of KHK slightly affected distribution of energy consumption between macronutrients.

**Body weight increase and insulin resistance caused by fructose consumption is blocked in the absence of KHK.** Fructose consumption by WT mice caused significant body weight gain comparing to control animals drinking water (Fig. 2,A). This increment was mostly due to accumulation of visceral fat (not shown), which is the most typical effect of fructose consumption (10). Body weight gain in response to drinking fructose-sweetened water was completely prevented in KHK-A/C-KO animals (Fig. 2,B).

To test how fructose consumption affects insulin sensitivity in WT and KHK-A/C-animals, we performed insulin tolerance test. Baseline for insulin and glucose (starving) for WT and KHK-A/C-KO mice at the beginning of the study were normal and not distinguishable statistically: glucose level in starving animals was 199±20 mg/dl in WT mice and 165±4.1 mg/dl
in KHK-A/C-KO while insulin level was 0.45±0.03 µg/ml in WT and 0.65±0.11 µg/ml KHK-A/C-KO mice. Therefore, we assume that insulin tolerance was normal in all animals at the baseline. The sensitivity to insulin was substantially reduced in WT mice treated with fructose compared to the control maintained only on the rodent diet (Fig. 2,C). KHK-A/C-KO mice treated with fructose responded to insulin almost as fast as mice maintained on the diet alone (Fig. 2,D) demonstrating that fructose did not cause loss in the insulin sensitivity in mice lacking KHK.

We also tested whether effect of fructose is different from the effect of glucose, which is metabolized via hexokinase/glucokinase and not via KHK. Glucose consumed with water caused some increase in body weight (Fig 1S, A), which was not observed in KHK-A/C-KO mice. The effect of KHK might be dependent on the pool of glucose converted to more lipogenic fructose in the liver, as shown recently (27). On the other hand, glucose feeding did not cause insulin resistance (Fig. 1S,B) demonstrating that KHK metabolism makes effects of similar sugar more detrimental, at least in terms of causing metabolic syndrome, which can be explained by dramatic lipogenic effect of fructose. Fructose pool derived from fructose was likely not sufficient to cause insulin resistance.

**KHK-dependent inflammation in the visceral adipose tissue in response to fructose consumption.** Visceral obesity is generally associated with macrophage infiltration in the adipose tissue, expression of inflammatory cytokines such as MCP-1, TNF-α (25; 28), and a endoplasmic reticulum (ER) stress as an underlying molecular mechanism (29). Fructose consumption with drinking water, indeed, caused a noticeable infiltration of macrophages in the visceral fat where they resided between adipocytes (Fig. 3, see inset for details) similarly to the previously reported macrophage infiltration in ob/ob mice (25; 28). Macrophage infiltration in response to fructose consumption was completely prevented in KHK-A/C-KO (Fig. 3). Average percentage of macrophages in the adipose tissue in response to fructose increased about two-fold in a KHK-dependent fashion (Fig. 3).

In addition to the macrophage infiltration, we tested the visceral adipose tissue mRNA expression for monocyte chemoattractant-1 (MCP-1), the pro-inflammatory chemokine, and a cytokine tumor necrosis factor-α (TNF-α) representing typical inflammatory adipokines expressed in the adipose tissue in obesity. Expression of both adipokines in WT animals was
significantly increased in response to fructose (Fig. 4A). At the same time, expression of MCP-1 and TNF-α was completely prevented in the adipose tissue from KHK-A/C-KO mice (Fig. 4,A).

These results demonstrated that fructose-induced inflammation in the visceral fat is dependent on the metabolism of fructose via KHK.

Next, we determined whether fructose consumption affects ER stress, specifically XBP-1-dependent branch of it, one of three branches of ER, which is activated in obesity and by nutrients, followed by triggering lipogenesis, inflammation, and insulin resistance (18; 26). XBP-1 is activated by splicing of the mRNA with a frame shift producing mRNA of the active form with smaller 26 nucleotides (30), which can be detected by PCR of both forms followed by electrophoretic separation in high density agarose gel. Active form of XBP-1 was barely detectable in control WT mice and become clearly visible in the fructose-fed animals (Fig. 4,B). In KHK-A/C-KO mice, there were no detectable XBP-1 activation in response to fructose consumption (Fig. 4,B).

Thus, XBP-1 activation in the visceral adipose tissue in response to fructose is mediated by the KHK-dependent metabolic pathway, similarly to the effect of fructose on the visceral obesity, inflammation and systemic insulin resistance.

**KHK-dependent decrease in the release of biologically active form of adiponectin in response to fructose.** Adiponectin is a major beneficial adipocyte-specific hormone with insulin-sensitizing and anti-inflammatory activity, and a role in glucose and lipid homeostasis (31; 32). In obesity, adiponectin production decreases with simultaneous increase in the production of pro-inflammatory adipokines (33; 34). Circulating adiponectin is not a monomer but forms trimers (low molecular weight, LMW), medium molecular weight (MMW), and high molecular weight (HMW) adiponectin forms, and only HMW adiponectin is biologically active (35; 36). Therefore, we examined effects of fructose in WT and KHK-A/C-KO mice on the expression of the adiponectin mRNA and its levels in blood including the total and HMW circulated adiponectin. Fructose did not affect the adiponectin mRNA expression in WT or KHK-A/C-KO mice (Fig.5,A) with a tendency (P=0.07) to slightly higher mRNA levels in mice lacking KHK (Fig. 5,A). ELISA for total adiponectin demonstrated that fructose causes surprising statistically unconfirmed increase in adiponectin in response to fructose in WT but not in KHK-A/C-KO (Fig. 5,,B). Measurement of HMW adiponectin by ELISA specific for the
HMW form of adiponectin showed that fructose caused significant drop in the relative content of the HMW adiponectin in WT mice demonstrating deficiency of biologically active adiponectin in fructose-consuming mice (Fig. 5,C). In KHK-KO mice fructose effect on HMW adiponectin was completely prevented (Fig. 5,C). Thus, fructose caused KHK-dependent decrease in the biological activity of adiponectin suggesting that all the beneficial effects of the hormone were decreased while the production of pro-inflammatory adipokines and macrophage infiltration in the visceral adipose tissue were elevated.

Assembly of HMW adiponectin is localized in ER, and secretion of active HMW form of adiponectin is regulated by two ER chaperons: ERp44, responsible for retention of adiponectin in ER via covalent binding to allow maturation of the HMW form, and Ero1-Lα, which is responsible for assembly and release of HMW adiponectin (36). Expression of both chaperons in obesity is decreased, which causes decrease in the levels of adiponectin and its HMW form (36). In the WT mice fed with fructose, level of the ERp44 mRNA in the visceral fat remained unchanged (Fig. 5,D) while the Ero1-Lα mRNA was significantly decreased (Fig 5E) indicating that mechanism of assembly and release the HMW complex was affected by high fructose. The levels of both ER chaperones remained unchanged by fructose treatment in KHK-A/C-KO mice (Fig. 5,D-E) indicating that fructose affected balance in ER chaperones responsible for the assembly of HMW adiponectin in KHK-dependent fashion resulting in decreased release of HMW adiponectin in WT mice.

Next we tested whether effects of fructose on the HMW adiponectin release and levels of ER chaperones responsible for HMW maturation could be reproduced in vitro by direct effect of fructose on adipocytes. However, we could not detect neither changes in the HMW adiponectin release in the medium or its intracellular content in response to fructose treatment for 72 h (Fig. S2,A HMW is separated in non-reducing non-denaturing conditions as an upper band with apparent MW about > 250 kD). The analysis was performed at the early and late stage of adipocyte differentiation to check the effect of differentiation on the sensitivity of adipocytes to fructose. In addition, expression of ERp44 and Ero1-Lα also remained unchanged by the presence of fructose in the culture medium (Fig. S2,B). This suggests that fructose-induced adiponectin resistance and suppression of all underlying beneficial effects of the HMW
adiponectin are, most likely, mediated by KHK-dependent metabolism and its effects in the liver as a predominant fructose-metabolizing organ.

**KHK-dependent downregulation of adiponectin-dependent signaling in response to fructose in the liver and kidney.** The results on fructose-induced decrease in the level of HMW adiponectin prompted us to analyze adiponectin receptors and the downstream signaling in the liver, a classical target of adiponectin, and kidney, an emerging adiponectin target. Fructose caused quite noticeable decrease in the level of both adiponectin receptors (AdipoR1 and AdipoR2) in the liver of WT mice (Fig 6A-C). Lack of KHK abrogated this effect and, interestingly, in the case of AdipoR2 even reversed it (Fig. 6,A-C). Similar results were obtained for the kidney (not shown). These results suggest that fructose caused adiponectin resistance in mice due to downregulation of AdipoR1 and AdipoR2 in addition to reducing levels of HMW adiponectin. Adiponectin resistance is a condition associated with insulin resistance in obesity and type 2 diabetes (37).

To further test the signaling to the post-receptor targets of adiponectin and detect whether or not it is consistent with the adiponectin resistance, we utilized immunoblotting to detect changes in phosphorylation of a lipogenic enzyme Acetyl-CoA carboxylase (ACC) on Ser79, which is one of the major metabolic targets of adiponectin. Phosphorylation of ACC on Ser79 blocks enzymatic activity of ACC and prevents use of acetyl-CoA from lipogenesis by preventing formation of malonyl-CoA, an important intermediate of fatty acid synthesis (38; 39). On the other hand, ACC is overexpressed in response to fructose load (40). As expected, ACC expression was substantially upregulated in WT mice and remained unchanged in KHK-KO mice (Fig. Fig. 6, D,F) showing that up-regulation of lipogenic enzymes in the liver is KHK-dependent. On the other hand, ratio of P-ACC/ACC was significantly decreased in WT mice treated with fructose and remained unchanged in KHK-A/C-KO mice (Fig. 6, D, E) demonstrating the weak or absent adiponectin effect on ACC via Ser79 in WT mice. These data suggest that active KHK not only triggers lipogenesis in the liver but also, by acting via visceral adipose tissue and decrease in the circulating HMW adiponectin and downregulation of hepatic AdipoR1 and AdipoR2, it prevents an essential block of lipogenesis in the liver at the critical ACC step, creating conditions even more favorable for lipogenesis.
Interestingly, in the kidney fructose also caused quite dramatic KHK-dependent loss of phosphorylated form of ACC without changes in total ACC (Fig. 6, G-I). Insufficient blocking of ACC in the kidney, one of major sites of KHK-C expression (see: Introduction) could potentially create condition for ectopic lipogenesis in a KHK-dependent fashion, which was not yet addressed in experiments. However, fructose-induced KHK-dependent injury in the kidney (41) and in the KHK-expression proximal tubular cells (12) is well-documented.

**DISCUSSION**

It is well documented that more than moderate consumption of fructose-sweetened drinks and foods causes hepatosteatosis, visceral obesity and decreased sensitivity to insulin including epidemiological and clinical studies (1; 22; 42). The most recent mechanistic insight (10; 12) demonstrated that detrimental effect of fructose consumption is mostly a result of rapid fructose metabolism via C-isoform of KHK but not by alternative pathways such as KHK-A or hexokinase, a starting point of the glycolytic pathway, which can also metabolize fructose (4).

In the present study, we demonstrated that fructose can cause not only visceral fat accumulation but also macrophage infiltration and production of pro-inflammatory cytokines in the visceral adipose tissue, decrease in the release of HMW adiponectin, and activation of the XBP-1 branch of the ER stress, which is a common scenario for the development of obesity of any origin (17; 19; 29). Moreover, we also showed that all these effects are mediated by KHK-dependent metabolism of fructose in the liver, which is, as we demonstrated in the present study and previously (10), a highly specific feature of obesity and the metabolic syndrome induced by fructose consumption. KHK deficiency does not protects from the fructose-independent obesity such as high fat diet (43) Interestingly, KHK-C expression is upregulated in fructose-induced obesity (10), which amplifies the detrimental effects of fructose even more, and in obesity caused by Western diet (high fat/high sucrose) (43) with fructose present in the form of a dimer with glucose, but it does not respond to obesity caused by fructose-independent mechanisms, such as high fat diet (43). Our results also suggest that direct effects of fructose on the adipose tissue via either KHK-A, which is a low activity KHK isoform expressed in adipocytes (5), or the adipose-specific version of hexokinase, which actively metabolizes fructose (11), or via KHK-C, which is expressed at minuscule level in WAT (Table S1), are less important. Based on the widely accepted concept that low-grade inflammation in the adipose tissue in obesity is a major cause of
insulin resistance and cardio-vascular risk (29; 44; 45), we believe that fructose-induced KHK-dependent pro-inflammatory changes in the adipose tissue (Fig. 3-4) are, in turn, an important contributor to the insulin resistance in the case of fructose consumption (Fig.2).

The reason why KHK-C might be such a powerful trigger of pro-diabetic changes locally in the liver (10) and, as we showed in the present study, remotely is that fructose metabolism via KHK-C is largely unregulated (4), in contrast to tightly regulated metabolism of glucose. This might have two important consequences. First, powerful metabolic flow generated by KHK-dependent fructose phosphorylation joins glycolytic pathway at the aldolase step and can overload it creating an abundance of acetyl-CoA, a substrate for fatty acid synthesis, followed by acceleration of lipogenesis de novo, which ultimately leads to the NAFLD (9; 46). Secondly, in hepatocytes, fructose is rapidly metabolized by KHK in the reaction of phosphorylation to fructose-1-phosphate causing ATP depletion due to high unregulated activity of the enzyme (47; 48) to the extent that it can trigger the AMP formation and activation of AMP deaminase followed by purine degradation via xanthine oxidoreductase (XOR) yielding uric acid (7). Elevated levels of uric acid is associated with a number of pathological conditions including insulin resistance, obesity, cardiovascular and kidney (1; 2; 4; 23).

The mechanism by which KHK-C-dependent metabolism of fructose in the liver affects adipose tissue is undefined but it is likely to be associated with the transport of triglycerides from the liver to the visceral adipose tissue. It is known that majority of triglycerides produced in the liver in response to fructose consumption is transported by lipoproteins, such as VLDL and LDL, to the visceral and not to the subcutaneous adipose tissue (49), which explains substantial accumulation of fat in the visceral adipose tissue in response to fructose feeding. All components of VLDL including its essential apolipoprotein ApoB (49) are induced in response to fructose consumption.

Our results also demonstrate that changes in the visceral adipose tissue in response to KHK-dependent metabolism of fructose in the liver can, in turn, affect the liver via adiponectin resistance. We detected several signs of the adiponectin resistance in the WT animals treated with fructose including: i) tendency to increase in the blood levels of total adiponectin with simultaneous decrease of the biologically active HMW form, ii) decrease in the expression of adiponectin receptor AdipoR1 and AdipoR2 in the liver, iii) downregulation of at least one
downstream postreceptor target of adiponectin (phosphorylation of ACC, which triggers inhibition of the key step of lipogenesis(38; 39)). Each component of adiponectin resistance was KHK-dependent. The mechanism underlying downregulation of the AdipoR1 and AdipoR2 in the liver and in the kidney in our study remains to be identified. It is believed that insulin resistance might be one of these factors (32). It is possible that adiponectin resistance might cause additional fructose-induced lipogenesis in the liver via unblocking the enzymatic activity of ACC and, possibly, can create a vicious cycle and, thereby, contribute to the progression to NAFLD.

Our experiments on assessing the fructose effect of the ER stress signaling were focused on the XBP-1-dependent signaling, which is activated by the metabolic overload and triggers lipogenesis (26), innate immunity and inflammatory responses (50). Fructose load caused activation of XBP-1 by a specific splicing mechanism producing mRNA that encodes active protein (Fig. 4B), and this mechanism was dependent on the KHK-dependent metabolism of fructose in the KHK-C-expressing tissues. Therefore, we can speculate that proinflammatory changes in the adipose tissue in fructose fed group were mediated via ER stress signaling, at least via the XBP-1-dependent branch. Since the drop in the production of HMW adiponectin is mediated by imbalance in the ER chaperones ERp44 and Ero-1α, it is plausible that this imbalance was also triggered by ER stress signaling. However, this question remains to be investigated.

Thus, in the present study we demonstrated that consumption of elevated amounts of fructose not only causes visceral obesity but also induces inflammatory changes in the visceral adipose tissue including macrophage infiltration, production of the inflammatory adipokines and decrease in the levels of circulating high molecular form of adiponectin. At the level of adipose tissue these changes might be mediated by ER stress signaling but only KHK-C-dependent metabolism of fructose in the liver can trigger all these changes in the visceral adipose tissue. In turn, adverse processes in the visceral fat induced by fructose can cause adiponectin resistance and thereby affected liver via downregulation of the liver targets of adiponectin. This may represent an important mechanism of insulin resistance and progression of type 2 diabetes and cardiovascular disease in the environment of increasing consumption of fructose-containing foods in the modern society.
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Author Contributions: G.M., V.P., P.S., B.P., D.M., and S.C. researched data. T.I. researched data, reviewed manuscript, and contributed to discussion. Y.S. designed the study, researched data, wrote manuscript, and is a guarantor of this submission.
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### TABLE 1

Primers for gene expression analysis by qRT-PCR and PCR assay for XBP-1 splicing

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Figure Legends

Fig. 1. Caloric intake from the rodent diet and fructose-containing drinking water in WT and KHK-A/C-KO mice fed ad libitum. A. Average caloric intake per animal per day for rodent diet and fructose was calculated based on the results of food and water intake experiment, correspondingly. We measured food and water consumption two weeks before the end of experiment for 3-5 days. Values are mean ± SE. B. Fructose-induced changes in the energy consumption for each of three major macronutrients by WT and KHK-A/C-KO mice.

Fig. 2. Fructose induced body weight and insulin resistance are dependent on the fructose metabolism by KHK. A-B. KHK-dependent body weight increase in WT but not KHK-A/C-KO mice. C-D. To assess level of insulin sensitivity, the insulin tolerance test was used. Insulin (1 unit/kg) was injected, and blood level was measured at 0, 15, 30, 60, 90, and 120 min after injection. Sensitivity to insulin was normal in control WT mice but significantly reduced in fructose–treated WT mice, and little changed from WT control in KHK-A/C-KO mice fed only with rodent. Fructose treatment almost did not change insulin sensitivity in KHK-A/C-KO mice. Values are mean ±SD (N=6-8).

Fig. 3. The effect of fructose on the macrophage infiltration in WT and KHK-A/C-KO mice. Samples of the adipose tissue from WT, WT treated with fructose, and KHK-A/C-KO mice treated or not with fructose were stained with F4/80 antibody and counterstained with hematoxylin. Because diaminobenzidine was used as a chromogen, macrophages (F4/80-positive cells) are stained in brown as indicated with arrowheads (see also magnified rectangular region). The percentage of F4/80-positive macrophages within the adipose tissue is significantly induced by fructose in WT but not KHK-A/C-KO mice. Counting positive and negative cells were performed in a blind fashion at least in three fields per animal. The values are mean ± SEM (N = 5–6, performed in triplicate). **P < 0.01 (U test), correspondingly, for the effect of fructose.

Fig. 4. KHK-dependent expression of proinflammatory adipokines in the visceral adipose tissue and activation of XBP-1-dependent ER stress signaling pathways in mice. A. Fructose-induced expression in the visceral adipose tissue is mediated by KHK. WT mice responded to drinking fructose water for twelve weeks by increased expression of the MCP-1 and TNF-α, two major inflammatory adipokines. This effect is prevented in KHK-KO mice. B. Fructose induced KHK-dependent activation of the transcriptional activator XBP-1 (X-box-binding protein 1) via mRNA splicing to the mRNA that encode an active protein. mRNA encoding active XBP-1 protein is produced by the specific endoribonuclease that cleaves 26 nucleotides. The short XBP-1 mRNA can be distinguished by performing PCR of the fragment surrounding 26 bp-region. Images of PCR products are shown for three representative animals per group. Shorter fragment is detected in WT mice treated with fructose. Densitometry of the fragments is presented as a ratio of spliced/unspliced forms. The values are mean ± SEM (N=6,
performed in duplicate) *,**, - P<0.05, P<0.01 (U-Test), correspondingly, for the effect of fructose.

**Fig.5. KHK-dependent effect of fructose on the levels of circulating high molecular weight prolactin and mRNA expression of ER chaperones ERp44 and Ero-1α.** A. The mRNA expression for adiponectin in the visceral adipose tissue in WT and KHK-A/C mice treated or not with fructose. Differences between the levels of the adiponectin mRNA in WT and KHK-A/C-KO mice is not confirmed (p = 0.07). B-C. Levels of total (B) and relative HMW adiponectin (HMW/total) in the blood. Adiponectin levels in the blood were measured by ELISA assays for total and HMW adiponectin. D-E. The mRNA expression of ER chaperones Erp44 and Ero-1α in the visceral adipose tissue of WT and KHK-A/C-KO mice treated or not with fructose. Effect of fructose on the relative level of HMW as well as on the mRNA expression of the ER chaperone responsible for the assembly of HMW adiponectin was dependent on the KHK. The values are mean ± SEM (N = 6, performed in duplicate or triplicate). ** - P<0.01 (U-test) for the effect of fructose.

**Fig. 6. KHK-dependent fructose induced decrease in the protein expression for the adiponectin receptors AdipoR1 and AdipoR2 in the liver and post receptor signaling to Acetyl CoA-carboxylase, a key lipogenic enzyme.** A. Fructose-induced decrease in the expression of AdipoR1 and AdipoR2 in the liver is mediated by KHK. D. Fructose-specific KHK-dependent stimulation of the expression and the absence of adiponectin-induced phosphorylation of ACC in the liver. G. Fructose-induced decrease in the ACC phosphorylation in the kidney is reversed by the absence of KHK activity in the kidney. Image analysis of bands and normalization to a reference protein and/or the same protein detected with phosphorylation-unspecific antibody is shown in B-C, E-F, H-I. In addition to GAPDH, additional reference protein detection is provided in the Fig. S3. Blots representing two or three separate animals per group are shown. The values are mean ± SEM (N=6, performed in duplicate) *,**, - P<0.05, P<0.01 (U-Test), correspondingly, for the effect of fructose.
Diabetes
Supporting data

Fig. S1. Glucose induced body weight but not insulin resistance in WT and KHK-A/C-KO mice. A-B. KHK-dependent body weight increase in WT but not KHK-A/C-KO mice. C-D. To assess level of insulin sensitivity, the insulin tolerance test was used. Insulin (1 unit/kg) was injected, and blood level was measured at 0, 15, 30, 60, 90, and 120 min after injection. Sensitivity to insulin was normal in both WT and KHK-A/C-KO mice treated or not with Glucose. Vales are mean ±SD (N=6-8).
Fig. S2. Absence of the effect of fructose on the release of HMW adiponectin the medium and protein expression of ERp44 and Ero-1α chaperones in 3T3-L1 adipocytes. A. HMW adiponectin along with biologically inactive MMW (medium molecular weight) and LMW (low molecular weight) forms was detected by Western blotting after protein separation in non-reducing non-denaturing conditions as described (1). 3T3-L1 cells were differentiated to adipocytes in vitro. Samples were collected at early (Dif1) and late (Dif2) stages of differentiation. B. Protein expression of the ER chaperones ERp44 and Ero-1α was detected by Western blot after separation by regular electrophoretic SDS-PAGE separation in denaturing and reducing conditions.

Reference

Table S1.

**Relative expression (KHK/β-Actin) of KHK isoforms in mouse liver and fat**

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<th>KHK-A (Mean+/−SE)</th>
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<td>1</td>
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<tr>
<td>2</td>
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Calculated ratio C/A = 0.15

Specific expression of KHK, A and C isoforms was detected with the sets of primers recognizing A and C subunits of KHK (1)

**Reference**

Fig. S3. Detection of GAPDH and β-Actin in the liver of WT and KHK-A/C-KO mice treated or not with fructose. A validation of the Fig.6 reference gene.