ORIGINAL ARTICLE

Basic Study

Lipogenesis in Huh7 cells is promoted by increasing the fructose: Glucose molar ratio

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Author contributions: Windemuller F performed all the experiments and prepared the initial manuscript draft; Xu J and Rabinowitz SS assisted in study design and interpretation of data, as well as review of the final document; all experiments were performed in the laboratory of Hussain MM, under his guidance; Schwarz SM provided the initial study design, conducted all statistical analyses and wrote the final version of the manuscript.

Institutional review board statement: N/A.

Informed consent statement: N/A.

Conflict-of-interest statement: The authors have no personal, professional or financial relationships relevant to this report.

Data sharing statement: All technical and statistical documents, as well as all data sets, are available from the corresponding author at steven.schwarz@downstate.edu. No additional data are available.

Manuscript source: Invited manuscript

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Abstract

AIM: To determine whether hepatocyte lipogenesis, in an *in vitro* cell culture model, is modulated by adjusting culture media monosaccharide content and concentration.

METHODS: Hepatocytes (Huh7), demonstrating glucose and fructose uptake and lipid biosynthesis, were incubated in culture media containing either glucose alone (0.65-0.72 mmol/L) or isosmolar monosaccharide (0.72 mmol/L) comprising fructose:glucose (F:G) molar ratios ranging from 0.58-0.67. Following a 24-h incubation, cells were harvested and analyzed for total protein, triglyceride (TG) and cholesterol (C) content. Significant differences ($P < 0.05$) among groups were determined using analysis of variance followed by Dunnett's test for multiple comparisons.

RESULTS: After a 24 h incubation period, Huh7 cell mass and viability among all experimental groups were not different. Hepatocytes cultured with increasing concentrations of glucose alone did not demonstrate a significant change either in C or in TG content. However, when the culture media contained increasing F:G molar ratios, at a constant total monosaccharide

concentration, synthesis both of C and of TG increased significantly [F:G ratio = 0.58, C/protein (μg/μg) = 0.13; F:G = 0.67, C/protein = 0.18, $P < 0.01$; F:G ratio = 0.58, TG/protein (μg/μg) = 0.06; F:G ratio = 0.67, TG/protein = 0.11, $P < 0.01$].

CONCLUSION: In an *in vitro* hepatocyte model, glucose or fructose plus glucose support total cell mass and lipogenic activity. Increasing the fructose:glucose molar ratio (but not glucose alone) enhances triglyceride and cholesterol synthesis. These investigations demonstrate fructose promotes hepatocellular lipogenesis, and they provide evidence supporting future, *in vivo* studies of fructose's role in the development of hepatic steatosis and non-alcoholic fatty liver disease.

Key words: Hepatocytes; Cholesterol; Triglycerides; Fructose; Glucose

Core tip: Employing an *in vitro* hepatocyte culture model, these data demonstrate fructose promotes intracellular synthesis both of cholesterol and of triglyceride. The results support the requirement for future, *in vivo* investigations to determine whether diets high in fructose are risk factors for hepatic steatosis and development of non-alcoholic fatty liver disease.

INTRODUCTION

Fructose, a five-carbon monosaccharide, comprises an increasing component of the Western diet, particularly in the form of high fructose corn syrup (HFCS). In the United States, this sweetener is both readily available from domestically grown corn and inexpensive, when compared to imported, granulated sugar. It was introduced in the 1960's, with subsequent expansion into a vast array of foods and beverages. HFCS is made from corn starch, processed by glucose isomerase to convert a portion of its glucose fraction into fructose. HFCS preparations contain approximately 25% water, fructose (up to 55% of the water-free fraction), glucose and 0%-5% unprocessed glucose oligomers. HFCS's use as a commercial sweetener has doubled in the last decade and, as a consequence, fructose intake in developed countries has increased five-fold[1].

In several human studies to date, increased fructose intake has been linked both to abnormal plasma lipid profiles and to the development of non-alcoholic fatty liver disease (NAFLD)[2-4]. However, because the effects of fructose, *per se*, are difficult to distinguish from the influences of other dietary carbohydrates and fats, current clinical evidence does not establish the precise contribution of fructose-containing food and beverage products to the etiology and/or the exacerbation of specific biochemical, clinical and histopathologic abnormalities. Further, since the causes of dietary carbohydrate and lipid-related problems (*e.g.,* hyperlipidemia, type Ⅱ diabetes, NAFLD, metabolic syndrome) are multifactorial and also related to age, gender and lifestyle, additional investigations are required to determine the relative contributions of dietary and other co-factors.

In attempting to identify the importance of fructose in hepatocellular lipid metabolism, available *in vitro* studies indicate fructose may preferentially promote *de novo* lipogenesis[5]. Since fructose bypasses the glycolytic rate-limiting enzyme phosphofructokinase, it is metabolized efficiently and provides a readily available substrate for hepatic lipid synthesis[6]. In addition, fructose has been shown to inhibit peroxisome proliferator-activated receptor (PPAR)-alpha mediated hepatocellular fatty acid beta-oxidation and lipid clearance[7].

The potential lipogenic role of fructose also may be related to its relationship to endogenous insulin activity. Insulin insensitivity has been implicated in the pathogenesis and progression of NAFLD[8]. Impaired insulin responsiveness to circulating carbohydrate is associated both with increased adipocyte lipolysis and with increased levels of circulating free fatty acids (FFA). Thus, insulin resistance promotes lipolysis, particularly in intra-abdominal, white adipose tissue. This phenomenon occurs as a consequence of dysregulation of lipid regulatory transcription factors (*e.g.,* PPAR-gamma), instability of adipocyte lipid and impaired lipogenesis. Released FFA lead to upregulation of inflammatory cytokines and chemokines[8,9]. Subsequently, liberated adipocyte FFA are taken-up by the liver and re-esterified to triglyceride. As fructose does not promote insulin secretion, it may therefore exacerbate the effects of insulin resistance on hepatic lipid synthesis, steatosis and the development of NAFLD[10]. Further evidence of fructose' role in the development of NAFLD derives from a recently reported rat model of hepatic steatosis. A high fructose-containing diet promoted not only lipogenesis leading to steatosis, but also increased expression of lipocalin-2, a ubiquitous glycoprotein involved in the response to inflammation and oxidative stress[11].

Despite evidence linking fructose to the development of altered lipid dynamics, hepatic steatosis and NAFLD, a recent meta-analysis concluded that studies examining the hepatocellular effects of fructose were confounded by the co-stimulation of lipogenesis resulting from increased total energy intake[12]. This observation suggests the deleterious effects of HFCS are merely a reflection of overall calorie excess in the Western diet.

In light of the above clinical and experimental data, the present study seeks to establish the influence of fructose on hepatocyte lipogenesis and provide a basis for future, translational investigations of fructose-

Table 1 Hepatocellular protein content			
Glucose (mmol/L)	0.65	0.68	0.72
Protein (μg/mL)	635.0 ± 52.7	608.6 ± 35.3	543.2 ± 126.6
Fructose:glucose (mmol/L: mmol/L)	0.58:1[1]	0.61:1[1]	0.67:1[1]
Protein (μg/mL)	626.6 ± 95.2	610.2 ± 30.6	521.9 ± 38.7

[1]Total monosaccharide concentration = 0.72 mmol/L, Huh7 cell homogenates, means ± SD after 24 h of incubation in culture media containing only glucose or glucose plus fructose as the nutrient monosaccharide(s). All experiments comprise samples at a fixed volume of 10 mL/plate.

mediated lipid biosynthesis. These experiments employ an established, immortal and metabolically active human hepatocellular carcinoma cell line, Huh7, used extensively in studies of hepatocyte metabolism[13-15]. Since facilitated uptake of glucose and fructose by the transmembrane GLUT2 transporter is demonstrated in Huh7 cells[16], these cells provide an excellent model for studies of carbohydrate-induced lipogenesis. Accordingly, the studies herein were carried out to determine whether hepatocyte lipogenesis, in an *in vitro* cell culture model, is modulated by adjusting culture media monosaccharide content and concentration.

MATERIALS AND METHODS

Cell culture
Huh7 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine, in a 37 ℃, 5% CO_2 cell culture incubator on 100 mm × 200 mm tissue culture dishes (BD Falcon Durham, NC). Once 80% confluence was achieved (cell count - 1 × 10^5), cells were incubated for 24 h in culture media (DMEM) with 10% FBS containing either glucose alone (0.65, 0.68 and 0.72 mmol/L) or isosmolar monosaccharide (0.72 mmol/L) comprising fructose:glucose (F:G) molar ratios of 0.58, 0.61, and 0.67. For all experiments, total media osmolality was 400 mOsm/L. All incubations were performed in triplicate. Following the 24 h incubation, as described above, culture media was removed, plates were washed with phosphate buffered saline, and cells were lysed and collected in 750 microliters isopropanol, as previously described[17]. The cell lysates were kept at 4 ℃ for 12 h. Each sample was then centrifuged at 4 ℃ for 10 min at 10000 rpm. Supernatants were removed for lipid studies and the remaining cellular precipitate was re-suspended in 0.1N NaOH for protein quantification, employing a previously validated method[17].

Protein and lipid analyses
Cholesterol and triglyceride were measured utilizing samples from the isopropanol supernatant[18]. The samples were placed in a 96 well plate for lipid quantification, using established spectrophotometric methods[19,20]. Spectrophotometric absorbancies of each cell lysate sample were compared to known lipid standards, and the concentrations of cholesterol and triglyceride

were calculated from the derived measurements[19,20]. Protein was quantified in triplicate utilizing samples from the NaOH suspension, employing standard spectrophotometric methods[21].

Cell mass determination
For these cell culture studies, estimates of cell mass in all experimental groups were made using total protein measurements in Huh7 lysates[22]. All single cell-line incubations were performed concurrently, under the same environmental conditions, thereby synchronizing cell cycles among experimental groups. Accordingly, as previously described, calculation of total cell protein content was employed to estimate relative cell biomass among incubations[23].

Statistical analysis
Differences among all experimental groups were assessed by analysis of variance, followed by Dunnett's test for multiple comparisons. A *P*-value of < 0.05 was considered significant.

RESULTS

Cell mass
Cultured Huh7 cells incubated in glucose-only supplemented media (0.65, 0.68, and 0.72 mmol/L) and in media containing varying F:G molar ratios (total monosaccharide concentration 0.72 mmol/L), did not show any statistically significant differences in protein content among all study groups. These data indicate total cell mass was not affected by varying the monosaccharide concentration or distribution (Table 1).

Glucose-mediated lipogenesis
As shown in Figures 1 and 2, triglyceride and cholesterol content (μg/mg cell protein) did not differ significantly among Huh7 cells incubated for 24 h in media containing 0.65, 0.68 or 0.72 mmol/L glucose per plate. Further increases (> 0.72 mmol/L) in glucose molar concentration did not result in any additional enhancement in cellular lipid content (data not shown).

Fructose-mediated lipogenesis
For these experiments, all cells were incubated in media containing 0.72 mmol/L monosaccharide (total media osmolality = 400 mOsm/L), the maximum sugar concentration employed in the "glucose-only" experiments, described above. To determine the effects of fructose on lipogenesis, cells were incubated in the presence of increasing molar ratios of F:G (0.58, 0.61 and 0.67). Following a 24 h incubation, Huh7 cell cholesterol content (μg/μg cell protein) increased significantly at a F:G molar ratio of 0.67:1 (Figure 3), compared both to a 0.61:1 ratio (0.18 μg/μg *vs* 0.14 μg/μg protein, *P* < 0.05) and to a 0.58:1 ratio (0.18 μg/μg *vs* 0.13 μg/μg protein, *P* < 0.01). Triglyceride analyses (Figure 4) demonstrated fructose-mediated Huh7 triglyceride synthesis also increased significantly in stepwise fashion. Thus, increased triglyceride content was
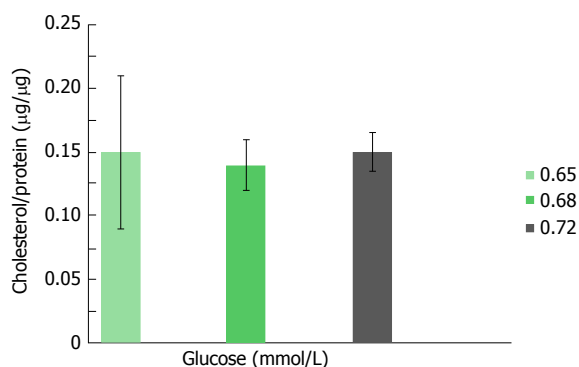
Figure 1 Cholesterol content of Huh7 cells (μg/μg cellular protein) following a 24-h incubation, in culture media containing 0.65, 0.68 and 0.72 mmol/L glucose.

Figure 2 Triglyceride content of Huh7 cells (μg/μg cellular protein) following a 24-h incubation, in culture media containing 0.65, 0.68 and 0.72 mmol/L glucose.
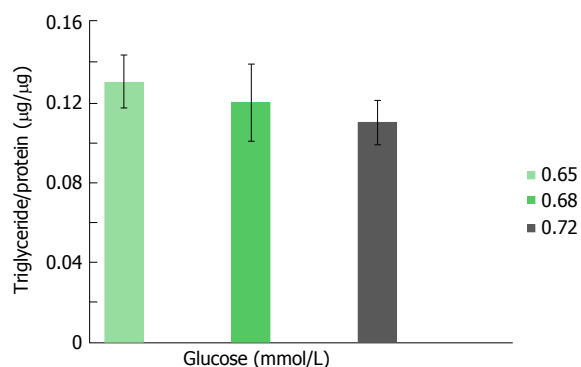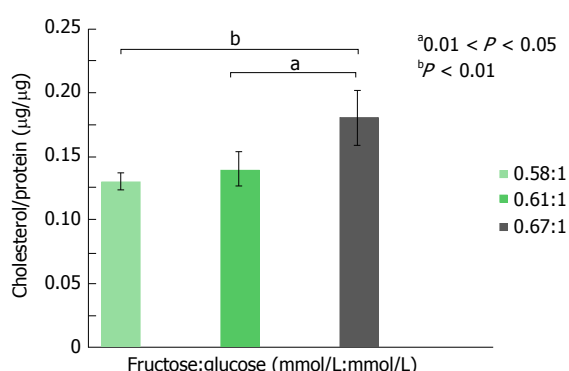

Figure 3 Cholesterol content of Huh7 cells (μg/μg cellular protein) following a 24-h incubation, in culture media containing fructose and glucose, at fructose:glucose molar ratios of 0.58:1, 0.61:1 and 0.67:1. All incubations were carried out in media containing 0.72 mmol/L total monosaccharide.

Figure 4 Triglyceride content of Huh7 cells (μg/μg cellular protein) following a 24-h incubation in culture media containing fructose and glucose, at fructose:glucose molar ratios of 0.58:1, 0.61:1 and 0.67:1. All incubations were carried out in media containing 0.72 mmol/L total monosaccharide.
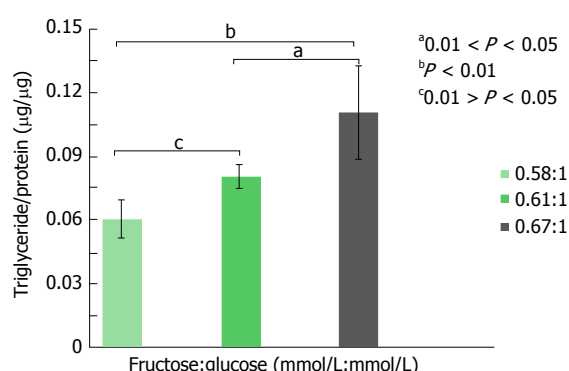
noted at a F:G ratio of 0.61:1 (compared to the ratio of 0.58:1, 0.08 μg/μg *vs* 0.06 μg/μg protein, $P < 0.05$) and at a ratio of 0.67:1 (compared to the ratio of 0.61:1, 0.11 μg/μg *vs* 0.08 μg/μg protein, $P < 0.05$; to 0.67:1, 0.11 μg/μg *vs* 0.06 μg/μg protein, $P < 0.01$).

DISCUSSION

The results presented in this report demonstrate Huh7 cells, an immortal hepatocellular carcinoma cell line, grown in standard culture media containing increasing glucose concentrations (up to 0.72 mmol/L), exhibit no differences in relative cellular TG and C, as a consequence of increased media glucose content. However, when the nutrient monosaccharides comprise fructose plus glucose (at increasing F:G molar ratios), significant promotion of lipogenesis is demonstrated by increased hepatocellular TG and C concentrations. For these studies, the cell culture media monosaccharide content of 0.72 mmol/L (glucose alone or glucose plus fructose) was found to maximize hepatocellular lipogenesis. This molar amount was determined following a series of experiments, employing a step-wise increase in sugar content and based on previous human studies showing serum total monosaccharide concentrations of approximately 0.50 mmol/L following a fructose-rich

meal[24]. Higher amounts of monosaccharide (> 0.72 mmol/L) in the Huh7 incubating media did not yield statistically significant increases either in cellular TG or in cellular C content, while further increases (> 400 mmol/L) in media osmolality resulted in decreased cell viability.

These results are consistent with a prior clinical report, suggesting hepatic fat (estimated by magnetic resonance imaging) in subjects fed fructose, in addition to a specific weight maintenance diet, was increased compared to a study group fed the same diet supplemented with glucose alone[4]. Conversely, another report failed to demonstrate any promotion of lipogenesis following four weeks of a fructose supplemented diet. As stated previously, a recent meta-analysis concluded the potential association between fructose and NAFLD was confounded by the concurrent consumption of hypercaloric diets[12]. The influence of fructose on lipogenesis, as a consequence of excess energy intake, may be mediated by this monosaccharide's direct attenuation of post-prandial ghrelin suppression[24]. On the other hand, another study examining the effects of dietary sucrose *vs* HFCS on endogenous hormone levels, failed to demonstrate any significant differences in serum insulin, leptin and ghrelin levels[25]. These combined *in vivo* studies therefore suggest the amount of carbohydrate taken up by hepatocytes is increased (thus leading

to enhanced lipogenesis) as a consequence of total calorie intake, rather than resulting directly from the lipogenic effects of any specific dietary substrate.

In contrast to available clinical data examining fructose mediated steatosis (often derived from imprecise imaging techniques), considerable biochemical evidence supports the role of fructose in promoting *de novo* lipogenesis. Since fructose is more efficiently utilized intracellularly, as compared with glucose, it may provide a more readily available substrate for lipid synthesis[26]. Interestingly, one recent study failed to demonstrate any effects of fructose on regulating hepatocellular lipogenic genes, thus providing further, indirect evidence linking the lipogenic role of this monosaccharide to its ability to provide increased amounts of carbon fragments for lipid synthesis[27]. The present results extend this observation by demonstrating that lipogenesis, in cultured hepatocytes, is not solely related to the provision of carbon precursors, since all incubations contained similar total monosaccharide concentrations. Thus, fructose, in this experimental model, appears to exert a direct effect on promotion of lipogenesis, and this effect is independent of carbon supply.

Because fructose metabolism is insulin-independent and does not stimulate pancreatic insulin secretion[28], this monosaccharide was previously thought to be a superior dietary substrate, as compared with other sugars[29]. However, more recent experimental data, while confirming these metabolic characteristics, also demonstrate fructose promotion of insulin resistance[12]. Fructose stimulates Jun-N-terminal kinase-1 (JNK-1), an intracellular mitogen activated protein kinase. JNK-1 in turn phosphorylates insulin receptor substrate-1 resulting in suppression of cellular glucose uptake, increased blood glucose levels and increased insulin secretion[30-32]. These findings, therefore, suggest another potential pathway for fructose-mediated steatosis.

Despite results presented in this report and published previously, the precise mechanisms by which fructose increases hepatic TG are not completely understood. Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein 1c (SREBP1c) are two transcription regulators of hepatic lipogenesis induced by glucose and insulin, respectively. Animal knockdown studies of peroxisome proliferator-activated receptor gamma co-activator-1 beta, a transcriptional co-activator of SREBP1c, have demonstrated improved hepatic lipid profiles in fructose fed rats. In a separate study[7], fructose fed rats showed increased expression of ChREBP hepatic mRNA, compared with a glucose fed group. Because recent data shows fetal bovine serum (FBS) contains factors that promote cellular lipogenesis, independent of insulin[33], and because FBS concentrations were held constant in our studies, stimulation of either SREBP1c or ChREBP represents an unlikely contributor to the observation of fructose-mediated *de novo* lipogenesis.

Our preliminary study certainly has several important limitations. These observations, in an *in vitro* cell culture model, cannot accurately predict the metabolic fate of fructose, in terms of its ability to enter lipogenic pathways. While the data clearly suggest direct effects of fructose on hepatocellular synthesis of triglyceride and cholesterol, they do not provide further elucidation of the underlying mechanisms leading to these findings. Nevertheless, the observation that fructose exerts a promoting influence on lipid synthesis, confirms prior studies suggesting the significant role of this monosaccharide in the development and/or exacerbation of hepatic steatosis. These data, therefore, warrant further investigations into the mechanisms and extent of fructose-mediated, *de novo* hepatic lipogenesis.

COMMENTS

Background

Available studies, both *in vitro* and in clinical trials, indicate the dietary monosaccharide, fructose, may be an important substrate for hepatic lipid synthesis, and may promote the development of hepatic steatosis. However, whether fructose-associated lipogenesis is related to dietary intake of fructose, *per se*, or merely reflects excess total energy consumption, remains unclear. The present study seeks to establish the effects of fructose on hepatocyte lipogenesis and provide a basis for future, translational investigations of fructose-mediated lipid biosynthesis. These experiments employ an established, immortal and metabolically active human hepatocellular carcinoma cell line, Huh7, used extensively in studies of hepatocyte metabolism. The studies herein were carried out to determine whether hepatocyte lipogenesis, in an *in vitro* cell culture model, is modulated by adjusting culture media monosaccharide content and concentration.

Innovations and breakthroughs

The results of these experiments clearly demonstrate, in a stable, *in vitro* hepatocyte culture model, at constant monosaccharide concentrations (glucose ± fructose), by increasing the culture medium fructose to glucose molar ratio, but not by increasing glucose alone, significant enhancement of lipogenesis.

Applications

The observation that fructose exerts a promoting influence on lipid synthesis, confirms prior studies suggesting the significant role of this monosaccharide in the development and/or exacerbation of hepatic steatosis. These studies, therefore, support the need for further investigations into the mechanisms and extent of fructose-mediated, *de novo* hepatic lipogenesis. Most important, these results provide a basis for future, clinical studies of fructose's role in development of hepatic steatosis and non-alcoholic liver disease.

Terminology

Huh7 cells, an immortal, stable hepatocyte line, derived from human hepatocellular carcinoma. These cells take-up both glucose and fructose, and have been used extensively in studies of hepatic metabolism.

Peer-review

This is an interesting study that reveals fructose is linked to lipogenesis in a concentration dependent way.

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