Clinical and Experimental Health Sciences

SREBP-1c Deficiency Attenuates Fructose-Induced Lipid Droplet Accumulation

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ABSTRACT

Objective: Sterol regulatory element binding protein 1c (SREBP-1c), a transcription factor involved in the biosynthesis of fatty acids, is critical in metabolic dysfunction-associated fatty liver disease (MAFLD) by promoting lipid accumulation and metabolic dysregulation that leads to hepatic pathologies. Fructose, becoming increasingly common in diets, activates SREBP-1c by increasing acetyl-CoA production. Present study aimed to sought the effect of SREBP-1c in fructose induced lipid accumulation.

Methods: A fructose-induced lipid accumulation model was developed in mouse hepatocyte cells (AML12), where SREBP-1c expression was inhibited through siRNA transfection. Following different fructose concentrations, viability was determined by MTT assay, and the protein expression of SREBP-1c protein was determined by western blotting. The number of lipid droplets (LDs) was quantified microscopically, and lipogenic mRNA expressions of FASN, SCD1, GPAM, ACLY, ACSL1 and ACACA were detected by qRT-PCR.

Results: Western blotting and microscopic analysis indicated that 25 mM for 72 hours of fructose increased total LDs, together with SREBP-1c levels, without affecting cell viability. The mRNA expression of SREBP-1c decreased in the presence of siRNA, confirming siRNA efficacy. SREBP-1c silencing reduced the number of fructose-induced total LDs. As lipogenic mRNA expressions, SREBP-1c silencing reduced SCD1 and ACLY, while other genes were unaffected.

Conclusion: Silencing of SREBP-1c in hepatocytes demonstrated its beneficial effect by reducing fructose-induced LD accumulation.

Keywords: Sterol regulatory element binding transcription factor 1c, fructose, lipid accumulation

1. INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD) is defined as lipid accumulation in hepatocytes of more than 5% of liver weight without a secondary cause such as alcohol or drugs (1). The progression of the disease varies considerably depending on the extent of damage to hepatocyte cells, the presence of inflammatory processes, and fibrosis development (2). Therefore, mechanisms leading to lipid accumulation in the liver are of crucial importance in steatosis development. Along with an irregular diet and sedentary lifestyle, excessive fructose consumption also promotes the development of MAFLD (3). This is due to the fact that fructose not only serves as a substrate but also induces lipogenesis in hepatic *de novo* lipogenesis (DNL). The glucose transporter 2 (GLUT-2), a transmembrane transporter protein that facilitates the transport of fructose to hepatocytes, is not regulated by insulin or another hormone. Furthermore, the direct incorporation of fructose into the glycolytic pathway, bypassing the regulatory phosphofructokinase step of glycolysis, results in an excess of glycolysis products and increased flux of fructose carbons to lipogenic precursors (3, 4). As a consequence of these effects, there may be a reduction in insulin sensitivity in tissues other than adipose tissue, potentially leading to lipotoxicity in these cells (5).

The main family of transcription factors that enable the activation of DNL are sterol response element binding proteins (SREBPs) (6). One of the three isoforms of the family, SREBP-1c, modulates the expression of genes associated with the biosynthesis of fatty acids, and is tightly controlled by insulin and glucose at both transcriptional and post-translational stages, especially in liver and adipose tissues (7). Studies demonstrated a direct interaction between SREBP-1c and MAFLD (8). In chronic fructose consumption, increased fructose metabolism and acetyl-coA production

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led to SREBP-1c activation, and thus, fructose behaves as a DNL inducer. Therefore, fructose is recognized as the most potent lipogenic carbohydrate inducing hepatic steatosis (9). Similarly, high fructose consumption is observed to be associated with an increase in liver triacylglycerols (TAG) levels. This is achieved by increasing DNL through the induction of expression of proteins, such as fatty acid synthase (FASN) (10) and acetyl-CoA carboxylase (ACC) (10, 11), and transcription factors, including SREBP-1c (11, 12).

Nowadays, the production and easy availability of fructose-containing sugar syrups have revealed that excessive consumption of fructose stimulates SREBP-1c expression and leads to fatty liver diseases through hepatic lipid accumulation (8). However, understanding fructose metabolism and its relationship with SREBP-1c may facilitate the exploration of therapeutic approaches aimed to mitigate lipid accumulation. In this context, we have investigated the influences of fructose-induced lipid metabolism in the connection of SREBP-1c using hepatocyte cells.

2. METHODS

2.1. Cell Culture and Treatments

Mouse hepatocyte cells (AML12, ATCC CRL 2254) were cultured in Dulbeco's Modified Eagle's Medium (DMEM) (Merck KGaA, Darmstadt, Germany) containing 10% FBS (Gibco) (Thermo Fisher Scientific, Massachusetts, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco) (Thermo Fisher Scientific, Massachusetts, USA) at a constant-temperature incubator (37°C and 5 % CO_2). Intracellular lipid accumulation was established by exposing the cells to fructose (Merck KGaA, Darmstadt, Germany, catalog No. 104007) as indicated in figure legends. All experimental procedures were approved by the Marmara University, School of Medicine Ethics Committee (protocol number 09.2019.188).

2.2. siRNA Mediated Gene Silencing

Cells were transfected with SREBP-1c targeting siRNA, as previously reported by our group (13). Briefly, AML12 cells were seeded at 80% confluency in 6-well plates, and transfection was conducted in accordance with the instructions provided by the manufacturer for the Lipofectamine RNAiMax Reagent (Thermo Fisher Scientific, Massachusetts, USA, catalog No. 13778075). Eighty pmol siRNA (Thermo Fisher Scientific, siRNA ID: 151861) was prepared at the ratio of 1:3 with Lipofectamine RNAiMax Reagent in serum-free culture medium (OptiMEM) (Thermo Fisher Scientific, Massachusetts, USA). Experiments were conducted after incubation for 24 h with siRNA.

2.3. BODIPY Staining

Bodipy staining was used to determine characteristic (size and number) differences in lipid droplets (LDs). AML12 cells were seeded at 25×10^3 density in 48-well plates, and treated with fructose and SREBP-1c targeting siRNA. Following the administrations, cells were rinsed with PBS and subsequently fixed with 4% formaldehyde. After washing with PBS, cells were incubated with BODIPY 493/503 (Thermo Fisher Scientific, Massachusetts, USA, catalog No. D3922) at 0.25 µg/ml for 20 min, then exposed to the nuclear stain DAPI. The images were acquired using a Zeiss LSM700 confocal microscope (Amsterdam, Netherlands). LDs were counted using ImageJ software. Data were obtained from at least 30 cells of each group.

2.4. Gene Expression Analysis

AML12 cells were seeded at 5×10^4 density in 6-well plates, and after the administrations, RNA isolation was performed with Pure Link RNA Mini Kit (Thermo Fischer). High-Capacity cDNA Reverse Transcription kit (Thermo Fischer) was used to synthesize cDNA according to the protocol recommended by the manufacturer. The amplification of the synthesized cDNA samples was performed using Power UP SYBR Green Master Mix kit (Thermo Fischer) and Rotor Gene Q-RT PCR system (Qiagen). After determining the threshold cycle (CT), relative gene expression was calculated as follows: fold change = $2^{-\Delta(\Delta CT)}$, where ΔCT = CT-CT target housekeeping (GAPDH) and Δ(ΔCT) = ΔCT-CT treated control. The primary sequences are presented in Supplementary Table 1.

2.5. Immunoblot Analysis

AML12 cells were seeded at 25×10^3 density in 48-well plates, and after the administrations, lysed with RIPA buffer (Cell Signalling Technology, Massachusetts, USA, catalog No. 9806), which allows for the isolation of proteins. Protein concentration was quantified using BCA assay (Thermo Fisher Scientific, Massachusetts, USA, catalog No. 23225), which was conducted in accordance with the manufacturer's instructions. Thirty µg of protein was subjected to SDS-PAGE, and subsequently, separated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA in TBST. Then, the membrane was incubated with primary antibodies against SREBP1c (Novus Biologicals, catalog No. NB100-2215, 1:500 dil) and GAPDH (Novus Biologicals, catalog No. NB300-221, 1:2000 dil) overnight. After 1 hour of incubation with HRP-conjugated secondary antibody, blots were visualized using a chemiluminescence kit (Thermo Fisher Scientific, Massachusetts, USA, catalog No. 34580). The intensity of the bands was measured using Image J software and normalized to GAPDH.

2.6. Statistical Analysis

The statistical analysis was conducted using the Prism 4 software package (GraphPad). To ascertain the statistical significance of observed differences, a one-way ANOVA was conducted, followed by multiple comparisons using the Student-Newman-Keuls test. A p-value of less than .05 is considered statistically significant.

3. RESULTS

3.1. Twenty-Five mM of Fructose Induces Lipid Accumulation and SREBP-1c Expression in Hepatocytes

Our study was performed in the AML12 mouse hepatocyte cell line, which is generally preferred in studies on the establishment of the fatty liver model (14, 15). Enhanced LD accumulation in hepatocytes is a characteristic feature of hepatic steatosis. Accordingly, the objective of our study was to initially observe the impact of fructose at different concentrations (0.5 mM, 5 mM, 25 mM) for 72 hours. We first checked the effect of fructose on cell viability, and all concentrations had no significant effect as expected (Figure 1A). To determine *in vitro* steatosis establishment, the accumulation of LDs was detected using BODIPY and confocal microscopy. As shown in Figure 1B, fructose at 0.5 and 5 mM showed no significant difference, whereas 25 mM of fructose increased the number of LDs. To further examine the involvement of SREBP-1c in fructose-induced lipid accumulation, we checked its protein expression by western blotting, and observed a significant increase in the 5 mM and 25 mM groups compared to control (Figure 1C). In this context, fructose administration at 25 mM for 72 hours was preferred to induce lipid accumulation for the remainder of the study.

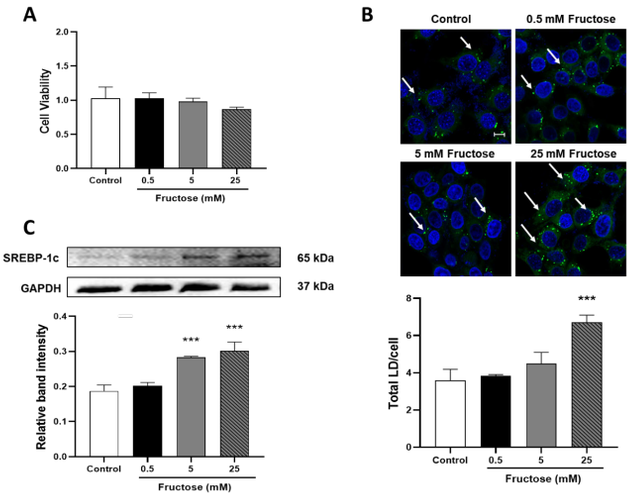

Figure 1. *Twenty-five mM of fructose induces lipid accumulation and SREBP-1c expression in hepatocytes*

*AML12 cells were treated with different concentrations of fructose (0.5 – 5 – 25 mM) for 72 hours. Cell viability was analyzed by MTT assay (A). Lipid droplets are labelled with BODIPY (green) and cell nuclei with DAPI (blue), followed by analysis of the number of LDs per cell. Representative images shows LD-positive areas in hepatocytes (arrowhead). Scale bar = 10 µm (B). SREBP-1c protein expressions in cells were measured by western blotting. Relative ratios were quantified and normalized to GAPDH (C). Data are expressed as mean ± S.D. One-way ANOVA, Student-Newman-Keuls test, (n=3). ***p < .001 vs. control*

3.2. SREBP-1c Mediates Fructose-Induced Lipid Accumulation

To identify the role of SREBP-1c in fructose-induced lipid accumulation, SREBP-1c was silenced by siRNA transfection in accordance with our previous studies (13). As expected, siRNA transfection significantly inhibited SREBP-1c expression at the transcriptional level in both fructose-treated and non-treated conditions (Figure 2A). However, in accordance with Figure 1B, fructose administration in normal conditions increased the number of LDs, which was prevented by siRNA transfection (Figure 2B). Present findings so far reveal the crucial role of SREBP-1c on fructose-induced LD accumulation in hepatocytes.

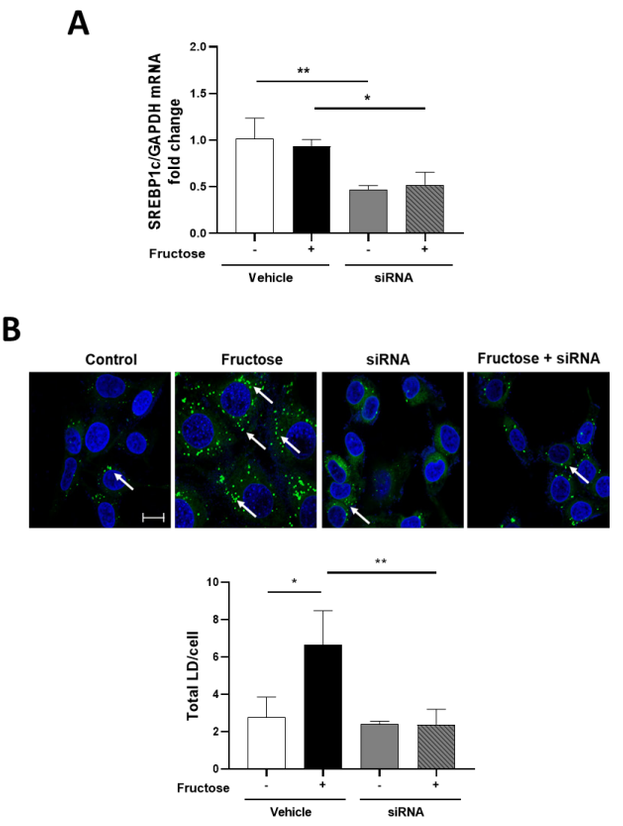

Figure 2. *SREBP-1c mediates fructose-induced lipid accumulation AML12 cells were treated with 25 mM fructose for 72 hours followed by SREBP-1c siRNA transfection. mRNA expression of SREBP-1c was determined by qRT-PCR and normalized to GAPDH (A). Lipid droplets were labelled with BODIPY (green) and cell nuclei with DAPI (blue), followed by analysis of the number LDs per cell. Representative images shows LD-positive areas in hepatocytes (arrowhead). Scale bar = 10 µm (B). Data are expressed as mean ± S.D. One-way ANOVA, Student-Newman-Keuls test, (n=3). *p< .05 and **p < .01*

3.3. SREBP-1c Dependent Lipid Accumulation in Fructose-Induced Hepatocytes is Lipid Metabolism Independent

To further evaluate the alterations in lipid metabolism, we examined the mRNA expression of well-known parameters of lipolysis and lipogenesis, including FASN, SCD-1, GPAM, ACLY, ACSL1, and ACACA. As shown in Figure 3, neither siRNA transfection nor fructose administration had a

significant effect on FASN, GPAM, ACSL1, and ACACA expressions. However, siRNA transfection under normal conditions exhibited a significant decrease in SCD1 and ACLY expressions, while fructose administration in SREBP-1c silenced hepatocytes did not alter any of the parameters (Figure 3).

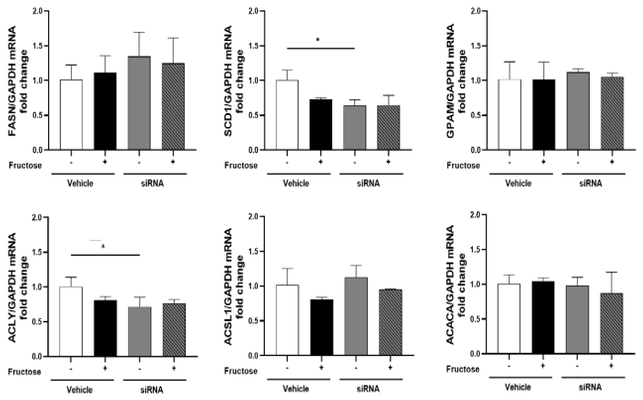

Figure 3. *SREBP-1c dependent lipid accumulation in fructose-induced hepatocytes is lipid metabolism independent*
*AML12 cells were treated with 25 mM fructose for 72 hours followed by SREBP-1c siRNA transfection. mRNA expressions of FASN, SCD1, GPAM, ACLY, ACSL1 and ACACA were determined by qRT-PCR and normalized to GAPDH. Data are expressed as mean ± S.D. One-way ANOVA, Student-Newman-Keuls test, (n=3). *p< .05*

4. DISCUSSION

The most fundamental pathological characteristic of MAFLD, the most prevalent metabolic disorder affecting about 25% of the worldwide population, is the accumulation of lipids in hepatocytes (16). Although MAFLD becomes incurable in its later stages, it is possible to control or reverse hepatosteatosis that develops due to unhealthy diets in the early stages (17). In particular, acute fructose metabolism causes the rapid formation of substrates required for DNL, leading to MAFLD. The consumption of fructose, a highly lipogenic sugar, has recently increased markedly. There is a developing body of findings associating its intake with components of metabolic syndrome, such as insulin resistance, abdominal obesity, dyslipidemia, and hypertension (18). Furthermore, fructose stimulates hepatic DNL more strongly compared to glucose (19-21), starch (22-24) or high-fat diets (25-27). In the short term (4-6 h), only a small percentage of the carbon skeleton of fructose is transformed into lipid in a span of 4-6 h (28). Furthermore, the long-term DNL-promoting impact of fructose was confirmed by labelled acetate infusion studies (19, 20, 29). Thereby, along with the increased level of DNL substrate with chronic fructose exposure, fructose also has an effect on prolipogenic mechanisms (30). Natural fructose from plant sources is relatively beneficial compared to fructose from industrial sources such as sucrose and high-fructose corn syrup. Its slower absorption and components, such as antioxidants and plant fiber, provide metabolic benefits. In contrast, industrial fructose, especially in liquid form, shows rapid absorption and may cause hepatic insulin resistance and MASLD (31).

In this study, we sought to investigate the effect of fructose on lipid metabolism and evaluate potential approaches for steatosis treatment. To establish an *in vitro* steatosis model, we used theAML12 cell line due to its stable phenotypes and replicative capacity and established fructose-induced lipid accumulation. There are various *in vitro* studies involving different concentrations in the fructose-induced steatosis model. For instance, studies in HepG2 cells and primary human hepatocytes have shown enhanced lipid accumulation by 8 mM (32) and 22.2 mM (33) fructose, respectively. Other studies in hepatocytes used 25 mM (34), 88.8 mM (35) and 100 mM (36) fructose for lipid accumulation. These studies have shown that in addition to the lipid accumulation-inducing effect of fructose, it also increases the production of inflammatory cytokines interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α), and the expression of Ubiquitin carboxyl-terminal hydrolase 2 (USP2), which plays a role in cell cycle and protein degradation (35). Due to the contradictions on dose, we performed an optimization process for the fructose-induced model in the initial part of our study. For this purpose, we aimed to establish our model under optimal conditions by comparing the effect of three different concentrations, 0.5, 5, and 25 mM, on lipid accumulation. Our findings confirmed that 25 mM fructose for 72h increased total LDs. In addition, our study indicated that 5 mM and 25 mM fructose upregulated the SREBP-1c expression without any effect on cell viability. Accordingly, 25 mM fructose was selected as the inducer of lipid accumulation in hepatocytes in the rest of our experiments.

SREBP-1c is a major regulatory factor of DNL-related genes and contributes to MAFLD development (37). The expression of SREBP-1c and its post-translational activation is markedly enhanced by insulin signaling. A diet high in fructose frequently results in insulin resistance and hyperinsulinemia, thereby inducing insulin-mediated SREBP-1c activation and promoting hepatic lipid synthesis (38, 39). In our previous study, silencing SREBP-1c reduced the number of LDs induced by oleic acid (13). Inconsistent, silencing of SREBP-1c caused a reduction in fructose-induced total LDs formation in the present study. These findings indicate that targeting SREBP-1c may serve as a therapeutic strategy against fructose-induced steatosis. Furthermore, various studies have demonstrated the efficacy of SREBP-1c silencing through therapeutic interventions. Ruiz et al. (40) revealed that silencing of SREBP-1 induced the gluconeogenesis genes, while decreasing the glycolysis and glycogen synthesis genes in an animal model of obesity and type 2 diabetes. In another study by Li et al. (41), SREBP-1 knockdown was observed to increase apoptosis and inhibit proliferation, migration and invasion in both MHCC97L and HepG2 cells. These findings suggest that SREBP-1 may contribute to tumor development by enhancing cell growth and metastasis, thus suggesting that it may be a potential therapeutic target for HCC (41).

However, further studies using animal and human samples are crucial to confirm these findings.

The mechanism by which SREBP-1c siRNA reduces fructose-induced LD accumulation might occur through the inhibition of lipogenesis. However, SREBP-1c activation was reported to be associated with increase in enzymes involved in DNL, including FASN (42) SCD1 (43), ATP citrate lyase (ACLY), glycerol-3-phosphate acyltransferase (GPAM) and acyl-CoA synthetase long chain family member 1 (ACSL1) (44). For this reason, the effect of siRNA on mRNA expressions of lipogenic genes was examined in our research. The siRNA only reduced the expression levels of SCD1 and ACLY, while the mRNA expression levels of the other lipogenic genes remained unchanged. However, the reducing effect on SCD1 and ACLY was not on fructose-induced hepatocytes. Therefore, it can be concluded that the therapeutic effect of siRNA on fructose-induced lipid accumulation is independent of lipid metabolism. However, further experiments, such as lipogenic proteins at the translational level, should be conducted to establish that this effect is independent of lipid metabolism.

According to our results, the silencing of SREBP-1c prevented the fructose-induced accumulation of LDs by alternative mechanisms other than lipogenesis suppression. Recent findings indicate that lipophagy, which is essential for maintaining cellular homeostasis, is an effective mechanism for lipid removal in hepatocytes (45). For instance, the inhibition of lipophagy resulted in increased TAG and LDs levels, decreased TAG degradation (46). It was also revealed that autophagy parameters such as Autophagy protein 5 (Atg5), Atg7, LC3II/I, and p62 were inhibited in the liver of mice subjected to a high fructose intake (47). Autophagic dysregulation has also been identified as a factor in the progression of human MAFLD (48, 49). Rather than autophagy, endoplasmic reticulum (ER) stress, which is known to be associated with SREBP in steatosis, might be involved in the therapeutic effect of siRNA(50). In our previous study, the silencing of SREBP-1c resulted in a notable reduction in ER stress (phospho-IRE1, ATF6) and ER stress-triggered apoptosis (JNK, CHOP) parameters in oleic acid-induced steatosis (51). Fructose is also known to increase ER stress in hepatocytes, triggering adverse processes such as inflammation, insulin resistance and apoptosis (17). Finally, siRNA might indirectly reduce fructose-induced LD accumulation by blocking signaling pathways of ER stress or reducing the production of inflammatory cytokines or preventing cellular damage, this should be examined in further studies. The present study provides novel insights into the function of SREBP-1c in fructose-induced lipid accumulation. The silencing of SREBP-1c was found to result in a notable reduction in the accumulation of LDs. Our findings provide promising evidence in developing novel treatment strategies against fructose-induced lipid accumulation and MAFLD.

5. CONCLUSION

It is evident that fructose, a prevalent dietary component nowadays, plays a contributory role in the progression of steatosis. This study investigates the role of SREBP-1c in regulating lipid accumulation induced by fructose. The findings of present study indicate that the silencing of SREBP-1c is an effective method for preventing fructose-induced lipid accumulation. The lack of impact on lipogenesis mRNAs suggests that SREBP-1c siRNA exerts its therapeutic effect through alternative pathways, such as lipophagy, and ER stress. A future examination of the mechanism of action of SREBP-1c siRNA through alternative pathways will provide a different perspective on approaches to reduce the development of MASH.

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Author Contributions: (Initials only)
Research idea: SMT, NKO, ES
Design of the study: SMT, ES
Acquisition of data for the study: SMT, II, BC, TDY
Analysis of data for the study: SMT, II, BC, TDY
Interpretation of data for the study: SMT
Drafting the manuscript: SMT, ES
Revising it critically for important intellectual content: SMT, ES, NKO
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