SH2 domain-containing inositol 5-phosphatase (SHIP2) inhibition ameliorates high glucose-induced de-novolipogenesis and VLDL production through regulating AMPK/mTOR/SREBP1 pathway and ROS production in HepG2 cells

Sattar Gorgani-Firuzjaee ¹, Reza Meshkani ¹,

1- Department of Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, I.R Iran.

Running title: SHIP2 regulates lipid metabolism in HepG2 cells

To whom correspondence should be addressed:

Reza Meshkani,

Department of Biochemistry,

Faculty of Medicine,

Tehran University of Medical Sciences,

Tehran, I.R. Iran.

Tel: +98-21-64432502;

Email: rmeshkani@tums.ac.ir

Abbreviations:

AMPK: AMP-activated protein kinase, ACC: Acyl COA Carboxylase, BSA: Bovine Serum Albumin, DHE: Dihydroethidium, DPI: Diphenylene iodonium, ERK: Extracellular signal-regulated kinase, FAS: Fatty Acid Synthase, FBS: Fetal bovine serum, FOXO1: Forkhead box protein O1, GFP: Green fluorescent protein, HDL-C: High density lipoprotein cholesterol, HG: High glucose (33 mM), LDL: Low density lipoprotein, JNK: c-Jun N-terminal kinase, Ins: insulin, MAPK: Mitogen-activated protein kinase, MTP: Microsomal transfer protein, mTOR: mammalian target of rapamycin, NG: Normal glucose (5.5 mM), p38: p38 mitogen-activated protein kinases, PI3-K: phosphatidylinositol 3-kinase, PIP2: Phosphatidylinositol diphosphate, PIP3: Phosphatidylinositol 3,4,5-trisphosphate, PTEN: Phosphatase and tensin homolog, ROS: Reactive oxygen species, SREBP1c: Sterol Regulatory Element-Binding Proteins, SHIP2: SH2 domain containing inositol 5-phosphatase, T2D: SHIP2-DN: SHIP2 Dominant negative mutant gene, SHIP2-WT: SHIP2 wild type gene, T2D: type 2 diabetes, TG: triglyceride, TSC1/2: tuberous sclerosis 1/2, VLDL: Very low density lipoprotein

Abstract

Hepatic de-novo lipogenesis and production of triglyceride rich very low density lipoprotein (VLDL) is increased in the state of insulin resistance, however, the role of a negative regulator of the insulin signaling pathway, the SH2 domain-containing inositol 5-phosphatase (SHIP2) in this process, remains unknown. In the present study, we studied the molecular mechanisms linking SHIP2 expression to metabolic dyslipidemia using overexpression or suppression of SHIP2 gene in HepG2 cells exposed to high glucose (33mM). The results showed that high glucose induced SHIP2 mRNA and protein levels in HepG2 cells. Overexpression of the dominant negative mutant SHIP2 (SHIP2-DN) ameliorated high glucose-induced de-novo lipogenesis and secretion of apoB containing lipoprotein in HepG2 cells, as demonstrated by a reduction in both secreted apoB and MTP expression, and decreased triglyceride levels and the expression of lipogenic genes such as SREBP1c, FAS and ACC. Overexpression of the SHIP2-DN decreased high glucose-induced apoB containing lipoproteins secretion via reduction in ROS generation and JNK phosphorylation and Akt activation. Furthermore, using the specific inhibitor and activator, it was found that the AMPK/mTOR/SREBP1 is the signaling pathway that mediates the effects of SHIP2 modulation on hepatic de-novo lipogenesis. Taken together, these findings suggest that SHIP2 is an important regulator of hepatic lipogenesis and lipoprotein secretion in insulin resistance state.

Keywords: De-novo lipogenesis, VLDL, SH2 domain containing inositol 5-phosphatase (SHIP2), Insulin resistance, hyperglycemia, HepG2, liver

Introduction

Dyslipidemia is one of the key risk factors for cardiovascular disease in type 2 diabetes (T2D) [1]. The dyslipidemia is characterized by increased plasma concentration of triglycerides (TG), reduced concentration of high density lipoprotein cholesterol (HDL-C) and an increased concentration of small dense low density lipoprotein (LDL) cholesterol [2]. Evidence from both animal and human studies suggests that insulin resistance (in adipose and liver tissues) is an important underlying cause of hypertriglyceridemia in subjects with metabolic syndrome and T2D [3, 4]. In the liver, the insulin controls lipid metabolism through its cell surface receptor and intracellular mediators such as the phosphatidylinositol 3-kinase (PI3-K) and serine-threonine kinase Akt. It was shown that the insulin inhibits apoB100 secretion via the activation of the PI3-K [5, 6]. Previous studies have also shown that the insulin signaling through PI3-K inhibits the maturation of VLDL lipoprotein particles by preventing the lipidation of VLDL precursor [7]. In insulin resistance state, hepatic VLDL production is increased. In the setting of insulin resistance, fatty acid flux through the plasma into the liver, increased de novo lipogenesis and decreased apoB degradation promote VLDL overproduction [4].

Activation of the PI3-K pathway enhances the production of the phosphatidylinositol 3,4,5- trisphosphate (PI(3, 4, 5)P₃) a lipid second messenger molecule that mediates many cellular responses to insulin [8]. PI(3, 4, 5)P₃ level is controlled by the activity of PI3-K and lipid phosphatases such as phosphatase and tensin homolog (PTEN) and SH2 domain containing inositol 5-phosphatase (SHIP2) [7, 9-11]. SHIP2 hydrolyzes the PI(3, 4, 5)P₃ to produce PI(3, 4)P₂. SHIP2 acts as a negative regulation of the insulin signaling both in vitro and in vivo [12, 13]. An increased expression of SHIP2 has been reported from the skeletal muscle and adipose tissues of db/db mice [14, 15]. It has been demonstrated that SHIP2 overexpression inhibits the insulin-induced Akt activation, glucose uptake, and glycogen synthesis in 3T3-L1 adipocytes, L6 myotubes [16] and tissues of animal models [17, 18]. In addition, liver-specific inhibition of SHIP2 improves the insulin signaling in mice [17, 19]. Furthermore, the heterozygous knockout mice of SHIP2 gene were resistant against high-fat diet-induced obesity and insulin resistance [13]. In this model, the insulin-induced Akt phosphorylation was enhanced in the liver and skeletal muscle, and surprisingly blood TG concentrations and non-esterified free fatty acids were decreased even when they were fed a normal chow diet [13, 20]. Taken together, these studies demonstrate that the alterations of SHIP2 expression and/or enzymatic function appear to have a profound impact on the development of insulin resistance. However, the function of SHIP2 gene in hepatocytes lipid and lipoprotein metabolism in insulin resistance state remains unknown. In this study we investigated the role of SHIP2 in hepatic de-novo lipogenesis and the secretion of apoB100 containing lipoproteins by modulating its expression in HepG2 cells exposed to high glucose concentration. Our data clearly showed that the modulation of SHIP2 in HepG2 cells could affect lipogenesis and lipoprotein secretion by different mechanisms including the ROS production, JNK activation, and the AMPK/mTOR/SREBP1c axis modulation.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin EDTA were purchased from Life Technologies (Life Technologies, Gaithersburg, USA). Tissue culture flasks and disposable plastic ware were purchased from Greiner Bio-One (Frickenhhausen, Germany). Phenyl methyl sulfonyl fluoride (PMSF), protease inhibitor cocktail, and NonidetP-40 were from Roche (Mannheim, Germany). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Schwalbach, Germany). ECL reagents were from Amersham Pharmacia Corp. (Piscataway, NJ, USA). DHE and MitoSOX red were from Invitrogen (San Diego, CA, USA). Polybrene, puromycin, rapamycin, and metformin were from Sigma. All other reagents and chemicals were from Sigma Aldrich (Taufkirchen, Germany).

Cell culture

HepG2 and HEK 293 packaging cells were purchased from the Pasteur Institute of Iran. Cells were maintained at 37 °C (in an atmosphere of 5% CO₂) in DMEM containing 10% FBS, 2mM glutamine, and 1% penicillin–streptomycin. In order to study lipid and lipoprotein metabolism in an insulin resistance state, HepG2 cells were treated with 33mM D-glucose. D-mannitol was used as an osmotic control. To study the role of the specific signaling pathways, HepG2 cells were pretreated with the appropriate activators (anisomycin, metformin) or inhibitors (MAPKs inhibitors, rapamycin, rotenone, diphenyleneiodonium (DPI) and ascorbic acid) for 2h.

Retroviral infection

In order to generate the retrovirus, the transfer and packaging (PMD2 and PUMVC) plasmids were transfected into 293 packaging cells. The plasmids, pBABE- SHIP2 (SHIP2-WT), and pBABE- dominant negative mutant (SHIP2-DN) which was mutated at the catalytic site of the enzyme, were kindly gifted by Dr. Kristopher Erneux [21]. Viral supernatants were collected during 48–96 h after transfection and filtered on 0.44 µm filters. HepG2 cells were infected with the viral supernatants in the presence of 10 µg/ml polybrene. Infected cells were selectively stabled with 2 µg/ml puromycin.

Western blot analysis

Cell lysate was prepared by homogenization in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, and 0.2% sodium deoxycholate, 0.2% SDS, 1 mM Na-EDTA, and 1 mM PMSF) supplemented with protease inhibitor cocktail. For detection of phosphoproteins, a buffer consisting of 50 mM HEPES, pH 7.5, 150mM NaCl, 100mM NaF, 10mM EDTA, 10mM Na₄P₂O₇, and 2mM NaVO₄, and protease inhibitor cocktail was used. After determining protein concentrations, equal amounts of protein were subjected to SDS–PAGE, followed by transfer onto PVDF membrane. Blocking was carried out through 2h incubation at room

temperature with 5% nonfat dry milk or BSA for p-proteins in TBS with 0.5% Tween 20. Blots were incubated overnight with antibodies against SHIP2 (R&D system), p-Akt (S473), Akt, SREBP1c, p-ACC, p-FOXO1, p-AMPK, p-mTOR, mTOR, p-JNK, p-ERK, p-P38, JNK and AMPK (Cell Signaling Technology, Beverly, MA, USA) and β -actin (Abcam, Cambridge, MA, USA) at 4 °C. The bands after incubating with second HRP-conjugated antibodies were visualized using an enhanced chemiluminescent substrate (ECL). Each experiment was performed at least three times.

Real-Time-PCR

After treatments, the cells were harvested and total RNA was extracted using RNeasy mini kit. Total RNA was reverse transcribed using the MMulv reverse transcriptase and random hexamer primer (Takara Tokyo, Japan). Gene expression was quantified using specific primers for ACC, FAS, SREBP1c, SHIP2 and MTP with SYBR Green PCR Master Mix (Takara Tokyo, Japan). The sequences of the primers used in the study are in Table 1, supplementary file. The levels of target genes transcript were normalized relative to β -actin. The amplification protocol for 40 cycles was as follows: 10s at 95 °C for initial activation, 5s at 95 °C for denaturation, and 20s at 60 °C for annealing/extension.

Oil red O staining

After 24 h treatment, the cells were washed in cold PBS and then fixed with 4% paraformaldehyde solution for 1 h at room temperature. The cells were washed twice with distilled water and incubated 5 min in 60% isopropanol. Cells were then stained for 20 min with fresh oil red O working solution (300 mg of oil red O powder and adding this to 100 ml of 99% isopropanol). Stained cells were washed thoroughly with distilled water prior to microscopic observation. Images were captured using an Olympus camera mounted on an Olympus upright microscope. In order to quantify, 250 μ l DMSO were added to the dried plates and then the optical density was measured in 510 nm. The results were normalized against total protein.

Determination of triglyceride

Intra and extra cellular triglycerides content were measured with the Biovision triglyceride quantification Colorimetric/Fluorimetric kit.

Determination of ApoB

Secreted apoB in cells supernatant was analyzed using the apolipoprotein B human Elisa kit (Abcam, USA) according to the manufacturer's instructions. The data were normalized to intracellular protein levels. Protein concentrations were determined by a BCA protein assay kit.

Reactive oxygen species (ROS) determination

Intracellular production of ROS was measured using the cell-permeable fluorescent dyes, CM-H₂DCFDA, dihydroethidium (DHE), and MitoSOX red. CM-H₂DCFDA was applied to monitor intracellular H₂O₂ and

hydroxyl radical production. DHE is oxidized by cytosolic superoxide anion to red fluorescent ethidium, while MitoSOX red is selectively oxidized by superoxide anion in the mitochondria. Briefly, HepG2 cells were grown to reach 50–75% confluences in 6-well plates. The cells were then washed twice with PBS and loaded with CM-H₂DCFDA (5 μ M) for 30 min in the dark at 37°C. Then, cells were washed twice with sterile PBS, resuspended in PBS and analyzed for fluorescence distribution using FACSCalibur flow cytometer. The cells were also plated in 96-well culture dishes. After treatment and then washing twice with PBS, the cells were incubated with DHE (2 μ M) for 30 min, and MitoSOX red (5 μ M) for 10 min in the dark at 37°C. The fluorescent intensity was then determined on a fluorescent plate reader.

Statistical analyses

All statistical analyses were performed using SPSS18.0. (SPSS, Chicago, IL, USA). Comparisons among all groups were performed with the one-way analysis of variance. If statistical significance was found, the Tukey posthoc test was performed. Values of $p < 0.05$ were considered statistically significant. Results are expressed as the mean $\pm$ SEM of at least three independent experiments.

Results

Importance of SHIP2 inhibition in high glucose-induced lipogenesis

Dyslipidemia is one of the important events in hyperglycemic state such as T2D [22, 23]. To show the role of SHIP2 in high glucose-induced de-novo lipogenesis and lipoprotein secretion, we first assessed SHIP2 expression in HepG2 cells treated with 33mM glucose. High glucose enhanced SHIP2 expression both at mRNA and protein levels more than 90% ($p < 0.001$) (Fig 1A-B). To verify the role of SHIP2 in high glucose-induced lipogenesis, we first generated the stable HepG2 cells overexpressing or suppressing the activity of SHIP2. These cells had 3.4 and 3.7 fold more SHIP2 protein level of the dominant negative (SHIP2-DN) and wild-type (SHIP2-WT), respectively, compared to the GFP control cells (Fig 2A). In order to confirm that our retrovirus systems carrying the SHIP2 genes work efficiently, the phosphorylation of Akt, a downstream protein of SHIP2 was evaluated. As shown in Fig 2B, overexpression of the SHIP2-DN and SHIP2-WT genes result in 1.66 and 0.52 fold changes in Akt phosphorylation, respectively, confirming the functionality of SHIP2 in stable HepG2 cells.

We then evaluated different factors related to lipid and lipoprotein metabolism in SHIP2-DN cells. Inhibiting the activity of SHIP2 in SHIP2-DN cells reduced total lipid content induced by high glucose (Fig 2C-D). Further experiments demonstrated that both intra and extracellular TG content significantly reduced in SHIP2-DN cells treated with high glucose (27 and 34%, respectively) (Fig 2 E-F). The amount of secreted apoB was also affected by SHIP2 modulation, as the SHIP2-DN cells treated with high glucose had lower apoB levels compared to control cells (Fig 2G). We confirmed above findings by investigating the expression of important genes involved

in lipid and lipoprotein metabolism such as MTP, ACC, FAS, and SREBP1c. The expression of all these genes significantly diminished in SHIP2-DN cells treated with high glucose (Fig 2 H and I, and supplementary file, Fig 1). Our data from western blot analysis also revealed that the inhibition of SHIP2 in SHIP2-DN cells could significantly attenuate high glucose-induced SREBP1c expression (Fig 2L). Furthermore, the inhibition of SHIP2 could prevent high glucose-reduced ACC phosphorylation in hepatocytes (Fig 2K). We finally studied the FOXO1 phosphorylation in SHIP2-DN cells. As shown in Figure 1M, SHIP2-DN cells obviously had more than 2 fold FOXO1 phosphorylation. On the other hand, overexpression of SHIP2-WT led to a significant increase in both secretion of apoB containing lipoproteins and de-novo lipogenesis, as demonstrated by an enhance in both secreted apoB and MTP expression, increased intra and extracellular TG levels and enhanced the expression of lipogenic genes such as SREBP1c, FAS and ACC. Totally, above findings demonstrate that SHIP2 inhibition ameliorates high glucose-induced lipogenesis and secretion of apoB lipoproteins in HepG2 cells.

Effect of SHIP2 inhibition on high glucose-induced ROS production

It has been previously shown that high glucose induces oxidative stress in different cells [24, 25]. To investigate the mechanism by which SHIP2 inhibition could ameliorate high glucose-induced lipogenesis in HepG2 cells, we hypothesized that the effects of SHIP2 on lipid and lipoprotein metabolism might be mediated through the ROS production. To evaluate this hypothesis, total lipid content and the expression of lipogenic genes were analyzed in HepG2 cells treated with high glucose and SHIP2-WT cells at the presence of 50 μ M ascorbic acid as a ROS scavenger. Ascorbic acid could significantly reduce lipid content in both HepG2 cells treated with high glucose and SHIP2-WT cells (Fig 3A). Similarly, ascorbic acid could prevent increased apoB secretion and the expression of MTP, SREBP1c, ACC and FAS in HepG2 cells treated with high glucose and the SHIP2-WT cells as well (Fig 3B-F).

In order to demonstrate the direct role of ROS in de novo lipogenesis, ROS production was measured by using the fluorescent probes CM-H₂DCFDA and DHE. As shown in Fig 3G and Fig 2 of the supplementary file, the SHIP2-DN cells treated with high glucose had significantly lower ROS production assessed by both CM-H₂DCFDA and DHE probes, whereas the SHIP2-WT cells had an increased ROS production compared to their controls. To demonstrate the source of ROS induced by high glucose and SHIP2 modulation in hepatocytes, we examined the cellular pathway involved in ROS production, including NADPH oxidase and mitochondria mediated pathways. We first treated the cells with different concentrations of diphenylene iodonium (DPI), an inhibitor of NADPH oxidase and rotenone, an inhibitor of mitochondrial respiratory chain complex I. Our results showed that 5 μ M DPI and 0.1 μ M rotenone have the highest capacity to attenuate total lipid induction in high-glucose treated and SHIP2-WT cells (Fig 3 I and K). We confirmed this data by measurement of TG concentrations (Fig 3 L and M). We also measured mitochondrial ROS level using MitoSOX Red, a

mitochondrial superoxide indicator. As shown in Fig 3H, high glucose and overexpression of SHIP2-WT led to a significant increase in mitochondrial ROS level compared to controls. Taken together, these results suggest that both NADPH oxidase and mitochondria constitute the major source of enhanced oxidative stress in high glucose-treated and SHIP2-WT cells.

The role of MAPK signaling pathway in SHIP2 mediated de novo lipogenesis

To address the signaling pathway which link SHIP2 to lipid and lipoprotein metabolism, we targeted Mitogen-activated protein kinase (MAPK) pathway. We first pretreated the cells with MAPKs inhibitors (SP600125, 20 μ M inhibitor of JNK, SB202190, 10 μ M inhibitor of p38 and PD98059, 20 μ M inhibitor of ERK) and then oil red o staining and real-time PCR for gene expression analyses were performed. The results showed that only JNK inhibitor significantly reduced total lipid content, apoB secretion and the expression of MTP, SREBP1c, ACC and FAS genes in HepG2 cells treated with high glucose and also SHIP2-WT cells compared to control cells (Fig 4A-C). We confirmed this finding by detecting the phosphorylation of the MAPKs proteins. Our results showed that only JNK phosphorylation significantly increased at the presence of high glucose in HepG2 cells (Fig 4D). Interestingly, SHIP2 modulation affects JNK phosphorylation, as the inhibition of SHIP2 in SHIP2-DN cells reduced JNK phosphorylation by 52%, whereas overexpressing SHIP2-WT led to 65% more phosphorylation of JNK in HepG2 cells (Fig 4D). To confirm these data, HepG2 cells were pretreated with anisomycin, a JNK activator. As shown in Fig 4E, anisomycin significantly induced the phosphorylation of JNK. Importantly, anisomycin significantly induced total lipid content in HepG2 cells treated with both normal and high glucose (Fig 4F). While total lipid content and apoB secretion reduced in SHIP2-DN cells treated with high glucose, adding anisomycin to the media prevented the effect of SHIP2 inhibition on these factors (Fig 4G). In addition, as shown in Fig 4H and Fig 3 of the supplementary file, the expression of MTP, ACC, FAS and SREBP1c significantly were induced by anisomycin in HepG2 cells. Furthermore, anisomycin could neutralize the effect of SHIP2 inhibition on high glucose-induced lipogenesis and secretion of apoB lipoproteins in HepG2 cells. Taken together, these data suggest that high glucose induces both lipogenesis and secretion of apoB lipoproteins by a mechanism that involves the activation of JNK in HepG2 cells. It also appears that the effects of SHIP2 on lipid and lipoprotein metabolism might be exerted through the induction of JNK phosphorylation in hepatocytes.

It has been previously shown that JNK is activated in response to ROS production [26]. To find the link between JNK activation and ROS production, we treated the cells at the presence of high glucose and ascorbic acid. High glucose could increase JNK phosphorylation by 2.2 fold, whereas ascorbic acid reversed the effect of high glucose on JNK phosphorylation. Interestingly, ascorbic acid also reduced JNK phosphorylation in SHIP2-

WT cells, suggesting the existence of a link between ROS production and JNK activation in SHIP2 modulated cells (Fig 4I).

Previous studies have shown a link between JNK and Akt. It has been reported that JNK activation leads to a reduction in Akt phosphorylation [26]. Accordingly, we hypothesized that the effects of SHIP2 modulation on lipid and lipoprotein metabolism might be exerted through this pathway. We first investigated the link between JNK and Akt phosphorylation. Using a specific inhibitor of JNK, we found that the inhibition of JNK leads to increased Akt phosphorylation in HepG2 cells treated with high glucose and the SHIP2-WT cells (Fig 4K). On the other hand, anisomycin decreased Akt phosphorylation in both the HepG2 and SHIP2-DN cells treated with high glucose (Fig 4L). Regarding the effects of SHIP2 modulation on Akt phosphorylation, we found that the inhibiting SHIP2 in SHIP2-DN cells could ameliorate high glucose-reduced Akt phosphorylation, whereas overexpression of SHIP2-WT led to decreased phosphorylation of Akt in HepG2 cells (Fig 4M). Altogether, these findings suggest that the effects of SHIP2 on lipid metabolism might be mediated by a mechanism involving the activation of ROS/JNK followed by the inhibition of Akt activity in hepatocytes.

Effect SHIP2 Inhibition on AMPK-mTOR-SREBP1c pathway in high glucose condition

Studies on hepatic lipogenesis have targeted nutrient-dependent activation of the AMPK/mTOR/SREBP1c pathway in hepatic *de novo* lipogenesis [27-31]. To investigate whether SHIP2 modulation influence these pathways, we first evaluated AMPK phosphorylation at the presence of high glucose. High glucose significantly reduced the phosphorylation of AMPK in HepG2 cells, whereas the phospho-AMPK levels were increased and decreased in SHIP2-DN cells treated with high glucose and the SHIP2-WT cells, respectively (Fig 5A). We then assessed the mTOR ser 2481 phosphorylation level in SHIP2 modulated cells. As shown in Fig 5B, high glucose induced the phosphorylation of mTOR 3.1 fold. Inhibiting SHIP2 in SHIP2-DN cells reduced mTOR phosphorylation by 30% and overexpression of SHIP2-WT induced phospho-mTOR level 2.2 fold versus the GFP control cells. To confirm the role of this pathway in lipogenesis, we treated the cells with high glucose and rapamycin as mTOR inhibitor. Rapamycin significantly reduced total lipid content, TG levels and SREBP1c protein level in the cells suggesting the importance of mTOR in hepatic lipogenesis (Fig 5D-E). To identify the role of AMPK in lipogenesis, we treated the cells with metformin as an activator of AMPK. Interestingly metformin was able to decrease lipogenesis in HepG2 cells, as demonstrated by changes in total lipid content, TG level, SREBP1c protein and ACC phosphorylation levels (Fig 5C, F). To study whether AMPK is the upstream of mTOR or vice versa, we measured phospho-AMPK and phospho-mTOR levels in the cells treated with rapamycin and metformin. Our results suggested that AMPK activates mTOR pathway. In this regard rapamycin did not significantly change the AMPK phosphorylation, (Fig

5G), while, metformin was able to significantly reduce high glucose-induced mTOR phosphorylation in HepG2 cells (Fig 5H).

We further focused on whether the effect of SHIP2 on hepatic lipogenesis is also mediated through the AMPK/mTOR pathway. To this aim we analyzed SREBP1c and phospho-ACC levels at the presence of metformin and rapamycin in SHIP2-WT cells. The results showed that rapamycin reduced SREBP1c protein and increased ACC phosphorylation levels in the SHIP2-WT cells (Fig 5G). Furthermore, metformin treatment resulted in a lower level of SREBP1c protein and a higher level of phospho-ACC in the SHIP2-WT cells (Fig 5H). Altogether, these data demonstrate that SHIP2 overexpression enhances lipogenesis in HepG2 cells via the activation of AMPK/mTOR/SREBP1c pathway.

Discussion

Hypertriglyceridemia a pathological feature of the metabolic syndrome and T2D, is characterized by augmented production and/or retarded clearance of TG-rich particles, such as VLDL [32, 33]. While the pathophysiology of hypertriglyceridemia is poorly understood, its close association with visceral adiposity and T2D implicates insulin resistance as a causative factor for hypertriglyceridemia [34]. VLDL production in the liver largely depends on substrate availability and sensitivity of the cells to the insulin. [35]. In T2D and metabolic syndrome, the aberrant insulin action in combination with increased influx of FFAs into the liver promotes excessive VLDL-TG production, contributing to the pathogenesis of hypertriglyceridemia [36]. In spite of many efforts, the underlying molecular mechanism of hepatic VLDL-TG overproduction and key players in this process is a matter of debate. In the present study we focused on SHIP2, an intermediate of the insulin signaling, and investigated its role in hepatic lipogenesis and secretion of apoB containing lipoproteins in a high glucose condition.

Many complications of chronic metabolic diseases such as metabolic syndrome and T2D can be attributed to hyperglycemia. One of the consequences of elevated glucose level is hepatic lipid accumulation and increase of VLDL secretion [37, 38]. Despite many studies, the molecular mechanism of hyperglycemia-induced lipogenesis is not well understood. The present data suggest that the effects of high glucose on lipid and lipoprotein metabolism may at least in part be mediated by SHIP2. High glucose could induce SHIP2 expression at mRNA and protein levels in HepG2 cells. It is worthy note that an increased expression of SHIP2 in the state of insulin resistance has been previously reported [15, 39, 40]. Furthermore, SHIP2 inhibition via the dominant negative expression significantly prevented hyperglycemia-induced de-novo lipogenesis as demonstrated by a reduced the expression of key lipogenic genes such as FAS, ACC and transcription factors including the SREBP1c, FOXO1. SHIP2 inhibition also decreased secretion of apoB containing lipoproteins. These findings suggest that the inhibition of SHIP2 via reduction of lipid (TG) and protein content of VLDL (apoB) and

suppressing the responsible protein in VLDL assembly (MTP) leads to decreasing VLDL production in high glucose condition. These observations suggest that a reduction in SHIP2 expression may prevent carbohydrate-induced changes in insulin sensitivity and the associated abnormalities in lipid and lipoprotein metabolism.

The mechanism by which SHIP2 influence lipid and lipoprotein metabolism is most likely regulated indirectly via the changes in insulin sensitivity. Insulin is an important regulator of lipid and lipoprotein metabolism, and hepatic lipogenesis and lipoprotein production are tightly controlled by the insulin action [41]. Insulin induces SREBP-1c expression by the PI3-K/Akt-dependent mechanism, but the downstream effectors are not clearly understood [42]. Insulin has also been shown to block apoB secretion by a mechanism involving the degradation of intracellular apoB [43], and it is thought that this effect can be blocked with the induction of hepatic insulin resistance. Previous studies have also reported that the insulin-suppressive effects on apoB secretion need PI3-K activation. Activation of PI3-K was thought to mediate the inhibition of apoB secretion possibly via the generation of the PI(3, 4, 5)P₃ [44]. In the present study SHIP2 suppression increases the PI3-K/Akt activity and therefore it is plausible to suggest that SHIP2 inhibition may promote the suppressive effect of insulin on apoB secretion by activating the PI3-K/Akt pathway. In addition, our data demonstrated that high glucose alone and SHIP2 overexpression despite of a reduction in Akt activation leads to an increased the expression of SREBP1c and its target genes, suggesting an Akt-independent pathway operating the lipogenic effect of high glucose and SHIP2 overexpression in HepG2 cells. Thus, it appears that SHIP2 can potentially affect the rate of de-novo lipogenesis, resulting in alterations of the secretory pool of lipid substrates available for VLDL assembly and secretion. In accordance with this notion, it has been reported that liver-specific inhibition of SHIP2 improve glucose tolerance in diabetic KKA mice by affecting glucose metabolism [17]. But this study, however, did not examine the alterations of lipid and lipoprotein metabolism associated with the inhibition of SHIP2 expression. Our finding appears to extend these observations and further suggest that reducing SHIP2 expression can significantly attenuate hepatic de-novo lipogenesis and lipoprotein secretion most likely as a result of enhanced hepatic insulin signaling.

Another mechanism linking SHIP2 to lipid metabolism is oxidative stress. In vitro and in vivo studies have demonstrated a correlation between the oxidative stress and lipid accumulation in liver tissue [45, 46]. However, the direct molecular mechanism linking the oxidative stress to enhanced lipogenesis remains unknown. A growing body of evidence suggests that the oxidative stress due to excessive production of ROS is the main contributor to the pathophysiology of diabetic complications under the hyperglycemic conditions [47, 48]. However, the exact source, and cellular location of the glucose-induced ROS is still unclear. Both mitochondria and NADPH oxidase have been suggested to be the major sources of ROS production in different cells treated with glucose [49, 50]. In the present study, we used DPI, an inhibitor of NADPH oxidase and

rotenone an inhibitor of mitochondrial respiratory chain complex I to explore the importance of mitochondria versus NADPH oxidase derived ROS in high glucose-induced lipogenesis of HepG2 cells. Our observations suggested that both mitochondria and NADPH oxidase are the sources of ROS in high glucose-induced lipogenesis of Hepatocytes. Consistent with our results, DPI was found to decrease ROS production by NADPH oxidase in HepG2 cells treated with high glucose concentration [51]. Our data also revealed that both mitochondria and NADPH oxidase are involved in lipogenesis induced by overexpression of SHIP2-WT in HepG2 cells.

The present data is first to suggest that high glucose-induced de-novo lipogenesis is mediated through ROS-dependent JNK activation. In this regard, a ROS scavenger and a JNK inhibitor prevented high glucose-induced de-novo lipogenesis and secretion of apoB containing lipoproteins. JNK activation then leads to inactivation of Akt in HepG2 cells. Inactivation of Akt, as stated earlier, mediates the induction of apoB secretion and VLDL overproduction [44]. Interestingly, SHIP2 modulation was able to affect the status of the oxidative stress and JNK activation in HepG2 cells. SHIP2 overexpression enhanced ROS production and JNK activation, whereas overexpression of the SHIP2-DN gene led to a reduction in ROS production and JNK phosphorylation in the cells treated with high glucose. These observations highlight that alterations in the oxidative status and JNK activation might be the link between high glucose, SHIP2 modulation and lipogenesis and VLDL production in HepG2 cells. In the present study we did not focus on the link between SHIP2 and JNK activation, however, it is possible that the effect of SHIP2 on JNK activation is mediated through its partner, JNK-interacting protein 1 (JIP1). SHIP2 positively regulates the tyrosine phosphorylation of JIP1. It has been previously reported that SHIP2 has a positive influence on mixed-lineage kinase3 (MLK3)/JIP1-induced JNK1 activity [52].

It is worth also noting that the effects of ROS/JNK pathway on de-novo lipogenesis is Akt-independent in both the SHIP2 modulated and HepG2 high glucose treated cells. While SREBP1c induction is usually dependent on the activation of Akt, we observed a decrease of Akt phosphorylation by JNK activation in SHIP2-WT and HepG2 cells treated with high glucose. However, it appears that the effect of ROS/JNK pathway on VLDL production occurs through an Akt-dependent mechanism.

The mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase that senses the nutrients and growth factors and regulates several biological processes required for the promotion of cell growth [53]. Aberrant mTOR signaling is involved in the pathogenesis of several diseases such as obesity, T2D, cancer and neurodegenerative disorders [53]. mTOR is present in two distinct complexes, mTOR complex 1 (mTORC1), which is partially inhibited by rapamycin and mTORC2. Growth factors and hormones (e.g. insulin) activate mTORC1 via the PI3-K/Akt pathway. Akt inactivates the tuberous sclerosis 1/2 (TSC1/2) to prevent the

inhibition of mTORC1 [54]. Alternatively, low ATP levels lead to AMP-activated protein kinase (AMPK)-dependent activation of TSC2 and phosphorylation of raptor to reduce the mTORC1 signaling. It is now clear that mTORC1 promotes hepatic lipogenesis by activating the SREBP1 by inducing the SREBP1 expression, processing, and nuclear accumulation[55]. In this regard, our data support the role of mTORC1 in hepatic lipogenesis, as mTORC1 phosphorylation (ser2481) increased in parallel with the SREBP1c expression in the state of high glucose. However, our results did not demonstrate an Akt-dependent activation of mTORC1 and it seems that a high glucose suppression of phospho-AMPK promotes the mTORC1 activation in HepG2 cells. It is of note that an Akt-independent regulation of mTORC1 by energy-sensing pathways (AMPK) has been mostly found in cancer cells [56]. AMPK communicates the energy status of the cell to the mTOR pathway by both indirect and direct inhibition of mTORC1. AMPK inhibits the mTOR indirectly by phosphorylating the tumor suppressor TSC2 at S1227 and S1345, or by direct phosphorylation on raptor (S722 and S792), a component of mTORC1[57]. Our data also revealed that the effect of SHIP2 on hepatic lipogenesis is mediated by an Akt-independent activation of mTORC1-SREBP1c pathway. While SHIP2-WT overexpression as expected resulted in a decreased Akt phosphorylation, we were not able to observe a parallel inhibition of mTORC1 in HepG2 cells. In addition, the inhibition of SHIP2 in SHIP2-DN cells exposed to high glucose caused AMPK activation leading to a decrease in the activity of mTOR-SREBP1c pathway. This Akt-independent lipogenesis in both high glucose and SHIP2-WT overexpression states appears to be unexpected. One explanation for this unexpected finding can be attributed to the nature of the cell line we used. HepG2 is a human hepatocellular liver carcinoma cell line and the activation of mTORC1 is important for the survival, proliferation and apoptosis resistance of this cell [58]. Furthermore, AMPK is dysfunctional in patients with hepatocellular carcinoma, and low phospho-AMPK staining is correlated with aggressive clinicopathologic features and poor prognosis [59]. Therefore, it appears that the AMPK inhibition accompanied by the mTOR activation is a dominant signaling pathway that might promote the proliferation of HepG2 cells. Further studies in primary hepatocytes might help to have a better conclusion on the role of high glucose and SHIP2 modulation on these pathways.

In summary, our study provides the evidence that SHIP2 regulates the de-novo hepatic lipogenesis and the secretion of apoB containing lipoproteins in HepG2 cells. We discovered that the effects of high glucose on lipid and lipoprotein metabolism are mediated through two mechanisms including the ROS/JNK and the AMPK/mTORC1/SREBP1 pathways. Interestingly, the inhibition of SHIP2 expression could ameliorate high glucose-induced hepatic lipogenesis by reversing the mentioned pathways (Fig 6). According to these findings, it appears that the inhibition of SHIP2 could potentially be a target in treatment of dyslipidemia in T2D and metabolic syndrome subjects. However, further in vivo and in vitro studies are needed to elucidate the potential role of SHIP2 in control of hepatic lipogenesis.

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Figure Legends:

Figure 1

Effect of high glucose on SHIP2 expression at mRNA and protein levels. HepG2 cells were treated with 33mM glucose for 24h and then the expression of SHIP2 at mRNA (A) and protein levels (B) were analyzed using the real-time PCR and western blotting, respectively. Represented data are from three independent experiments and are means $\pm$ SEM. HG, high glucose (33mM); NG, normal glucose (5.5mM). *: $p < 0.0001$

Figure 2

Importance of SHIP2 inhibition in high glucose-induced lipogenesis. A-B):Generation of stable HepG2 cells. HepG2 stable cells were generated by infecting the cells with the viral supernatants followed by stable selection with 2 μ g/ml puromycin. Real-time PCR and western blotting were used to detect SHIP2 mRNA and protein levels in HepG2 stable cells. A: Protein levels of SHIP2 in HepG2 stable cells. B) Analysis of Akt phosphorylation for assessment of the SHIP2 functionality.C-I): The influence of SHIP2 inhibition on high glucose-induced lipogenesis and the secretion of apoB. HepG2 cells and the cells expressing the SHIP2-DN gene were used in the absence or presence of high glucose (33mM) for 24h. C-D:Qualitative and quantities analyses of lipogenesis by oil red o staining, E-F: the amount of intra and extracellular TG levels, respectively,G:apoB secretion, H and I: the expression of the lipogenic genes such as MTP and ACC, K-M: the changes of SREBP1c protein and phosphorylation of ACC and FOXO1. Represented data are from three independent experiments and are means $\pm$ SEM.DN: overexpresseddominant negative SHIP2 gene, WT: overexpressed wild typeSHIP2 gene, NG: normal glucose (5.5mM), HG: High glucose (33mM), BSA: Bovine Serum Albumin, Ins: insulin 100 nM, TG: triglyceride, *: $p < 0.0001$

Figure 3

Effect SHIP2 modulation on ROS production. HepG2 cells and the cells expressing GFP as control and DN and WT SHIP2 genes were used in these experiments. A: oil red o staining after treatment of the cells with 50 μ M ascorbic acid [34], as ROS scavenger, B-E: the expression of the key lipogenic genes such as SREBP1c, ACC, FAS and MTP at the presence of ascorbic acid, E): apoB secretion at the presence of ascorbic acid in SHIP2 modulated cells. G: ROS level in the SHIP2 modulated cells using DHE probe. H): Mitochondrial ROS level in SHIP2 modulated cells using MitoSOX red dye. I and L): Total lipid (oil red o staining) and TG content

at the presence of NADPH oxidase inhibitor (DPI 10 μ M). K and M): Total lipid (oil red o staining) and TG content at the presence of ETC inhibitor (rotenone 1 μ M). Represented data are from three independent experiments and are means $\pm$ SEM. DN: overexpressedd dominant negative SHIP2 gene, WT: overexpressed wild typeSHIP2 gene, Asc: ascorbic acid (50 μ M), NG: normal glucose (5.5mM), HG: High glucose (33mM), R: rotenone, DPI: Diphenylene iodonium *: $p < 0.0001$

Figure 4

The effect of SHIP2 inhibition on MAPK pathway in high glucose condition. HepG2 cells and the cells expressing the GFP as control and DN and WT genes were used in the absence or presence of high glucose (33mM) for 24h. A): total lipid content at the presence of MAPKs pathway inhibitors (JNK, P38 and ERK), B: the expression of the key lipogenic genes at the presence of JNK inhibitors, C: apoB100 secretion at the presence of JNK inhibitors, D: MAPKs protein phosphorylation in SHIP2 modulated cells, E: Effect of SHIP2 inhibition on high glucose-induced JNK phosphorylation at the presence anisomycin (50 μ M), F: total lipid content at the presence anisomycin (50 μ M) as JNK activator, G: apoB100 secretion at the presence of anisomycin (50 μ M). H: The expression of MTP gene at the presence of anisomycin (50 μ M), I: JNK phosphorylation in the SHIP2-WT and GFP control cells at the presence of ascorbic acid (50 μ M), K): phospho-Akt level at the presence of high glucose and JNK inhibitor in the GFP and SHIP2-WT cells, L): phospho-Akt level in the GFP and SHIP2-DN cells at the presence of anisomycin, M): the phosphorylation changes in SHIP2 modulated cells. Represented data are from three independent experiments and are means $\pm$ SEM. DN: overexpressedd dominant negative SHIP2 gene, WT: overexpressed wild typeSHIP2 gene, NG: normal glucose (5.5mM), HG: high glucose (33 mM), Ins: insulin 100 nM, In: inhibitors, JNK In: inhibitor of JNK, A: Anisomycin (50 μ M), *: $P < 0.001$

Figure 5

The effects SHIP2 inhibition on the AMPK-mTOR-SREBP1c pathway in the high glucose condition. HepG2 cells and the cells expressing GFP as control and SHIP2-DN and SHIP2-WT genes were used in the absence or presence of high glucose (33mM) for 24h. A: the AMPK phosphorylation in SHIP2 modulated cells, B: the mTOR ser2481 phosphorylation in the cells treated with high glucose, C: total lipid content at the presence of rapamycin (20 nM) and high glucose, D: TG level of the cells at the presence of rapamycin (20 nM) and high glucose, E) total lipid content at the presence of metformin (5 mM) and high glucose, F: TG level of the cells at the presence of metformin (5 mM) and high glucose, G: phospho- AMPK, phospho-mTOR, SREBP1c and phospho-ACC levels at the presence of high glucose and rapamycin (20 nM), I) phospho- AMPK,

phospho-mTOR, SREBP1c and phospho-ACC levels at the presence of high glucose and metformin (5 mM). Represented data are from three independent experiments and are means $\pm$ SD. DN: overexpressed dominant negative SHIP2 gene, WT: overexpressed wild type SHIP2 gene, Normal glucose (5.5mM), HG: High glucose (33mM), *: $P < 0.001$

Figure 6

Fig 6: Molecular mechanisms linking SHIP2 to hepatic lipid homeostasis in the state of high glucose. High glucose appears to induce the de-novo lipogenesis and the secretion of apoB containing lipoproteins in HepG2 cells by affecting two signaling pathways including the ROS/JNK/Akt and the AMPK/mTOR/SREBP1c. Inhibition of SHIP2 expression could ameliorate high glucose-induced secretion of apoB containing lipoproteins by an ROS/JNK/Akt-dependent mechanism. SHIP2 inhibition could also prevent high glucose-induced de-novo lipogenesis by an Akt-independent mechanism involving the AMPK/mTOR/SREBP1c signaling pathway. Altogether, a decreased de-novo lipogenesis leading to a lower TG content along with a decreased expression of MTP results in a reduced secretion of apoB containing lipoproteins (VLDL) in SHIP2-DN cells.

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