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Cholesterol accumulation in hepatocytes mediates IRE1/p38 branch of endoplasmic reticulum stress to promote nonalcoholic steatohepatitis

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD), based on the elevating obesity incidence, is one of the major health issue worldwide. Transition from NAFLD to non-alcoholic steatohepatitis (NASH) is driven by increased apoptosis and is relevant to higher morbidity rates. In regard to limited understanding on cholesterol mediated hepatocyte alterations in NALFD/NASH transition, we investigated endoplasmic reticulum (ER) stress and related apoptosis. Our findings suggest that cholesterol upregulates ER stress and enhances C/EBP homologous protein (CHOP) either in hypercholesterolemic rabbits or in hepatocytes treated with liposome-cholesterol complex. Mechanistically, cholesterol accumulation in hepatocytes activates IRE1/p38 branch of ER stress, stimulating CHOP levels. In liver tissues of cholesterol fed rabbits, α-tocopherol supplementation decreased IRE1/p38/CHOP activation and prevented NASH development. Thus, our study provides a critical role of hepatocyte cholesterol in inducing IRE1/p38/CHOP pathway and suggests novel candidates for therapeutic targets against NASH.

1. Introduction

In regard to elevating obesity prevalence, non-alcoholic fatty liver disease (NAFLD) is reported as the leading cause of liver disease worldwide [1]. However, uncompleted understanding of pathophysiology has resulted to limited therapeutic options that may reverse or halt the progression to nonalcoholic steatohepatitis (NASH), hepatic fibrosis and hepatocellular carcinoma (HCC) [2]. Activated apoptosis in hepatocytes associates with clinical outcome observed in NASH patients [3]. Cholesterol is a sterol-like substance that is essential in modulating cell functions, including the fluidity and permeability of membrane and synthesis of steroid hormones. However, accumulation of hepatic free cholesterol is suggested as an important process in NASH development by inducing inflammatory pathways in hepatocytes and enhancing fibrosis in hepatic stellate cells [4]. Elevation of liver cholesterol content in humans is also found to be associated with NASH development and is reported to be crucial in pathogenesis [5,6].

These studies emerge the critical question of the mechanisms by which cholesterol increases apoptosis during NASH progression. Previous reports have determined that endoplasmic reticulum (ER) stress and related apoptosis act in sequence to enhance various liver diseases including NAFLD, viral hepatitis and cholestatic liver disease [7,8]. Mechanistically, accumulated free cholesterol in ER lumen results with ER stress and subsequently, disassociation of GRP78 from ER stress sensors (IRE1, PERK and ATF6) activates transcription factors and signaling pathways through different mechanisms [9]. As an initial response, PERK activation involves in the inhibition of protein synthesis [10], while IRE1 enhances the expression of a variety of genes, including chaperones [11]. However, if the stress is severe enough to exceed the capacity of response to restore ER homeostasis, ER stress may trigger apoptosis and drive NAFLD/NASH transition. CHOP is the best characterized ER stress mediated pro-apoptotic transcription factor that is regulated via IRE1 and PERK branches of ER stress. Following activation, serine/threonine-protein kinase domain of IRE1 leads to the induction of p38 and JNK, and subsequently enhances apoptotic cell death through CHOP expression [12,13].

ER stress mediated signaling pathways are shown to have crucial role in both the steatosis and NASH progression [14]. Increased expression of

Abbreviations: ATF6, Activating Transcription Factor 6; ASK1, apoptosis signal-regulating kinase 1; CHOP, C/EBP Homologous Protein; ER, Endoplasmic Reticulum; Grp78, Glucose Regulated Protein 78; Grp94, Glucose Regulated Protein 94; IRE1, Inositol Requiring Kinase 1; JNK, c-Jun N-terminal kinase; PERK, RNA-activated Protein Kinase-Like Endoplasmic Reticulum Kinase; ROS, Reactive Oxygen Species; UPR, Unfolded Protein Response.

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proteins involved in PERK signaling was found to increase in liver samples of NASH patients [15]. Besides PERK signaling, proteins related to IRE1 branch of ER stress response, JNK and XBP-1, were also reported as increased in adipose tissue of obese human subjects [16]. Another relevant finding in humans is the correlation of GRP78/CHOP ratio with liver damage [17].

Previous study from our group demonstrated that rabbits fed high cholesterol diet have elevated levels of inflammation and apoptosis, enhancing the progression from steatosis to NASH [18]. By using the same liver tissues of rabbits and cholesterol-accumulated hepatocytes, present study targeted to investigate ER stress activation and revealed a crucial process in NASH by determining that cholesterol activates IRE1 branch of ER stress in hepatocyte and through this pathway enhances apoptosis. Mechanistically, cholesterol acts through IRE1–p38 signaling to activate CHOP expression. Our findings reveal a novel mechanism regarding to the effect of hepatocyte cholesterol in enhancing a critical apoptosis stimulant, CHOP, and suggest potential molecules in therapeutic approaches against NASH development.

2. Materials and methods

2.1. Cell culture and cell treatment

AML12 cells (mouse hepatocytes) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco), 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco) at 37 °C with 5% CO_2. To obtain the cholesterol delivery, cells were treated with cholesterol-enriched phospholipid liposomes (Lip-Chol) with or without SP600125 (JNK inhibitor), 4-phenylbutyrate (4-PBA; ER stress inhibitor) or SB203580 (p38 inhibitor). All three chemicals were purchased from Sigma-Aldrich. Following pretreatment with 1 mM 4-PBA for 24 h, 30 μM SP600125 for 1 h or 0.5 μM SB203580 for 6 h, cells were treated with Lip-Chol at the indicated concentrations and time periods.

2.2. Preparation of liposomes

Liposomes (Lip) and cholesterol-enriched liposomes (Lip-Chol) was prepared as described in literature [19]. Briefly, DMPC (Avanti Polar Lipids, cat no: 850345) and cholesterol (Sigma Aldrich, cat no: C8667) were dissolved in chloroform at a ratio of 1:2 (w/w). Only DMPC was used in preparing liposomes. Following the removal of solvent via a stream of nitrogen gas, lipids were resuspended in PBS, mixed, and sonicated on ice for 5 min using 10-s on-off intervals. Following the centrifuge at 10,000×*g* for 10 min, supernatant was filtered by using a 100-nm polycarbonate filter (Avanti, cat no: 610000-1Ea). Final solution was stored at 4 °C and used within 2 weeks after kept under argon.

2.3. Filipin staining

To determine cholesterol accumulation in cells, Filipin staining was applied. Briefly, AML12 was fixed in 4% formaldehyde, washed with PBS, and 0.25 mg/ml Filipin (Sigma Aldrich, F9765) for 2 h at room temperature was applied. Images were taken using a Leica flouresence microscope (Wetzlar, Germany) and quantified with ImageJ software by recording the average number of fluorescence intensities per cell. At least thirty cells for each group was used for quantification.

2.4. Immunofluoresence analysis

After inhibitor and/or Lip-Chol administrations, AML12 was fixed in 4% formaldehyde, and incubated for overnight at 4 °C using following antibodies; phospho IRE1 (Thermo Fisher, catalog No. PA5-10524), phospho PERK (Thermo Fisher, PA5-40294), JNK (Novus Biologicals, NBP2-22209), CHOP (Novus Biologicals, NBP2-13172) and p38 (Cell Signalling, 8690). After administrating Alexa Fluor 488 or Alexa Fluor 594 secondary antibodies, nucleus was visualised using DAPI and images were captured using Zeiss LSM700 confocal microscope (Amsterdam, Netherlands). At least thirty cells for each group were recorded in analyzing fluorescence intensities per cell using ImageJ software.

2.5. Rabbits and treatments

All animal experiments were performed in accordance with Marmara University Experimental Animals Research and Implementation Committee (protocol number 49.2015.mar). Male albino rabbits (2–3 months old) were fed α-tocopherol poor diet or α-tocopherol poor diet containing 2% cholesterol and divided into following groups; i) control, ii) cholesterol, iii) cholesterol + α-tocopherol and iv) α-tocopherol. α-tocopherol (Evigen, Aksu Pharmaceutical) was applied intramuscularly to cholesterol+α-tocopherol and α-tocopherol groups at a daily dose of 50 mg/kg. Our previous studies were utilized in determining α-tocopherol concentration used in the present study [20,21]. After eight weeks, rabbits were sacrificed and blood was collected to measure cholesterol and α-tocopherol, while the liver was rapidly frozen and stored at −80 °C for immunoblotting.

2.6. Immunoblot analysis

Fifteen mg of liver was lysed in RIPA buffer (Cell Signaling) via Ultra Turrax homogenizator in accordance with manufacturer's protocol. After the measurement of protein amount with Bradford, twenty μg of protein was applied to SDS-PAGE gels, and immobilized on nitrocellulose membrane. After the blocking with 5% skim milk in TBS, membranes were incubated with primary antibodies as follows: Grp94 (Cell Signaling, 2104), Grp78 (Cell Signaling, 3177), phospho PERK (Cell Signaling, 3179), phospho IRE1 (Abcam, ab104157), CHOP (Cell Signaling, 5554), JNK (AnaSpec, AS-54390), p38 (Cell Signaling, 8690) and β-actin (Cell Signaling, Catalog No: 4967). Following the HRP-conjugated secondary antibody and chemiluminescence substrate incubations, blots were quantified by Image J.

2.6.1. Statistical analysis

Prism 4 (Graph-Pad) software was used in performing statistical analysis. Statistical signifance was estimated using One-Way ANOVA followed Student-Newman-Keuls test for multiple comparisons. P-value less than 0.05 was considered statistically significant.

3. Results

3.1. High cholesterol diet in rabbits enhances endoplasmic reticulum stress and related apoptosis

Excess of factors involved in the NAFLD pathogenesis as well as their interactions with each other complicate the findings on the treatment of disease. Previous study from our group demonstrated the capacity of high cholesterol diet in inducing NASH development through inflammation and apoptosis, as well as the beneficial effect of α-tocopherol, most active form of vitamin E [18]. By using the same liver tissues, we further investigated the involvement of ER stress and related apoptosis through the measurement of well-identified UPR parameters, Grp78, Grp94, phospho IRE1, phospho PERK and CHOP. As shown in Fig. 1, protein expression of a number of ER stress parameters were significantly affected either by high cholesterol diet or α-tocopherol supplementation. Cholesterol group exhibited an increase in Grp94 and phospho IRE1, while Grp78 and phospho PERK were not changed significantly. However, α-tocopherol supplementation with cholesterol diet decreased Grp94 and phospho IRE1 to control levels (Fig. 1A-D). In cases where the unfolded protein response (UPR) is insufficient, apoptotic cell death occur mainly through the CHOP, most studied pro-apoptotic protein regulated by ER stress. As we published before [18] 8 weeks of high cholesterol diet induced CHOP expression, while

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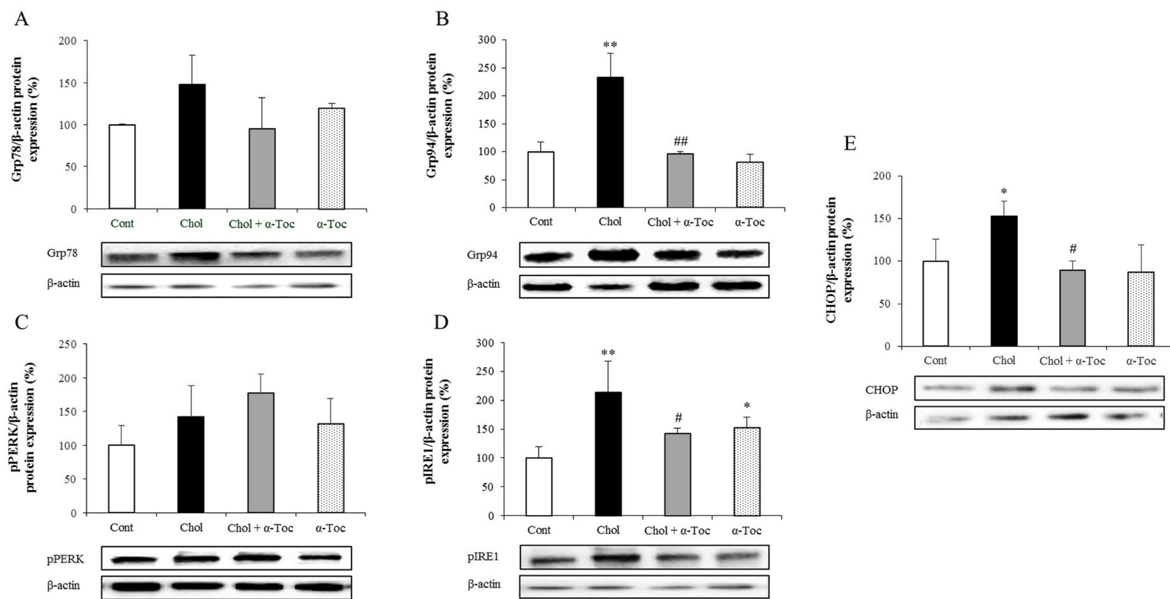

Fig. 1. High cholesterol diet in rabbits enhances endoplasmic reticulum stress and related apoptosis
Protein expressions in liver tissues were measured by western blotting. Bands were quantified and normalized to β-actin against Grp78 **(A)**, Grp94 **(B)**, pPERK **(C)**, pIRE1 **(D)** and CHOP **(E)** antibodies.
Data are expressed as mean ± S.D.
**$p < 0.01$, and *$p < 0.05$ vs. control group,
##$p < 0.01$, and #$p < 0.05$ vs. cholesterol group ($n = 5$).

α-tocopherol supplementation attributed this induction (Fig. 1E).

3.2. Cholesterol enrichment in hepatocytes induces phospho IRE1 and CHOP, while phospho PERK is unaffected

After verifying the involvement of ER stress and related apoptosis in liver tissues of rabbits fed high cholesterol diet, we switched into cell culture experiments to investigate its molecular mechanism. To mediate cholesterol enrichment, we incubated AML12 cells with liposome (Lip) and cholesterol-enriched liposome (Lip-Chol) complexes and evidenced the cholesterol content by filipin staining. As shown in Fig. 2A, Lip-Chol administration for 6, 8 and 10 h exhibited a significant induction in cholesterol content. The optimum time was determined by evaluating the phospho IRE1, phospho PERK and CHOP levels via immunflouresence. While a marked increase in phospho IRE1 expression was observed for all time periods, only 8 h of Lip-Chol incubation was induced CHOP. However, phospho PERK expression was not affected by Lip-Chol administration, which is in consistent with our *in vivo* findings. Microscopy images of selected parameters are supplied in Supplementary Fig. 1.

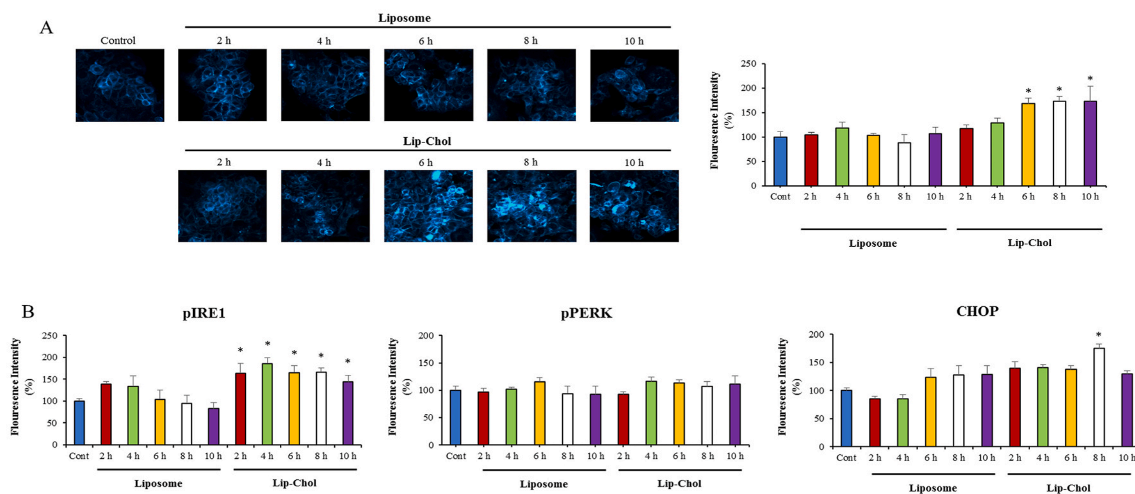

Fig. 2. Cholesterol enrichment in hepatocytes induces phospho IRE1 and CHOP, while phospho PERK is unaffected
AML12 cells were incubated for various time points between 0 and 10 h with liposome or Lip-Chol complex. Cholesterol content was analyzed by Filipin staining following the quantification of fluorescence intensities **(A)**. pIRE1, pPERK and CHOP levels were visualised by confocal microscopy (Supplementary Fig. 1) and relative fluorescence intensities were quantified **(B)**.
Data are expressed as mean ± S.D.
*$p < 0.05$ vs. control group, ($n = 3$).

3.3. Inhibition of ER stress in cholesterol enriched hepatocytes attenuates CHOP, JNK and p38 induction

In order to observe the effect of ER stress response, cholesterol-enriched hepatocytes were treated with ER stress inhibitor, 4-PBA. Analysis of images revealed that pretreatment with 4-PBA reduced cholesterol mediated IRE1 phosphorylation and CHOP induction (Fig. 3B and C). JNK and p38 are two crucial molecules belonging to the MAPK family and involve in IRE1-CHOP signaling [13]. In consistence with phospho IRE1 and CHOP findings, the major effect of Lip-Chol administration on JNK and p38 levels was determined at 8 h (Supplementary Fig. 2). Additionally, cholesterol-enriched hepatocytes pretreated with 4-PBA exhibited a decrease in JNK and p38 expressions (Fig. 3C and D), suggesting that the CHOP reduction following ER stress inhibition might be mediated by JNK and p38.

3.4. IRE1/p38 branch of ER stress regulates cholesterol mediated CHOP induction

In regard to ER stress mediated alterations in JNK and p38 following Lip-Chol administration, we decided to test JNK and p38 expressions in our hypercholesterolemic rabbit model. In this context, we observed higher JNK and p38 expressions in livers of rabbits fed high cholesterol diet. Additionally, the increase in JNK and p38 was abrogated by α-tocopherol supplementation (Fig. 4A and B). To further examine the involvement of JNK and p38 in ER stress related CHOP induction, we treated cholesterol enriched cells with SB203580 or SP600125, well used inhibitors of p38 and JNK. As shown in Fig. 4D, JNK inhibition had no significant effect on cholesterol mediated CHOP induction. On the other hand, we observed that the p38 inhibitor SB203580 prevented the induction in CHOP in cholesterol-loaded AML12 cells (Fig. 4D), consistent with α-tocopherol mediated p38/CHOP reduction in rabbits. Our combined findings suggest that cholesterol accumulation in hepatocyte enhances ER stress mediated CHOP expression by promoting IRE1 phosphorylation and consequent p38 activation.

4. Discussion

Non-progressive steatosis observed in developed countries and in individuals who adopt a sedentary lifestyle can progress into NASH, and if not treated, hepatic fibrosis, cirrhosis, and carcinoma may occur, resulting in liver transplantation as the inevitable end [22]. Therefore, understanding the transition from steatosis to NASH in a mechanistic level has the capacity to prevent tissue injury and identify novel therapeutic targets for this devastating and widespread disease. Cholesterol-CHOP interaction identified herein has implications for the NASH and cellular cholesterol signaling. Our results demonstrate reasonable mechanisms linking increased hepatocyte cholesterol to ER stress and ER stress to increased CHOP, and enhanced the possibility of novel therapeutic molecules against NASH. Based on the cell biology aspects, our study demonstrates a crucial role of hepatocyte cholesterol in the modulation of IRE1 phosphorylation, p38 and CHOP.

A number of studies linking liver cholesterol to NASH have determined excessive and complex mechanisms that interact with each other. Oxygenated derivatives of cholesterol, oxysterols, may lead the activation of mechanisms that are toxic to the cell, including inflammation and apoptosis [23]. Among these oxysterols, 7-ketocholesterol, mainly formed by the oxidation with ROS, is identified to enhance phosphorylated IRE1 and eIF2α levels with consequent involvement of the C/EBP homologous protein (CHOP) in endothelial cells [24], while the elevated levels of dietary oxysterols are shown to contribute intestinal inflammation through NOX1/p38 MAPK/NF-κB signaling [25]. In NASH pathogenesis, various reports using *in vitro/in vivo* experimental models and human samples suggested oxysterols as crucial players in inducing biochemical reactions [26–30]. Previous work from our laboratory using rabbits has determined the potential of high cholesterol diet in inducing NASH development based on comprehensive findings, including the induction of oxidative stress, inflammation, apoptosis and fibrosis [18]. In this study, the alteration of 7-ketocholesterol, associated with disease progression, might reflect its importance as possible reactive intermediate of cholesterol [18]. Other studies using cholesterol induced NASH models in mice also proposed a couple of mechanisms including, the activation of apoptosis and inflammatory pathways in hepatocytes [19, 31–33] and elevated fibrosis in hepatic stellate cells [34–36]. Epidemiological studies were also provided supporting evidence for an association between dietary cholesterol intake and NASH progression [15, 37]. Although the exact mechanism that link cholesterol to NASH development remains unclear, perturbation of ER functions in hepatocyte promises potential [19,38].

In regard to its high levels in the liver, hepatocytes are crucial in mediating tissue homeostasis and proper function. Since the ER hosts many metabolic processes and has a high protein folding capacity, it has a crucial effect in the development of liver-related diseases [39,40]. Involvement of ER stress parameters in NASH pathogenesis has

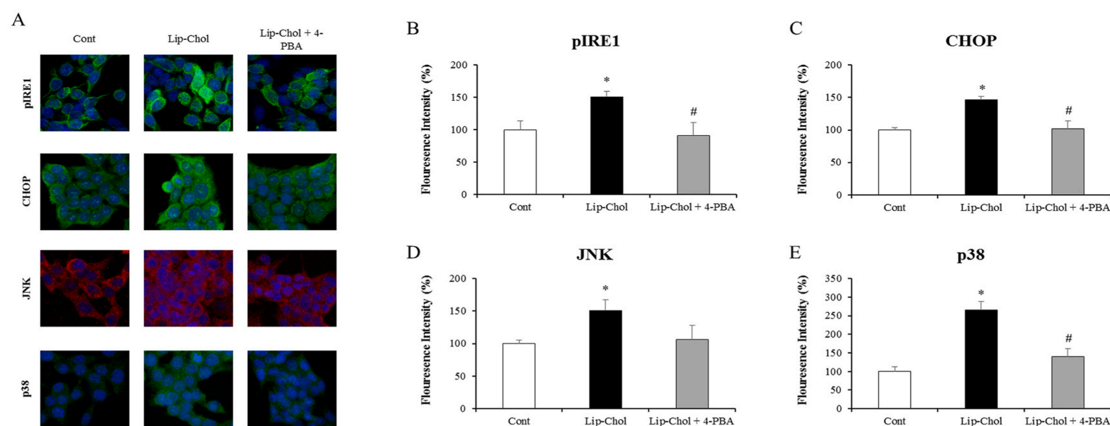

Fig. 3. Inhibition of ER stress in cholesterol enriched hepatocytes attenuates CHOP, JNK and p38 induction
AML12 cells were incubated for 8 h with Lip-Chol complex with or without 1 mM of 4-PBA (ER stress inhibitor). Cells were stained for pIRE1, CHOP, p38 (green), JNK (red) and nuclei by DAPI (blue). pIRE1, pPERK and CHOP levels were visualised by confocal microscopy **(A)** and relative fluorescence intensities were quantified **(B–E)**.
Data are expressed as mean ± S.D.
*$p < 0.05$ vs. control group, #$p < 0.05$ vs. Lip-Chol group ($n = 3$). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

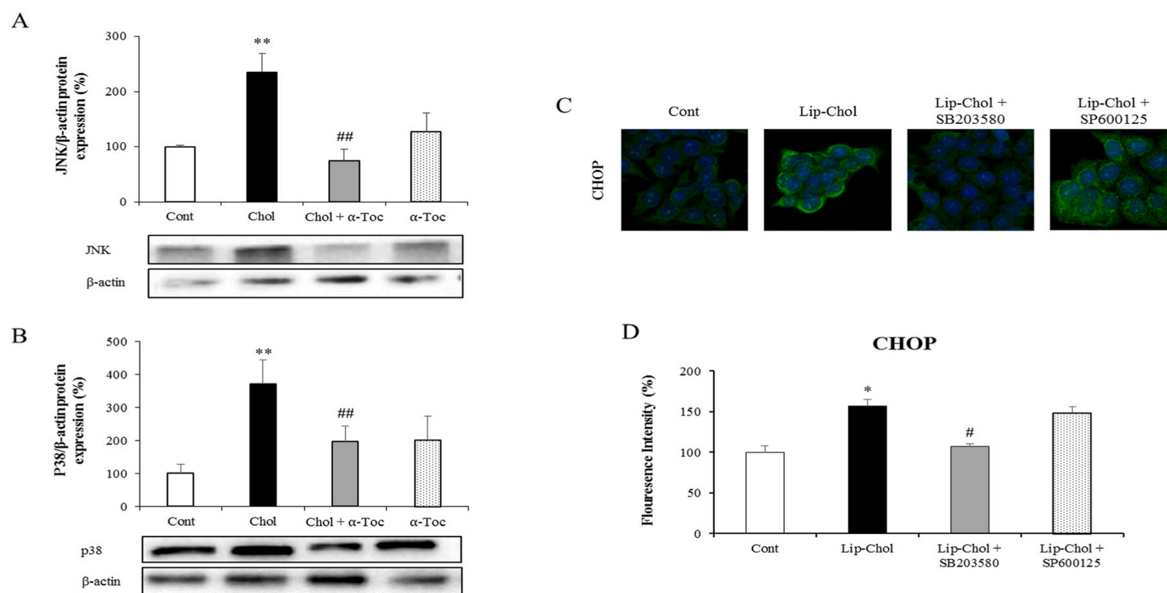

Fig. 4. IRE1/p38 branch of ER stress regulates cholesterol mediated CHOP induction
Protein expressions in liver tissues were measured by western blotting. Bands were quantified and normalized to β-actin against JNK **(A)** and p38 **(B)** antibodies. AML12 cells were incubated for 8 h with Lip-Chol complex with or without 0.5 μM of SB203580 (p38 inhibitor) or 30 μM of SB203580 (JNK inhibitor). Cells were stained for CHOP (green) and nuclei by DAPI (blue). CHOP levels were visualised by confocal microscopy **(C)** and relative fluorescence intensities were quantified **(D)**.
Data are expressed as mean ± S.D.
**$p < 0.01$, and *$p < 0.05$ vs. control group,
##$p < 0.01$ vs. cholesterol and #$p < 0.05$ vs. Lip-Chol group ($n = 5$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

identified in both mice [41–43] and humans [44,45]. Disruption of ER homeostasis due to abnormal lipid accumulations observed in NAFLD pathogenesis and signaling mechanisms modulated via ER stress are associated with lipotoxicity, inflammation and apoptotic cell death, crucial processes in NAFLD/NASH transition. As an initial and preventative step of UPR, IRE1, PERK and ATF6 transmembrane proteins activate unique pathways to prevent cellular damage through reducing protein synthesis and increasing chaperone levels. In prolonged ER stress conditions that exceed the capacity of initial response, UPR induces CHOP mediated apoptosis signaling [46]. Studies using hepatocytes have reported an association between apoptosis NASH, and reported the crucial role of IRE1 and PERK-mediated CHOP and Bax/Bak activation in this process [47,48]. In the present study, by using the same liver tissues of hypercholesterolemic rabbits developing NASH [18], we determined a significant induction in a couple of ER stress and related apoptosis parameters including Grp94, phospho IRE1 and CHOP. Unexpectedly, high cholesterol diet in rabbits did not affect hepatic PERK phosphorylation. Our *in vivo* finding of phospho IRE1 mediated CHOP induction (without affecting PERK phosphorylation) is also observed in *in vitro* cholesterol accumulation model using hepatocytes.

JNK and p38 are mitogen-activated protein kinases (MAPKs) belonging to serine/threonine protein kinase family and are activated by various ER stress mediated signaling pathway in the cell [32,49]. Activated JNK and p38 are shown to modulate a couple of signaling process in maintaining cellular integrity, including proliferation, apoptosis and inflammation [50–52]. Therefore, JNK and p38 inhibitors might have a therapeutic potential by reducing apoptotic cell death in liver injury. Inhibition of hepatic ASK1, an upstream activator of JNK and p38, is reported to prevent NAFLD and liver fibrosis in mice fed high fat diet [53,54]. Additionally, mice fed high fat diet have developed steatosis via JNK activation, while methionine-choline deficient diet increased hepatic injury by inducing TNF activation through JNK/c-Jun signaling [55].

In regard to the intensity and the duration of stress, activated IRE1 might result in the induction of p38 and JNK, enhancing CHOP mediated apoptotic cell death. Early studies reported that obesity induced ER stress results in IRE1-mediated JNK and p38 activation, leading to liver damage and hepatocyte apoptosis [56–58]. Additionally, p38/JNK activators resulted in increased levels of transaminases, while inhibitor administrations reduced hepatic damage [59]. Another study reported a direct link between p38 expression and NASH in liver tissues of NAFLD patients and in mice fed methionine-choline deficient diet, high fat diet or high cholesterol/high fat diet [60]. *In vitro* studies using primary hepatocytes also determined that cholesterol administration increased apoptotic and necrotic cell death via JNK signaling in an independent manner of ER stress [32]. In the present study, after determining the role of IRE1 branch of ER stress in cholesterol mediated CHOP induction, we tested the status of p38 and JNK, and found that both parameters are increased either in liver tissues of rabbits or hepatocytes. However, α-tocopherol supplementation in hypercholesterolemic rabbits reduced JNK and p38 levels in correlation with the NASH development, whereas only p38 inhibition reversed cholesterol mediated CHOP induction in hepatocytes.

ER stress mediated apoptotic cell death is regulated by multiple pathways in which many factors interact. Considering the importance of fibrosis in NASH development, UPR-mediated apoptosis is emphasized as a key therapeutic goal [61]. In this context, we have recently determined that supplementation of rabbits fed high cholesterol diet with α-tocopherol prevents apoptosis and reverses fibrosis in NASH [18]. Current findings now reveal that CHOP activation induced by cholesterol accumulation occurs in the presence of ER stress and in a p38-dependent manner. We believe that the finding of p38-dependent regulation in CHOP induction, one of the critical processes in the transition from steatosis to NASH, will contribute to the development of novel therapeutic strategies against hypercholesterolemia. Together with our previous findings [62], current study provides convincing results that cholesterol activates ER stress in a manner of IRE1 branch. However, there is still a gap in understanding why high cholesterol diet

stimulate the IRE1/JNK signaling in the heart while acting on the IRE1/p38 in the liver. In addition, further studies are needed to identify the molecular mechanism of cholesterol mediated IRE1 induction.

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Declaration of competing interest

The authors have no conflict of interest to declare.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.08.024.

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