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Hesperetin protects against palmitate-induced cellular toxicity via induction of GRP78 in hepatocytes

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ABSTRACT

Lipotoxicity plays a critical role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Hesperetin, a flavonoid derivative, has anti-oxidant, anti-inflammatory and cytoprotective properties. In the present study, we aim to examine whether hesperetin protects against palmitate-induced lipotoxic cell death and to investigate the underlying mechanisms in hepatocytes. Primary rat hepatocytes and HepG2 cells were pretreated with hesperetin for 30 min and then exposed to palmitate (1.0 mmol/L in primary rat hepatocytes; 0.5 mmol/L in HepG2 cells) in the presence or absence of hesperetin. Necrotic cell death was measured via Sytox green nuclei staining and quantified by LDH release assay. Apoptotic cell death was determined by caspase 3/7 activity and the protein level of cleaved-PARP. The unfolded protein response (UPR) was assessed by measuring the expression of GRP78, sXBP1, ATF4 and CHOP. Results show that hesperetin (50 μmol/L and 100 μmol/L) protected against palmitate-induced cell death and inhibited palmitate-induced endoplasmic reticulum (ER) stress in both primary rat hepatocytes and HepG2 cells. Hesperetin (100 μmol/L) significantly activated sXBP1/GRP78 signaling, whereas a high concentration of hesperetin (200 μmol/L) activated p-eIF2α and caused hepatic cell death. Importantly, GRP78 knockdown via siRNA abolished the protective effects of hesperetin in HepG2 cells. In conclusion, hesperetin protected against palmitate-induced hepatic cell death via activation of the sXBP1/GRP78 signaling pathway, thus inhibiting palmitate-induced ER stress. Moreover, high concentrations of hesperetin induce ER stress and subsequently cause cell death in hepatocytes.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized as increased accumulation of lipids in the liver, has emerged as one of the major health problems around the world. In the pathogenesis of NAFLD, lipotoxicity has been shown to play a key role, especially in the progression of non-alcoholic steatohepatitis (NASH) (Cusi, 2012; Friedman et al. (2018); Fuchs and Sanyal (2012); Neuschwander-Tetri (2010)). The surge of toxic lipids, e.g. palmitic acid (the most abundant dietary and plasma fatty acid), can cause organelle dysfunction (e.g. mitochondrial dysfunction and endoplasmic reticulum (ER) stress) and mal-activation of intracellular signaling that could lead to cell death (Biden et al., 2014; Leamy et al., 2013; Musso et al., 2018).

ER stress is believed to be one of the most important factors in the etiology of NAFLD (Cnop et al., 2012; Friedman et al., 2018; Fu et al., 2011; Puri et al., 2008). Both NAFLD patients and high-fat diet animal models demonstrated the occurrence of ER stress and the subsequent activation of the unfolded protein response (UPR) (Cao et al., 2012; Puri et al., 2008; Wang et al., 2006). Under normal conditions, the UPR is prevented from being activated due to the binding of the ER chaperone protein glucose-regulated protein 78 (GRP78) to three ER transmembrane proteins (inositol-requiring kinase 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor-6α (ATF6α)). In conditions of ER stress, GRP78 is released, allowing activation of PERK, IRE1α, and ATF6 and their downstream signaling pathways. The initiation of the UPR serves as an adaptive response via stimulating the expression of ER chaperone proteins, reducing protein translation and activating specific signaling pathways

Abbreviations: Non-alcoholic fatty liver disease, NAFLD; Unfolded protein response, UPR; Endoplasmic reticulum stress, ER stress; Non-alcoholic steatohepatitis, NASH; Hesperetin, Hst; Glucose-regulated protein 78, GRP78; Eukaryotic initiation factor 2α, eIF2α; C/EBP homologous protein, CHOP; Spliced X-box binding protein 1, sXBP1; c-Jun N-terminal kinases, JNK; Lactate dehydrogenase, LDH; Triglyceride, TG; Activating transcription factor 4/6, ATF4/6; inositol-requiring kinase 1α, IRE1α; PKR-like endoplasmic reticulum kinase, PERK

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(Hetz, 2012). However, prolonged or severe ER stress can also lead to the activation of apoptotic UPR signaling pathways, which have been shown to involve the activation of the p-eIF2α/CHOP and TRAF2/ASK1/JNK pathways (Cazanave et al., 2010; Hetz, 2012; Sano and Reed, 2013).

Hesperetin, a flavonoid derivative from citrus fruits, exhibits many beneficial actions including anti-inflammatory, anti-oxidative and cytoprotective effects. Our group has previously shown that hesperetin protected against Concanavalin A (ConA)-induced fulminant hepatitis in mice and reduced cytokine-induced inflammation in hepatocytes in vitro (Bai et al., 2017). Moreover, several studies reported that hesperetin also protected against hypoxia/reoxygenation (H/R) injury and doxorubicin-induced cardiotoxicity (He et al., 2017; Trivedi et al., 2011). Mechanistically, most of the studies attributed the beneficial effects of hesperetin to the activation of the PI3K/Akt signaling pathway and/or inhibition of activation of NF-κB and/or pro-apoptotic JNK (He et al., 2017; Trivedi et al., 2011). Moreover, our previous studies found that hesperetin induces HO-1 expression, thus reducing oxidative stress in the liver (Bai et al., 2017). However, the protective mechanisms of hesperetin are still largely unclear. Besides, it is not known whether hesperetin protects against lipotoxicity. Therefore, in the present study, we aim to examine the protective effect of hesperetin against lipotoxicity in hepatocytes and explore the underlying mechanisms.

2. Materials and methods

2.1. Primary rat hepatocyte isolation, cell culture and siRNA transfections

Hepatocytes were isolated from male specified pathogen free Wistar rats (180-250 g; Charles River Laboratories Inc. (Wilmington, MA, USA)) by the two-step collagenase perfusion method as described previously (Moshage et al., 1990) and cultured in William's E medium (Invitrogen, Breda, the Netherlands) supplemented with 50 μg/mL gentamicin (Invitrogen, Breda, the Netherlands), 100 U/mLpenicillin, 100 μg/mL streptomycin, 250 ng/mL fungizone (1% PSF, Lonza, Verviers, Belgium) at 37 °C in a 5% (*v*/v) CO_2 containing atmosphere. All experiments were performed according to the Dutch law on the welfare of laboratory animals and guidelines of the ethics committee of the University of Groningen for care and use of laboratory animals. Experiments were started after an attachment period of 4 h.

HepG2 cells and PLC/PRF/5 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HepG2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Breda, the Netherlands) and PLC/PRF/5 cells were maintained in RPMI 1640 medium (Invitrogen). Both media were supplemented with 10% fetal calf serum (Invitrogen, Breda, the Netherlands), 100 U/mLpenicillin, 100 μg/mL streptomycin, 250 ng/mL fungizone (1% PSF, Lonza, Verviers, Belgium). Cells were cultured at 37 °C in a 5% (v/v) CO_2 containing atmosphere.

For the siRNA transfection experiments, HepG2 cells were seeded in plates at 60% confluency. After attachment, cells were transfected with siRNA targeting GRP78, p53 or ALLStar negative control siRNA (Qiagen, Hilden, Germany) using Lipo2000 (Invitrogen, Breda, the Netherlands) according to the manufacturer's protocol. The final concentrations of siRNA were 75 nmol/L. Experiments were started 48 h after transfection and the efficiency of siRNA was assessed by Western-blot analysis.

2.2. Preparation of palmitate-BSA

BSA-conjugated palmitate solution was prepared as described before (Geng et al., 2020). In our previous studies, we have shown that palmitate dose- and time-dependently induces cell death in HepG2 cells (Geng et al., 2020). However, in primary rat hepatocytes, palmitate mainly induces necrotic cell death and exhibits a clear induction of necrotic cell death at 1.0 mmol/L after 24 h incubation (Geng et al., 2020). Therefore, A final pathophysiological relevant palmitate concentration of 0.5 mmol/L or 1.0 mmol/L was achieved by the addition of serum-free cell culture medium and were used in HepG2 cells and primary rat hepatocytes, respectively, in the present study. Of note, these final palmitate concentrations are of 22–45% of the average concentration of blood palmitate in obese individuals (Feng et al., 2017; Nakamura et al., 2003).

2.3. Caspase 3/7 enzyme activity assay

Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS, Invitrogen, the Netherlands) before the addition of caspase cell lysis buffer. Total cell lysates were prepared and caspase-3/7 activity was determined as described previously (Woudenberg-Vrenken et al., 2013).

2.4. Western blot analysis and antibodies

Cell lysates were prepared and subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Western blot method was performed as described by Woudenberg-Vrenken et al. (Woudenberg-Vrenken et al., 2013). The monoclonal antibodies against PARP/cleaved-PARP (Cell Signaling Technology, Leiden, The Netherlands), GRP78 (Cell Signaling Technology), p-eIF2α (Cell Signaling Technology), eIF2α (Cell Signaling Technology), p-JNK (Cell Signaling Technology), JNK (Cell Signaling Technology) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Calbiochem, Amsterdam, The Netherlands) were used to detect specific protein expression. GAPDH was used to ensure equal protein loading. Protein expression was quantified from 3 to 5 independent immunoblots by densitometry with ImageJ (ImageJ; National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/).

2.5. Sytox green nuclear staining

Necrosis was determined after 24 h of treatment. Cells were incubated with Sytox green nucleic acid dye (Invitrogen, Breda, the Netherlands) for 15 min. Fluorescent nuclei were visualized using a Leica DMI6000 fluorescence microscope (Leica, Amsterdam, the Netherlands) at 450–490 nm.

2.6. Lactate dehydrogenase release assay

To quantify necrotic cell death, the lactate dehydrogenase (LDH) released into the medium was determined as described by Woudenberg-Vrenken et al (Woudenberg-Vrenken et al., 2013). Briefly, the percentage of LDH released in the medium vs. the total LDH (in both the medium and cell lysates) was calculated.

2.7. Luminescent-based ATP assay

Cellular ATP level was measured with luminescent ATP detection assay kit (Promega, Leiden, The Netherlands). In brief, primary rat hepatocytes (1×10^4 cells/well) were seeded into black 96 well plates. Experimental procedures were according to manufacturer's instructions. Luminescence was measured and recorded using a Bio-Tek FL600 microplate fluorescent reader (Bio-Tek, Winooski, VT, USA).

2.8. Oil red O staining and triglyceride measurement

Lipid droplets were visualized by Oil red O staining and quantified by measuring intracellular triglyceride (TG) level using a TG measurement kit (Abcam, Cambridge, UK). Staining method and the TG measurement were performed as described before (Geng et al., 2020).

Table 1
Sequences of human and rat primers and probes used for real-time PCR Analysis.

Gene	Sense 5′-3′	Antisense 5′-3′	Probe 5′-3′
18S (human and rat)	CGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	CGCGCAAATTACCCACTCCCGA
Grp78 (human)	TGGTGATCAAGATACAGGTGACCT	GTGTTCCTTGGAATCAGTTTGGT	TCCCCTTACACTTGGTATTGAAACTGTGGG
Grp78 (rat)	AAAGAAGGTCACCCATGCAGTT	CAATAGTGCCAGCATCCTTGT	ACTTCAATGATGCACAGCGGCAAGC
Chop (human)	GGAAATGAAGAGGAAGAATCAAAAAT	GTTCTGGCTCCTCCTCAGTCA	TTCACCACTCTTGACCCTGCTTCTCTGG
Chop (rat)	TCCTGTCCTCAGATGAAATTGG	TCAAGAGTAGTGAAGGTTTTTGATTCT	CACCTATATCTCATCCCCAGGAAACGAAGA
sXbp1 (human and rat)	GCTGAGTCCGCAGCAGGT	CCCAAAAGGATATCAGACTCAGAATC	CCCAGT TGTCACCTCCCCAGAACATCT
Atf4 (human)	CAGCAAGGAGGATGCCTTCT	CCAACAGGGCATCCAAGTC	CCATTTTCTCCAACATCCAATCTGTCCC
Atf4 (rat)	CGGCAAGGAGGATGCCTTT	ACAGAGCATCGAAGTCAAACTCTTT	CCATTTTCTCCAACATCCAATCTGTCCC
P53 (human)	TTCTGTCCCTTCCCAGAAAA	GTTGGCAAAACATCTTGTTGAG	
P53 (rat)	CCATGAGCGTTGCTCTGATG	CAGATACTCAGCATACGGATTTCCT	CGGCCTGGCTCCTCCCCAAC

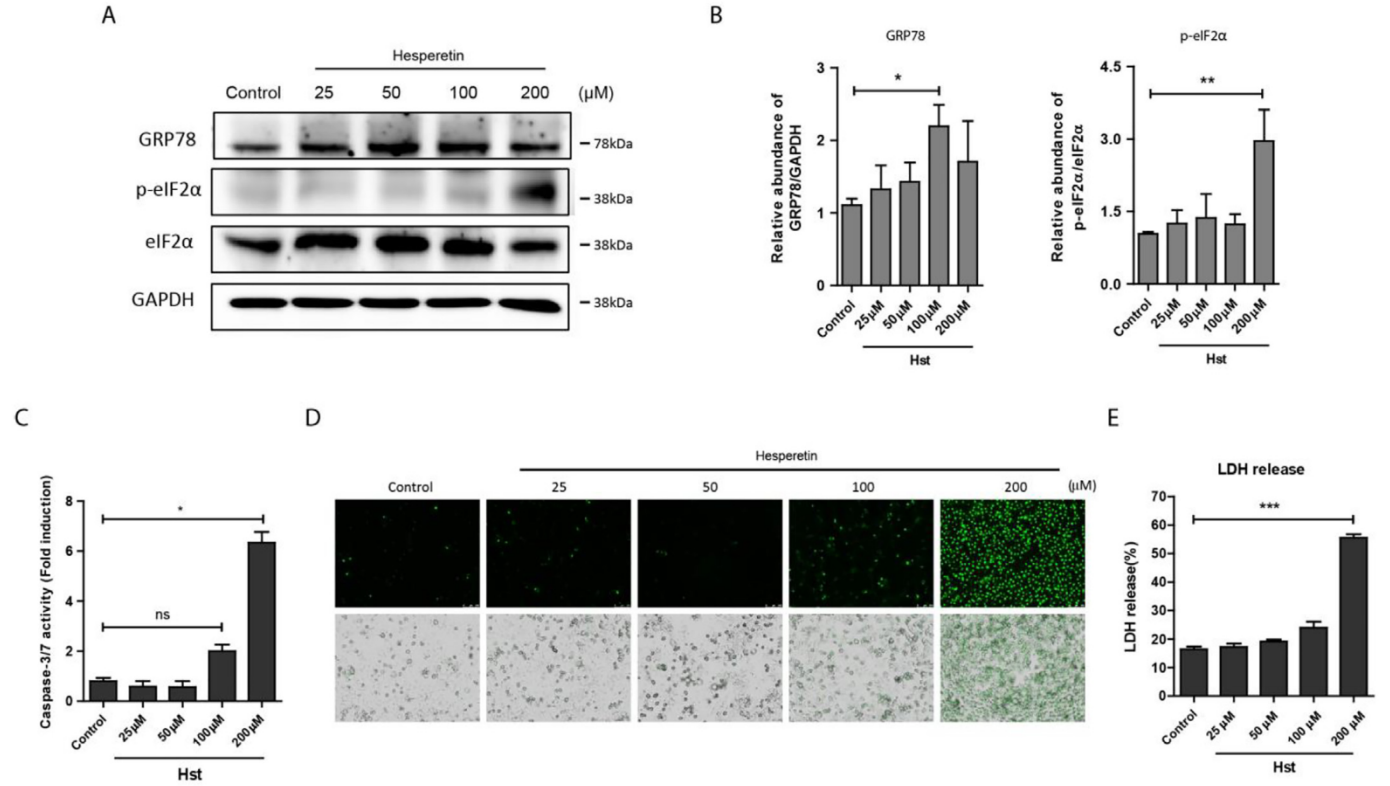

Fig. 1. Hesperetin (Hst) activates UPR signaling and induces cell death at high concentrations in primary rat hepatocytes.
Primary rat hepatocytes were treated with different concentrations of Hst (25, 50, 100, 200 μmol/L) for the time indicated in each experiment. (A and B) After 6 h of treatment, cells were harvested and protein levels of GRP78, p-eIF2α, JNK, eIF2α and GAPDH were measured via Western-blot and quantified from the immunoblots by densitometry with ImageJ. (C) Caspase 3/7 activity was measured after 12 h of treatment. (D) Necrotic cell death was determined using the Sytox green fluorescent dye. Scale bar: 100 μm (E) Necrosis was quantified by measuring LDH release shown as the percentage of total LDH released in the medium after 24 h treatment. Data are shown as mean ± S.D. ($n \geq 3$). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.9. RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated using Tri-reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to manufacturer's instructions. The RNA quantity and quality were measured with the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 2.5 μg RNA was used for reverse transcription. Quantitative real-time PCR was performed on the 7900HT Fast Real-time System (Thermo Fisher Scientific) using the TaqMan method (Eurogentec, Maastricht, The Netherlands) or SYBR Green method (Thermo Fisher Scientific). mRNA levels were normalized to 18S and then further normalized relative to the expression of control groups. The primers and probes are shown in Table 1.

2.10. Statistical analysis

All results are presented as the mean of 3–5 independent experiments ± S.D. For each experiment, statistical analyses were performed using Kruskal-Wallis test, followed by Dunn's multiple comparison tests or Mann-Whitney *U* test; $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Hesperetin activates UPR signaling and a high concentration induces cell death in primary rat hepatocytes

Different concentrations (0, 25, 50, 100, 200 μmol/L) of hesperetin (Hst) were given to primary rat hepatocytes. After 6 h of incubation, Hst

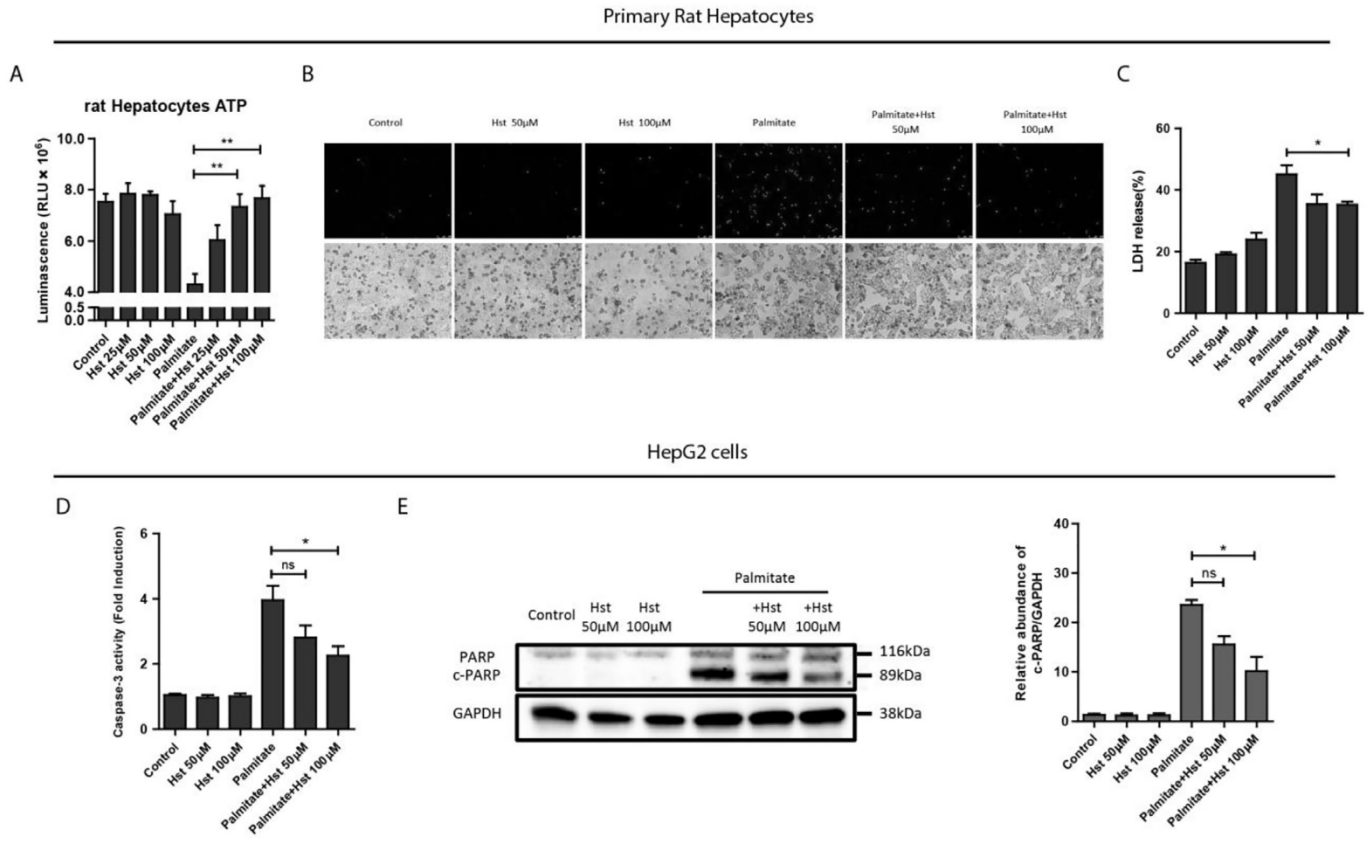

Fig. 2. Hesperetin protects against palmitate-induced cell death in both primary rat hepatocytes and HepG2 cells.
Primary rat hepatocytes or HepG2 cells were pre-treated with Hst (25, 50, 100 μmol/L) for 30 min, then exposed to palmitate with or without Hst. (A) Cellular ATP levels were measured in primary rat hepatocytes after 12 h of exposure to palmitate (1.0 mmol/L). (B and C) Necrotic cell death was determined using the Sytox green fluorescent staining and quantified by measuring LDH release after 24 h of exposure to palmitate (1.0 mmol/L). Scale bar: 100 μm. (D and E) In HepG2 cells, apoptotic cell death was determined after 16 h of exposure to palmitate (0.5 mmol/L) by measuring caspase 3/7 activity and cleaved-PARP (c-PARP) expression via Western-blot and quantification of the immunoblots by densitometry with ImageJ. Data are shown as mean ± S.D. ($n \geq 3$). * indicates $p < 0.05$, ** indicates $p < 0.01$, ns indicates $p > 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

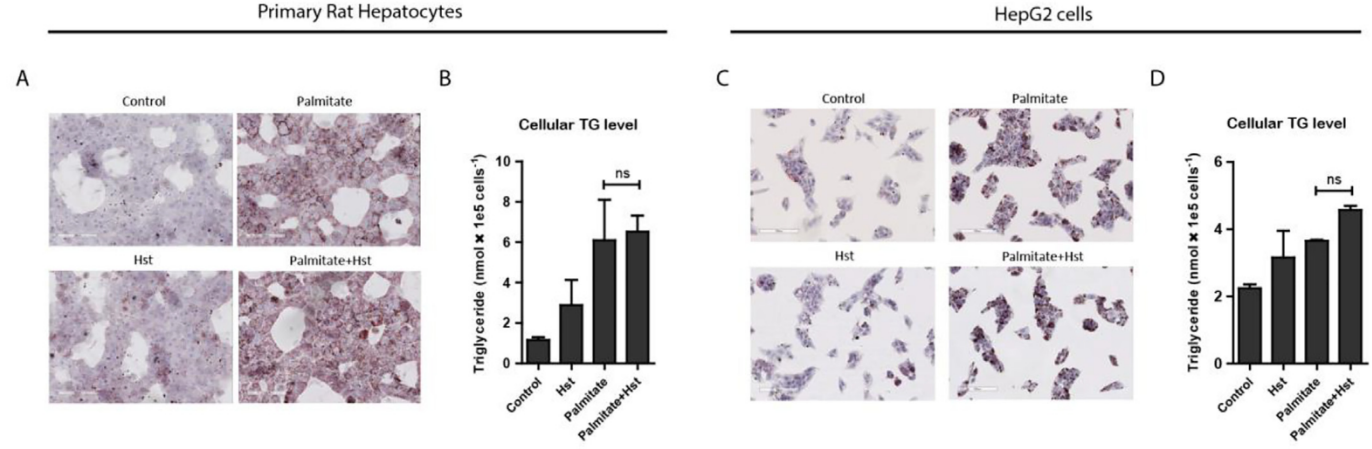

Fig. 3. Hesperetin does not reduce palmitate-induced lipid accumulation in both primary rat hepatocytes and HepG2 cells.
Primary rat hepatocytes or HepG2 cells were pre-treated with Hst (100 μmol/L) for 30 min, and then exposed to palmitate with or without Hst for 12 h. (A and C) Intracellular lipid accumulation was determined by Oil red O staining and (B and D) quantified by measuring cellular TG levels. Scale bar: 100 μm. Data are shown as mean ± S.D. (n ≥ 3). ns indicates $p > 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activated UPR signaling, demonstrated by the increased protein levels of GRP78 and p-eIF2α (Fig. 1A and B). Specifically, Hst increased the protein level of GRP78 statistically significant at 100 μmol/L ($p < 0.05$). However, at 200 μmol/L, the abundance of GRP78 was lower than that at 100 μmol/L. This could be due to the toxicity of Hst at 200 μmol/L, which is indicated by the observation that Hst only significantly increased the level of p-eIF2α at 200 μmol/L ($p < 0.01$). Moreover, after 12 h of treatment, Hst at 200 μmol/L induced apoptotic

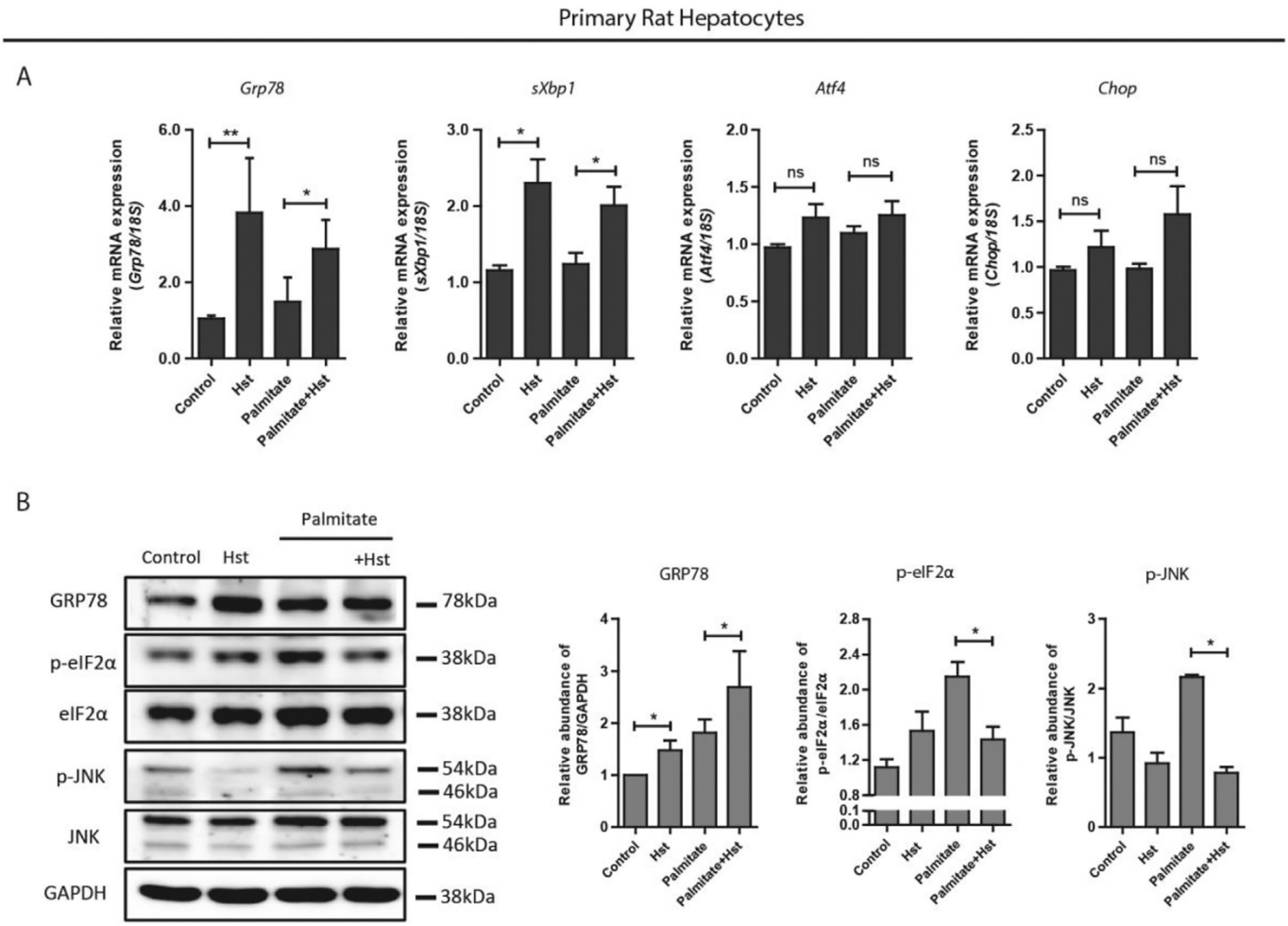

Fig. 4. Hesperetin activates sXbp1/GRP78 signaling pathway but inhibits palmitate-induced ER stress in primary rat hepatocytes.
Primary rat hepatocytes were pre-treated with Hst (100 μmol/L) for 30 min, and then exposed to palmitate (1.0 mmol/L) with or without Hst for 6 h. (A) mRNA levels *of Grp78, sXbp1, Atf4* and *Chop* were measured and normalized to *18S*. (B) Protein levels of GRP78, p-eIF2α, eIF2α, p-JNK, JNK and GAPDH were determined by Western-blot and quantified from the immunoblots by densitometry using ImageJ. Data are shown as mean ± S.D. (n ≥ 3). * indicates $p < 0.05$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

cell death, as shown by a significantly increased caspase 3/7 activity ($p < 0.05$, Fig. 1C). In addition, after 24 h of treatment, Hst at 200 μmol/L also induced necrotic cell death, as demonstrated by the positive Sytox Green nuclear staining (Fig. 1D) and significantly increased LDH release ($p < 0.001$, Fig. 1E). These results indicate that Hst activates UPR signaling pathways and induces hepatic cell death at high concentrations (≥ 200 μmol/L).

3.2. Hesperetin protects against palmitate-induced cell death in hepatocytes

Our previous studies showed that palmitate induces mainly necrosis, but not apoptosis in primary rat hepatocytes, whereas palmitate induces mainly apoptotic cell death in HepG2 cells (Geng et al., 2020). In the present study, we found that Hst (50 μmol/L and 100 μmol/L) protected against palmitate-induced cell death in both primary rat hepatocytes and HepG2 cells. In primary rat hepatocytes, Hst (50 μmol/L and 100 μmol/L) significantly preserved cellular ATP levels that were depleted after incubation with palmitate (1.0 mmol/L) ($p < 0.05$, Fig. 2A). Hst (100 μmol/L) also inhibited palmitate-induced necrosis, as shown by the reduced Sytox green nuclear staining (Fig. 2B) and significantly decreased LDH release ($p < 0.05$, Fig. 2C). In HepG2 cells, the protective effect of Hst (100 μmol/L) is demonstrated by the significantly reduced caspase 3/7 activity and decreased generation of cleaved-PARP that induced by palmitate ($p < 0.05$, Fig. 2D and E).

3.3. Hesperetin does not reduce palmitate-induced cellular lipid accumulation

Since lipotoxicity is related to cellular lipid accumulation, we determined the intracellular lipid content via Oil red O staining and cellular TG measurement. As shown in Fig. 3A and C, hesperetin (100 μmol/L) did not influence lipid accumulation in both primary rat hepatocytes and HepG2 cells. Similarly, Hst also did not affect cellular TG level in both primary rat hepatocytes and HepG2 cells ($p > 0.05$, Fig. 3B and D). The results indicate that the protective effect of Hst is not mediated via modulating cellular lipid accumulation.

3.4. Hesperetin activates sXBP1/GRP78 signaling pathway and inhibits palmitate-induced ER stress in primary rat hepatocytes

As indicated before, Hst stimulates UPR in primary rat hepatocytes. On the other hand, palmitate also stimulates UPR and induces ER stress in hepatocytes. As shown in Fig. 4A and B, Hst (100 μmol/L) significantly upregulated GRP78 expression, at both mRNA and protein levels ($p < 0.05$), while it inhibited palmitate-induced ER stress, as demonstrated by significantly reduced p-eIF2α and p-JNK levels ($p < 0.05$). Moreover, Hst (100 μmol/L) also significantly increased the expression of *sXbp1* ($p < 0.05$) but did not increase the expression of *Atf4* and *Chop* ($p > 0.05$, Fig. 4A), indicating a selective activation

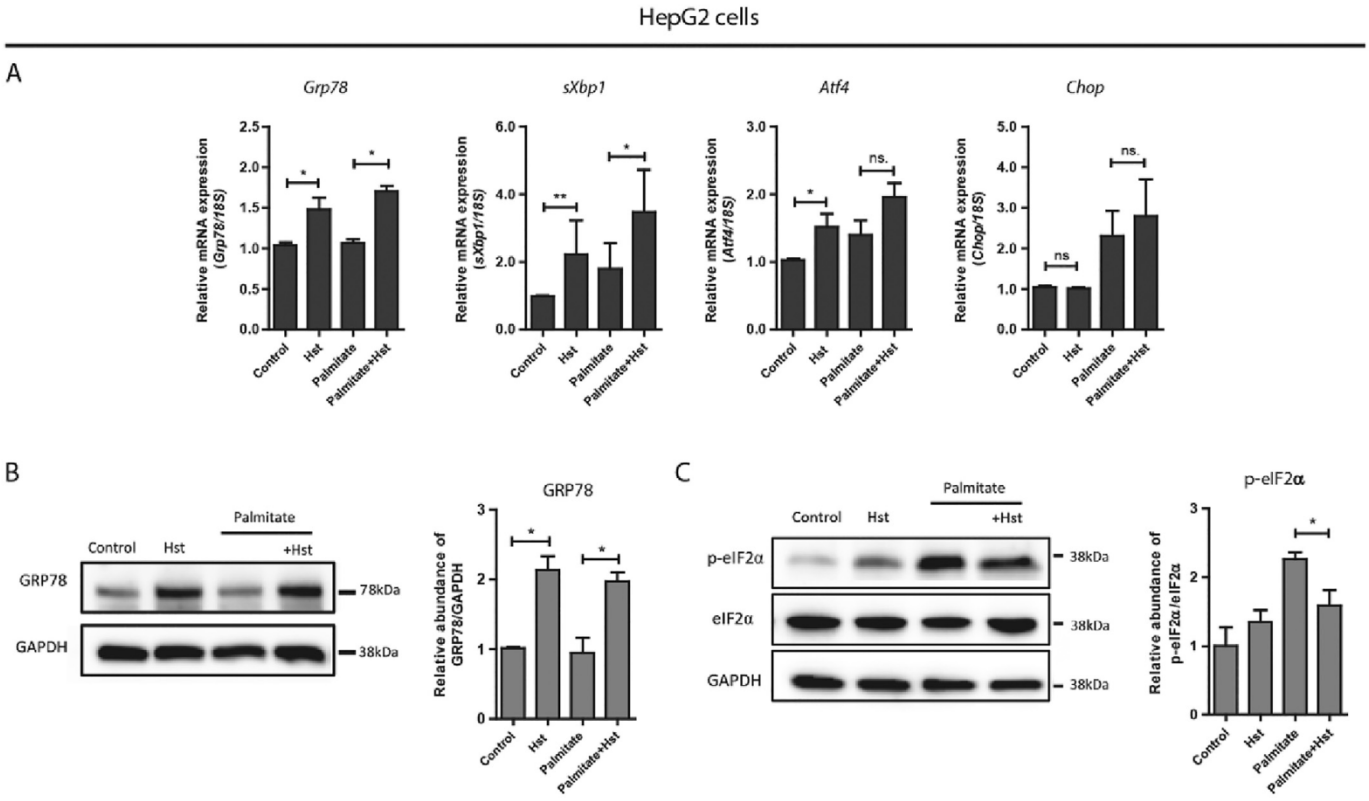

Fig. 5. Hesperetin activates sXbp1/GRP78 signaling pathway and inhibits palmitate-induced ER stress in HepG2 cells.
HepG2 cells were pre-treated with Hst (100 μmol/L) for 30 min, and then exposed to palmitate (0.5 mmol/L) with or without Hst. After 6 h of exposure to palmitate, (A) mRNA levels *of Grp78, Sxbp1, Atf4* and *Chop* were measured and normalized to *18S*. (B) Protein levels of GRP78 and GAPDH were determined by Western-blot and quantified from the immunoblots by densitometry using ImageJ. After 12 h of exposure to palmitate, (C) protein levels of p-eIF2α, eIF2α, and GAPDH were determined by Western-blot and quantified from the immunoblots by densitometry using ImageJ. Data are shown as mean ± S.D. (n ≥ 3). * indicates $p < 0.05$, ** indicates $p < 0.01$, ns indicates $p > 0.05$.

of UPR branches.

3.5. Hesperetin activates sXBP1/GRP78 signaling pathway and inhibits palmitate-induced ER stress in HepG2 cells

In line with the results observed in primary rat hepatocytes, Hst (100 μmol/L) also induced the expression of GRP78 in HepG2 cells, at both mRNA and protein levels ($p < 0.05$, Fig. 5A and B) and significantly increased the expression of *sXbp1* ($p < 0.05$), but not *Atf4* or *Chop* ($p > 0.05$, Fig. 5A). Since in HepG2 cells palmitate (0.5 mmol/L) time-dependently induced ER stress, shown as a clear induction of GRP78 and p-eIF2α after 12 h of treatment (Supplemental Fig. 1), we investigated ER stress markers at 12 h. As shown in Fig. 5C, Hst (100 μmol/L) significantly reduced p-eIF2α induced by palmitate ($p < 0.05$, Fig. 5C). The results in both primary rat hepatocytes and HepG2 cells indicate that Hst may protect against palmitate-induced cell death by inhibiting ER stress via activating the sXBP1/GRP78 signaling pathway.

3.6. Silencing GRP78 abolishes the protective effect of Hesperetin in HepG2 cells

In order to check whether the protective effect of Hst is dependent on the induction of the ER chaperone protein GRP78, we blocked GRP78 expression via siRNA transfection in HepG2 cells. As shown in Fig. 6A and B, after transfection with siGRP78 (75 nmol/L) for 48 h, HepG2 cells exhibited a 62% reduction of GRP78 expression. Importantly, Hst (100 μmol/L) did not protect against palmitate-induced cell death in siGrp78-transfected cells, demonstrated by the loss of its ability to reduce palmitate-induced caspase 3/7 activity as compared to control siRNA-transfected cells (Fig. 6B). This result implies that the protective effects of hesperetin are dependent on its induction of GRP78 expression.

4. Discussion

In this study, we showed that hesperetin protected against palmitate-induced cell death in hepatocytes. Its protective effects depend on the induction of GRP78 expression and the subsequent inhibition of palmitate-induced ER stress. Moreover, the increased expression of GRP78 is mainly mediated via activation of sXBP1/GRP78 signaling. Meanwhile, high concentrations of hesperetin lead to cell death.

As a dynamic signaling network aiming at restoring homeostasis, the UPR can lead to opposite cell fates: survival or death, via selective activation of its downstream signaling pathways (Hetz, 2012; Sano and Reed, 2013; Szegezdi et al., 2006; Walter et al., 2015). The classic UPR consists of three signaling pathways, initiated by three ER stress sensors. Several studies have suggested that these three UPR signaling pathways could be selectively activated and that they show differential sensitivities to specific inducers, thus leading to context-dependent cellular responses. For example, IRE1 and PERK are more sensitive to the release of ER Ca^{2+} than ATF6 (DuRose et al., 2006), while only the ATF6 arm was selectively activated in response to the expansion of ER membrane (Maiuolo et al., 2011). In the present study, we showed that hesperetin dose-dependently activated sXBP1/GRP78 signaling at the non-toxic concentrations (25–100 μmol/L), whereas at a higher concentration (200 μmol/L), it significantly activated p-eIF2α and caused cell death (Fig. 1). Importantly, the protective effect of hesperetin against palmitate-induced cell death was dependent on the increased GRP78 expression. Similarly, Chan et al. also observed the beneficial

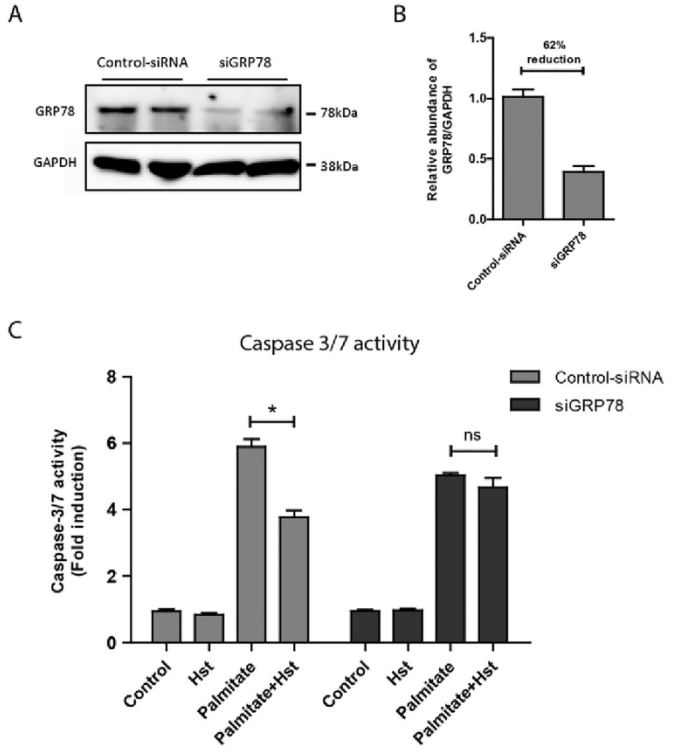

Fig. 6. Silencing GRP78 abolishes the protective effects of hesperetin in HepG2 cells.

HepG2 cells were transfected with control-siRNA or siGRP78 (75 nmol/L) for 48 h, then pre-treated with Hst (100 μmol/L) for 30 min and exposed to palmitate (0.5 mmol/L) with or without Hst for another 16 h. (A and B) Protein levels of GRP78 and GAPDH were measured after transfection by Western-blot and quantified from the immunoblots by densitometry using ImageJ. (C) Caspase 3/7 activity was measured after treatment with Hst and palmitate. Data are shown as mean ± S.D. (n ≥ 3). * indicates $p < 0.05$, ns indicates $p > 0.05$.

actions of sXBP1 against palmitate or pro-inflammatory cytokine-induced cell death in pancreatic β-cells (Chan et al., 2015). Specifically, inhibition of XBP1 could potentiate palmitate or pro-inflammatory cytokine-induced cell death and this response could be inhibited by CHOP inhibition, which indicated a reciprocal regulation between XBP1-mediated adaptive and CHOP-dependent apoptotic UPR signaling. Moreover, the protective response of sXBP1 could also relate to the reduced cellular oxidative stress via transcriptionally induced catalase expression or alleviated protein aggregation (Liu et al., 2009; Pirog et al., 2019).

Although we showed that hesperetin could selectively activate the UPR signaling pathways, it is not clear yet how the sXBP1/GRP78 arm is stimulated. It has been shown that loss of p53 could enhance GRP78 expression and activate the IRE1α/XBP1 pathway (Lopez et al., 2017; Namba et al., 2015). In our study, we observed that in p53 mutant human hepatoma PLC/PRF/5 cells, which have a dysfunctional p53 gene, hesperetin indeed did not activate sXBP1/GRP78 signaling and could not protect against palmitate-induced cell death, as demonstrated by the lack of induction of sXBP1 or GRP78 and did not reduce palmitate-induced caspase 3/7 activity and c-PARP level (Supplemental Fig. 2), which implies the need of intact p53 for the protective actions of hesperetin. Meanwhile, although both primary rat hepatocytes and HepG2 cells possess wild-type p53, hesperetin significantly reduced the expression of *p53* in primary rat hepatocytes but not in HepG2 cells (Supplemental Fig. 3A), which might be caused by differences in the metabolism of Hst in primary rat hepatocytes and HepG2 cells. It has been shown that the levels of cytochrome P450 enzymes are reduced in HepG2 cells compared to primary human hepatocytes (Gerets et al., 2012; Westerink and Schoonen, 2007). In addition, knockdown of p53 via siRNA did not lead to increased *Grp78* expression in both primary rat hepatocytes and HepG2 cells (Supplemental Fig. 3B). Thus, whether the activated UPR signaling is related to decreased *p53* expression and how hesperetin exactly activates UPR signaling still need to be investigated in more detail.

An interesting finding in our study is that low concentrations of hesperetin are protective whereas high concentrations are toxic. It is possible that hesperetin, at low doses, induces a low level of ER stress, which protects against a subsequent, more severe induction of ER stress, in our case palmitate. This phenomenon has also been observed in other settings, e.g. ischemic preconditioning, in which exposure to a low dose of oxidative stress (pre-conditioning) protects against a subsequent, more severe exposure to oxidative stress (ischemia). In previous studies, we have also shown this protective pre-conditioning in bile acid-induced toxicity (Verhaag et al., 2016). In general, this phenomenon is known as hormesis (Bhakta-Guha and Efferth, 2015; Li et al., 2019; Matai et al., 2019; Zhou et al., 2019) and it is possible that hesperetin also acts in a similar way.

Hesperetin belongs to the flavonoid family, which has long been known for its biomedical potentials (Bai et al., 2017; He et al., 2017; Trivedi et al., 2011; Yang et al., 2012). Its beneficial actions have been related to the activation of PI3K/Akt signaling pathway, inhibition of NF-κB signaling and induction of HO-1 expression. In our study, we show for the first time that hesperetin also protects against palmitate-induced cell death via modulating UPR signaling pathways, specifically via stimulating the sXBP1/GRP78 arm. These results suggest that hesperetin might have therapeutic potentials in the treatment of NAFLD. However, as shown in our results, high concentrations of hesperetin can also activate the apoptotic UPR signaling (p-eIF2α) arm and induce hepatic cell death. Therefore, the use of hesperetin in the treatment of NAFLD still needs more detailed evaluation.

In conclusion, in our present study, we show that hesperetin protects against palmitate-induced hepatic cell death via activation of the sXBP1/GRP78 signaling pathway and inhibiting ER stress. Meanwhile, high concentrations of hesperetin could activate apoptotic UPR signaling and induce hepatic cell death.

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Declaration of Competing Interest

The authors acknowledge no conflicts of interest with this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2020.115183.

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