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Scopoletin and umbelliferone protect hepatocytes against palmitate- and bile acid-induced cell death by reducing endoplasmic reticulum stress and oxidative stress

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ABSTRACT

Background: The number of patients with non-alcoholic fatty liver disease (NAFLD) is rapidly increasing due to the growing epidemic of obesity. Non-alcoholic steatohepatitis (NASH), the inflammatory stage of NAFLD, is characterized by lipid accumulation in hepatocytes, chronic inflammation and hepatocyte cell death. Scopoletin and umbelliferone are coumarin-like molecules and have antioxidant, anti-cancer and anti-inflammatory effects. Cytoprotective effects of these compounds have not been described in hepatocytes and the mechanisms of the beneficial effects of scopoletin and umbelliferone are unknown.

Aim: To investigate whether scopoletin and/or umbelliferone protect hepatocytes against palmitate-induced cell death. For comparison, we also tested the cytoprotective effect of scopoletin and umbelliferone against bile acid-induced cell death.

Methods: Primary rat hepatocytes were exposed to palmitate (1 mmol/L) or the hydrophobic bile acid glycochenodeoxycholic acid (GCDCA; 50 μmol/L). Apoptosis was assessed by caspase-3 activity assay, necrosis by Sytox green assay, mRNA levels by qPCR, protein levels by Western blot and production of reactive oxygen species (ROS) by fluorescence assay.

Results: Both scopoletin and umbelliferone protected against palmitate and GCDCA-induced cell death. Both palmitate and GCDCA induced the expression of ER stress markers. Scopoletin and umbelliferone decreased palmitate- and GCDCA-induced expression of ER stress markers, phosphorylation of the cell death signaling intermediate JNK as well as ROS production.

Conclusion: Scopoletin and umbelliferone protect against palmitate and bile acid-induced cell death of hepatocytes by inhibition of ER stress and ROS generation and decreasing phosphorylation of JNK. Scopoletin and umbelliferone may hold promise as a therapeutic modality for the treatment of NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has become an increasingly common liver disease in parallel with the increasing prevalence of obesity and metabolic syndrome. Non-alcoholic steatohepatitis (NASH) is the inflammatory stage of NAFLD and is characterized by steatosis (lipid accumulation in hepatocytes), chronic inflammation and liver injury (Neuschwander-Tetri, 2017). NAFLD is frequently associated with metabolic syndrome and diabetes mellitus type 2 (Geng et al., 2020a). Lipotoxicity caused by saturated fatty acids (SFA) plays an important role in the pathogenesis of non-alcoholic fatty liver disease (Malhi et al., 2006). The excess supply of circulating free fatty acids causes changes in metabolic pathways in hepatocytes leading to oxidative stress, mitochondrial dysfunction and endoplasmic reticulum (ER) stress (Geng et al., 2020a). Irreversible damage to hepatocytes leads to cell death and aggravates inflammation. In a murine model of high fat diet-induced NAFLD, steatotic hepatocytes were shown to be more sensitive to TNF-α induced apoptosis via the ASK1-JNK signaling

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; SCO, Scopoletin; UMB, Umbelliferone; GCDCA, Glycochenodeoxycholic acid; ROS, Reactive oxygen species; SFA, Saturated fatty acids; ER, Endoplasmic reticulum; MAPKs, Mitogen-activated protein kinases; GRP78, Glucose-regulated protein 78 kDa; ATF4, Activating transcription factor 4; CHOP, C/EBP homologous protein; UPR, Unfolded protein response; ASK1, Apoptosis signal-regulating kinase-1; CYP, Cytochrome P450; HO-1, Heme oxygenase-1.

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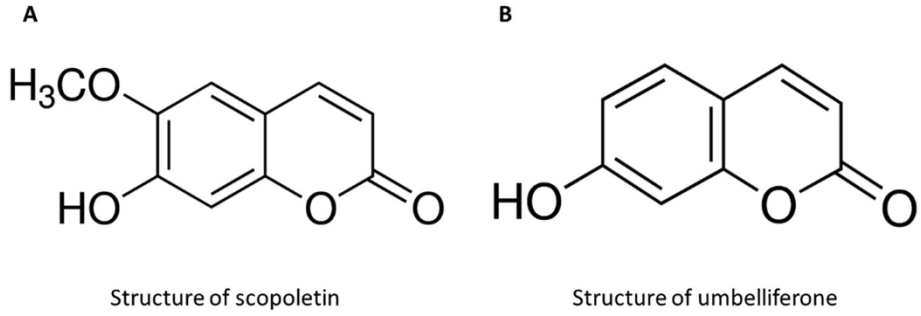


Fig. 1. Structure of scopoletin (A) and umbelliferone (B).

pathway (Zhang et al., 2010a). In addition, steatotic hepatocytes release extracellular vesicles that promote hepatic inflammation and fibrogenesis (Hernández et al., 2020). Mitogen-activated protein kinases (MAPKs) signaling pathways have long been recognized as central pathways that mediate metabolic stress-induced cellular responses (Jiménez-Castro et al., 2019). Many of these stress pathways lead to the phosphorylation and activation of JNK. Activated p-JNK causes cell death and has been shown to play a role in liver damage of various etiology, e.g. acetaminophen-induced liver injury, TNFα-induced liver injury and ischemia-reperfusion (I-R) injury (Kang et al., 2011; Lee et al., 2006). The role of the MAPK kinase ERK1/2 in cell death is controversial. Activation of ERK1/2 has been reported to both inhibit as well as aggravate cell death. It has been reported that the ERK1/2 pathway promotes caspase-3 mediated apoptosis in a murine model of cisplatin-induced acute renal failure (Jo et al., 2005). On the other hand, it has been shown that bee venom-induced apoptosis in human leukemic U937 cells is triggered by activation of p38 MAPK and JNK via down-regulation of the ERK and Akt signaling pathways (Moon et al., 2006). Previously, we have shown that ERK1/2 activation is important in the protective effect of the hydrophilic bile acid tauroursodeoxycholic acid against hydrophobic bile acid-induced apoptosis (Schoemaker et al., 2004).

We have previously shown that extracts of the plant *Ipomoea stolonifera* protect against inflammation (Cai et al., 2014). Five components have been identified in these extracts: hesperetin, esculetin, curcumin, umbelliferone and scopoletin. We have investigated the cytoprotective effect of hesperetin in models of liver damage in vivo and in vitro (Bai et al., 2017). More recently, we have investigated the protective action of hesperetin against palmitate-induced hepatocyte death and demonstrated that hesperetin prevents palmitate-induced ER stress (Geng et al., 2020b). However, little is known about the protective effects of umbelliferone or scopoletin. Hesperetin is a flavonoid, whereas umbellifeone and scopoletin are coumarin-like compounds Fig. 1. Scopoletin (SCO) is a naturally occurring coumarin and has been shown to have antioxidant, anti-inflammatory, anti-diabetic, anti-microbial and anti-cancer properties (Sakthivel et al., 2021). The mechanisms of action of scopoletin are very diverse and dependent on the cell type, organ and experimental model or disease studied: scopoletin has been proposed to act via the antioxidant Nrf2 pathway, the pro-inflammatory NF-κB pathway, the Stat-3 pathway, P53 signaling, hypoxia signaling, Wnt-β signaling, Notch signaling, autophagy, etc. (Sakthivel et al., 2021). In a rat model of spinal cord injury, it was shown that SCO improved functional recovery by activation of the AMPK/mTOR pathway and the induction of autophagy. In this model, SCO also decreased caspase-3 activity (Zhou et al., 2020). Moreover, SCO ameliorated hepatic steatosis and inflammation in a model of high fat diet fed diabetic mice. These beneficial effects were due to down-regulation of genes involved in triglyceride and cholesterol synthesis as well as inhibition of the inflammatory TLR4-MyD88-NF-κB pathway (Choi et al., 2017). Similar properties and mechanisms of action have been proposed for the coumarin umbelliferone (Garg et al., 2020). UMB has been shown to significantly alleviate alcoholic fatty liver and reduce alcohol-induced liver injury by inhibiting SREBP-1c and PPAR-γ-mediated lipogenesis and increasing PPAR-α-induced fatty acid oxidation. Moreover, UMB reduced CYP2E1 activity, thus reducing generation of reactive oxygen species and hepatocyte injury (Kim et al., 2014). In addition, UMB has renoprotective effects in a mouse model of cisplatin-induced acute kidney injury via suppression of RIPK1/RIPK3/MLKL-mediated necroptosis (Wu et al., 2020). Although hepatoprotective effects of SCO and UMB and other coumarin-like molecules have been described before, the protective mechanisms of SCO and UMB, in particular with regard to alleviating oxidative and ER stress have not been extensively described yet (Kang et al., 1998; Shalkami et al., 2021; Mahmoud et al., 2017; Mahmoud et al., 2019; Atmaca et al., 2011).

In the present study, we investigated the effect of umbelliferone and scopoletin on palmitate-induced hepatocyte death. For comparison, we also investigated the effect of UMB and SCO on glycochenodeoxycholic acid-induced apoptosis.

2. Materials and methods

2.1. Animals, primary hepatocyte isolation and HepG2 cell line

Specified pathogen-free male Wistar rats (180–250 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). Rats were housed under standard laboratory conditions with free access to standard laboratory chow and water. All experiments were performed according to the Dutch law on the welfare of laboratory animals and guidelines of the ethics committee of University of Groningen for care and use of laboratory animals.

Hepatocytes were isolated by two-step collagenase perfusion method as described previously (Moshage et al., 1990). Primary rat hepatocytes were plated at 1.125×10^6 cells per well in 6-well plates corresponding to a confluency of 90%. The cells were cultured in William's E medium (Invitrogen, Breda, the Netherlands) supplemented with 50 μg/ml gentamicin (Invitrogen), 100 U/ml penicillin (Lonza, Vervier, Belgium), 10 μg/ml streptomycin (Lonza) and 250 ng/ml fungizone (Lonza) at 37 °C in an atmosphere containing 5% (*v*/v) CO_2. The experiments were started after an attachment period of 4 h.

The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml fungizone. During experiments, cells were cultured in serum-free medium. HepG2 cells were only used to study the protective effect of umbelliferone and scopoletin against palmitic acid-induced toxicity, since HepG2 cells lack the bile salt importer NTCP and are therefore not susceptible to GCDCA-induced toxicity. HepG2 cells were exposed for 16 h to 0.5 mmol/l palmitate as described previously (Geng et al., 2020a).

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

Gene	Sense 5′-3′	Antisense 5′-3’	Probe 5′-3’
18 s	CGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	CGCGCAAATTACCCACTCCCGA
CHOP	TCCTGTCCTCAGATGAAATTGG	TCAAGAGTAGTGAAGGTTTTTGATTCT	CACCTATATCTCATCCCCAGGAAACGAAGA
Grp78	AAAGAAGGTCACCCATGCAGTT	CAATAGTGCCAGCATCCTTGT	ACTTCAATGATGCACAGCGGCAAGC
ATF4	CTATCTCCATTCTACTACTACCAGATCGA	CCTGGGCCTCAGCTTCTCAT	CCCTGGAAGACCCACATCTGG CAG
HO-1	CACAGGGTGACAGAAGAGGCTAA	CTGGTCTTTGTGTTCCTCTGTCAG	CAGCTCCTCAAACAGCTCAATGTTGAGC

2.2. Reagents

The following reagents were used at the doses described in the text and figure legends: umbelliferone (cat. # 93979), scopoletin (cat. # 92615), sodium palmitate (cat. # 408–35-5), glycochenodeoxycholic acid (cat. # 16564–43-5) and the ERK inhibitor U0126 (cat. # 19–147). All reagents were purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands. Palmitate was complexed to bovine serum albumin as previously described (Geng et al., 2020a).

2.3. Sytox green nuclear staining

After exposure to SCO, UMB, palmitic acid and GCDCA for 24 h, hepatocytes were incubated with Sytox green nucleic acid dye (Invitrogen) for 30 min. Sytox green is only taken up by necrotic cells with a compromised plasma membrane. Fluorescent nuclei were visualized using a Leica DMI6000 microscope at 450–490 nm (Amsterdam, The Netherlands).

2.4. Caspase-3 activity assay

Caspase-3 fluorimetric assay was used to determine apoptosis. Caspase-3 enzyme activity was determined as described previously (Schoemaker et al., 2004). Specific inhibitor of ERK U0126 (Sigma-Aldrich, Zwijndrecht, The Netherlands) was prepared as a 10 mmol/l stock solution and 10 μmol/l was used as final concentration in this study. Cells were harvested and washed twice with cold PBS before addition of caspase cell lysis buffer. Arbitrary units of fluorescence (AUF) were quantified by Synergy-4 (Bio-Tek, Winooski, VT, USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

2.5. RNA isolation and Real-time RT-PCR

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

2.6. Measurement of reactive oxygen species (ROS)

Intracellular reactive oxygen species (ROS) levels were measured using dihydroethidium (DHE) (Thermo). Primary hepatocytes were seeded on 96-well plates. After 4 h attachment, the medium was replaced with medium containing SCO and UMB and cells were treated with GCDCA and palmitate. After treatment, cells were incubated with 5 μmol/l DHE for 30 min in the dark. Fluorescence was recorded at Ex/Em wavelengths of 518/605 nm using a Bio-Tek FL600 microplate fluorescence reader (Bio-Tek).

2.7. Western blot analysis and antibodies

After attachment, cells were treated for 4 h with GCDCA and for different time intervals with palmitate. Protein samples were prepared lysis buffer (HEPES 25 mmol/l, KAc 150 mmol/l, EDTA pH 8.0, 2 mmol/l, 0.1% NP-40, NaF 10 mmol/l, PMSF 50 mmol/l, aprotinin 1 μg/l, pepstatin 1 μg/ml, leupeptin 1 μg/μl, DTT 1 mmol/l). Protein concentration was quantified by Bio-Rad protein assay. Gel electrophoresis was performed on precast 4–15% gels (BioRad, Veenendaal, The Netherlands), followed by transblotting to 0.2 μm nitrocellulose membrane (BioRad). Membranes were blocked with 5% nonfat dry milk and 1% BSA and then incubated with primary antibody overnight at 4 °C. Proteins were detected using the primary antibodies listed in Table 2.

2.8. Statistical analysis

Data were presented as mean ± standard deviation (mean ± SD) or mean ± standard error of mean (mean ± SEM) of at least three independent experiments. For each experiment, statistical analyses were performed using the one-way ANOVA test, followed by Tukey's multiple comparison tests of Mann Whitney test; $p < 0.05$ was considered as statistically significant. $P < 0.001$ was considered highly significant. Analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Scopoletin and umbelliferone protect against palmitic acid-induced necrosis of primary rat hepatocytes

Different concentrations of SCO (6.25 μmol/l to 200 μmol/l) and UMB (6.25 μmol/l to 200 μmol/l) were used to assess toxicity and optimize the dosage. Sytox green penetrates disrupted membranes and visualizes cell death. Umbelliferone and scopoletin alone at concentrations below 100 μmol/l did not induce significant necrosis. On the contrary, higher concentrations of UMB and SCO were clearly toxic, leading to cell death. At 200 μmol/l both UMB and SCO induced significant necrosis. At 100 μmol/l both UMB and SCO started to induce necrosis. For these reasons, we used SCO and UMB at concentrations of 6.25 μmol/l to 50 μmol/l to investigate the protective effect. Palmitic acid alone induced significant necrosis (Figure 2). Palmitic acid-induced necrosis was significantly inhibited by both UMB and SCO at concentrations from 12.5 to 50 μmol/l.

3.2. Scopoletin and umbelliferone protect against glycochenodeoxycholic acid-induced apoptosis in primary rat hepatocytes

Primary rat hepatocytes were treated with glycochenodeoxycholic acid (GCDCA; 50 μmol/l) for 2 to 6 h. GCDCA increased caspase-3 activity in a time-dependent manner (Figure 3C). Previous studies have shown that caspase-3 activity peaks at 4 h of GCDCA treatment (Schoemaker et al., 2004). Therefore, all subsequent studies were performed at 4 h of GCDCA treatment. Cells were pretreated for 30 min with different concentrations of SCO (25 μmol/l, 50 μmol/L, 100 μmol/l) and UMB (25 μmol/l, 50 μmol/l, 100 μmol/l) followed by the addition of GCDCA for 4 h. Both SCO and UMB protect against GCDCA-induced

Table 2
Primary antibodies.

Protein	Species	Dilution	Company
GAPDH	Polyclonal mouse	1:1000	CB1001, Calbiochem (Amsterdam, The Netherlands)
Phospho-JNK	Polyclonal rabbit	1:1000	44-682G, Cell Signaling (Leiden, the Netherlands)
Phospho-ERK	Polyclonal mouse	1:2000	9106, Cell Signaling
JNK	Polyclonal rabbit	1:1000	9252, Cell Signaling
ERK	Polyclonal rabbit	1:1000	Sc-94, Santa Cruz

caspase-3 activity at all doses tested (Figure 3 F, G). Both at 4 h and 24 h of exposure to GCDCA, the combinations with SCO or UMB did not induce necrosis as shown by Sytox green staining (Figure 3 A-B, D-E), demonstrating that SCO and UMB did not shift the mode of cell death from apoptosis to necrosis.

3.3. Scopoletin and umbelliferone inhibit glycochenodeoxycholic acid and palmitic acid-induced endoplasmic reticulum stress

CHOP, ATF4 and GRP78 expression were used as parameters of the unfolded protein response (UPR), indicative of ER stress (Zheng et al., 2014). Both GCDCA and palmitic acid induced ER stress as demonstrated by increased mRNA expression of CHOP and ATF4 at 4 h and 8 h respectively Fig. 4. Both SCO and UMB significantly decreased GCDCA-induced mRNA expression of CHOP and ATF4. Only SCO decreased palmitic acid-induced mRNA expression of CHOP and ATF4. Both SCO and UMB induced mRNA expression of the chaperone protein GRP78 Fig. 4.

3.4. Scopoletin and umbelliferone decrease palmitate- and glycochenodeoxycholic acid induced oxidative stress

GCDCA and palmitate generate ROS (Figure 5 A, B). UMB and SCO both reduced GCDCA- and palmitic acid-induced ROS generation (Figure 5 A, B). The reduction of palmitic acid-induced ROS generation by UMB and SCO was visible at all time points tested (6–24 h of palmitic acid treatment).

3.5. Scopoletin and umbelliferone attenuate palmitate- and glycochenodeoxycholic induced phosphorylation of JNK and ERK

Palmitate and GCDCA increase phosphorylation of JNK and ERK protein in primary rat hepatocytes. JNK activation is induced by the activation of Apoptosis signal-regulating Kinase-1 (ASK1) (Dhanasekaran and Reddy, 2017). GCDCA and palmitate-induced activation of JNK (p-JNK) and ERK (p-ERK) was markedly decreased by SCO and UMB (Figure 6 A-D), suggesting that the decreased death of hepatocytes is due to reduced JNK activation. In addition, Western blot demonstrated that both GCDCA and palmitate activate ERK1/2, which can be blocked with the ERK inhibitor U0126 (Figure 6 *E*-H). The ERK inhibitor U0126 did not significantly decrease GCDCA-induced caspase-3 activity (Figure 6 I).

3.6. Scopoletin and umbelliferone protect against palmitate-induced toxicity in the human hepatoma cell line HepG2

To verify whether scopoletin and umbelliferone are also protective in a human liver cell line, we tested the protective effect of umbelliferone and scopoletin in the human hepatoma cell line HepG2. Since HepG2 cells are partly dedifferentiated and lack the bile salt importer NTCP, HepG2 cells are not susceptible to GCDCA-induced cell death. We have previously shown that HepG2 cells are susceptible to palmitate-induced cell death and that palmitate induces apoptotic cell death in HepG2 cells (Geng et al., 2020a). As shown in Supplemental fig. 1, palmitate induces caspase-3 activity in HepG2 cells which is attenuated by SCO and UMB (Supplemental fig. 1). Slightly higher concentrations of SCO and UMB were necessary to obtain protection, which may be due to less efficient uptake of SCO and UMB by HepG2 cells compared to primary rat hepatocytes or to species differences. SCO and UMB also significantly

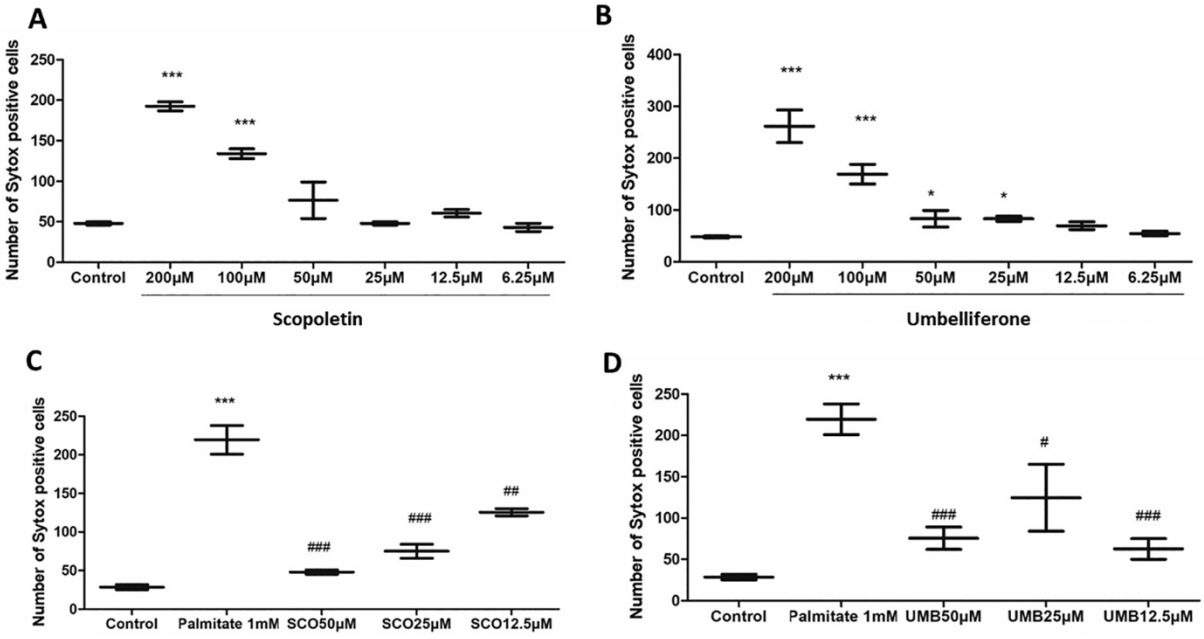
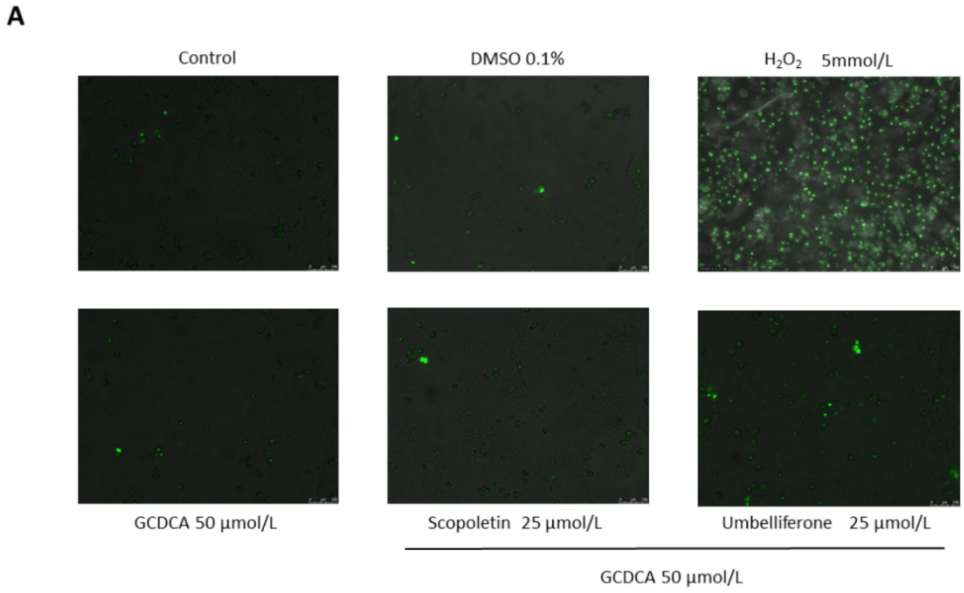
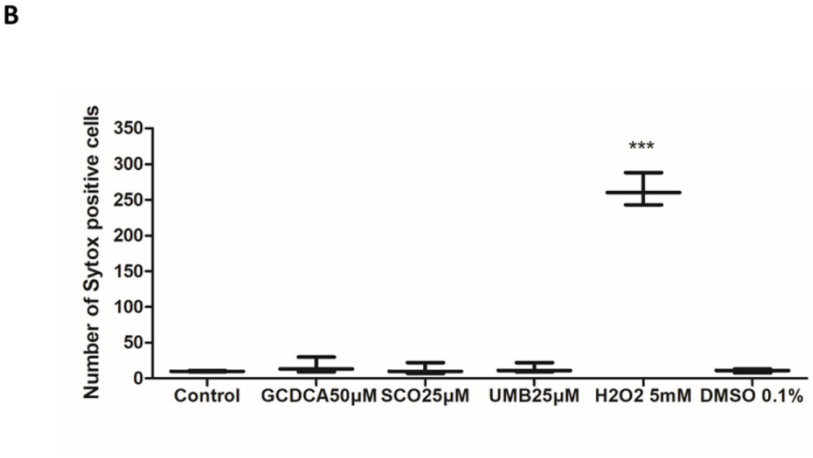
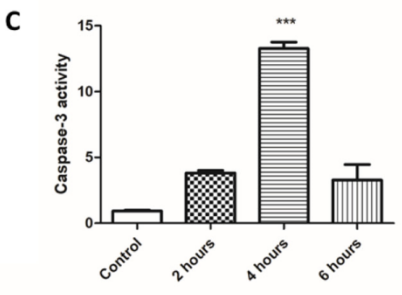
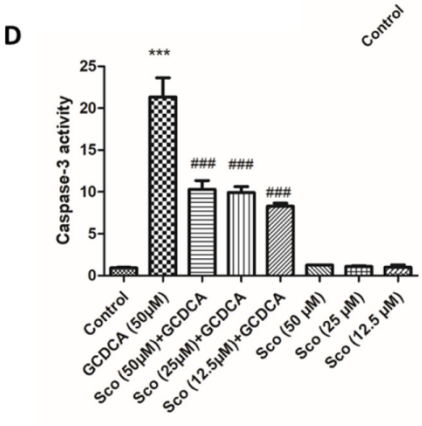


Fig. 2. Scopoletin and umbelliferone protect against palmitate-induced necrosis at concentrations of 100 μmol/L or lower in primary rat hepatocytes but induce toxicity at higher doses. 5 mmol/l H_2O_2 is used as positive control for necrosis. (A-B) Number of Sytox positive cells with SCO (A) or UMB alone (B). (C—D) Number of Sytox positive cells after 24 h treatment with palmitate in the presence and absence of different concentrations SCO (C) or UMB (D). Sytox green positive cells were counted using Image J image analysis software and shown as the total number of Sytox positive cells per microscopic field. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)









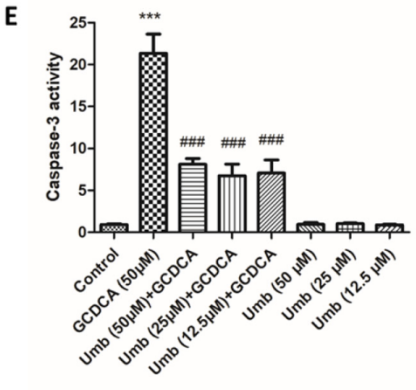


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Fig. 3. Scopoletin and umbelliferone inhibit GCDCA-induced cell death (A) Sytox green staining of hepatocytes treated for 24 h with GCDCA with and without SCO or UMB. 5 mmol/l H_2O_2 is used as positive control for necrosis. 0.1% DMSO was used as solvent control. (B) Quantitative representation (number of Sytox positive cells) of panel A. (C) Caspase-3 activity in primary rat hepatocytes treated with GCDCA (50 μmol/l for 2 h, 4 h and 6 h. (D) Caspase-3 activity in hepatocytes pretreated with different concentrations of scopoletin (25 μmol/l, 50 μmol/l, 100 μmol/l) for 30 min, followed by treatment with GCDCA for another 4 h. (E) Caspase-3 activity in hepatocytes pretreated with different concentrations of umbelliferone (25 μmol/l, 50 μmol/l, 100 μmol/l) for 30 min, followed by treatment with GCDCA for another 4 h. ($n \geq 3$). Caspase-3 activity is expressed relative to control (non-treated) values.
* indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$. ### indicates $P < 0.001$ compared to ***. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

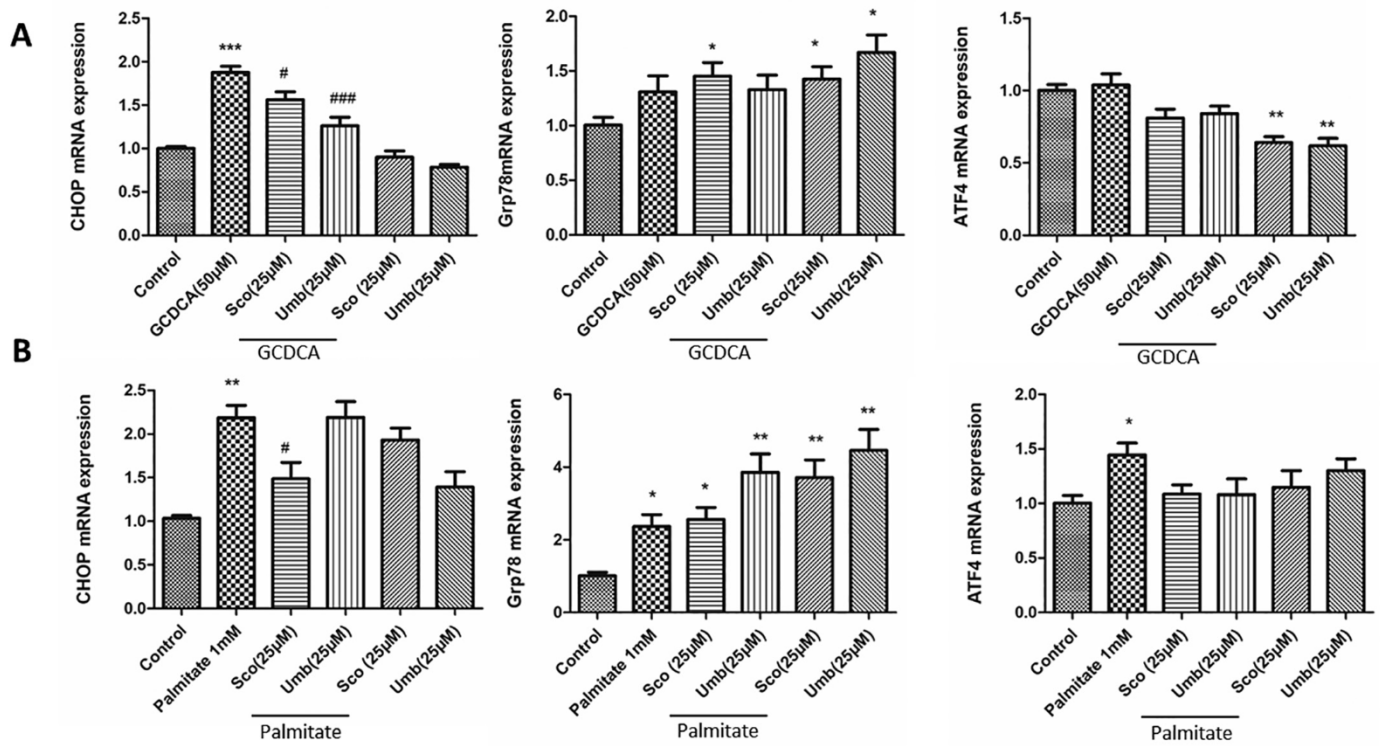

Fig. 4. Umbelliferone and scopoletin reduce GCDCA- and palmitic acid-induced ER stress. mRNA levels of CHOP, ATF4 and GRP78 were evaluated after 4 h and 8 h of exposure to GCDCA and palmitate, respectively, with and without 30 min pre-treatment with UMB or SCO. (A) mRNA levels of CHOP, GRP78 and ATF4 were measured and normalized to 18S after 4 h treatment with or without GCDCA. (B) mRNA levels of CHOP, GRP78 and ATF4 were measured and normalized to 18S, after 8 h treatment with or without palmitate.
*indicates P < 0.05; **indicates P < 0.01; ***indicates P < 0.001. ### indicates P < 0.001 compared to ***.

reduced palmitate-induced ROS production in HepG2 cells (Supplemental fig. 2).

4. Discussion

Lipotoxicity plays an important role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (Arroyave-Ospina et al., 2021). Lipotoxicity causes cell death of hepatocytes in the liver (Geng et al., 2021). In this study, we demonstrate that scopoletin (SCO) and umbelliferone (UMB) protect hepatocytes from saturated fatty acid (palmitic acid)-induced cell death by reducing ER and oxidative stress and attenuating activation of the pro-apoptotic MAPK JNK in hepatocytes. In addition, the protective effect of SCO and UMB was also observed in hydrophobic bile acid (GCDCA)-induced hepatocyte cell death. This is an important observation, since palmitic acid induces mainly necrotic cell death in hepatocytes (Geng et al., 2021), whereas GCDCA induces mainly apoptotic cell death (Schoemaker et al., 2004). These observations not only indicate that SCO and UMB are potentially protective in a wide range of liver diseases, but also that UMB and SCO act on signaling intermediates that are shared by apoptotic and necrotic cell death pathways, e.g. pathways involving mitochondrial dysfunction. Interestingly, we also show that umbelliferone and scopoletin protect against palmitate-induced apoptotic cell death of HepG2 cells.

ER stress is accompanied by the Unfold protein response (UPR). The UPR is a stress response that is activated to alleviate ER stress. However, when ER stress is chronic or excessive, the UPR may also lead to cell death. ER stress may be induced by protein misfolding as a result of protein overload and/or protein mutations leading to misfolding (van der Kallen et al., 2009). However, ER stress may also be caused by disturbed lipid metabolism and/or increased drug metabolizing activity by cytochrome P450 (CYP) family members, since both lipid biosynthesis as well as drug metabolism take place to a large extent in the ER (Yalcinkaya et al., 2020). Therefore, the ER is very sensitive to disturbances in lipid metabolism that occurs in NAFLD.

Both palmitate and GCDCA were shown to induce the expression of the ER stress marker CHOP and to increase ROS generation. It is known that ER stress can induce ROS generation due to impaired mitochondrial metabolism as a result of impaired lipid metabolism and fatty acid oxidation in mitochondria (Arroyave-Ospina et al., 2021). However, ER stress may also generate ROS as a result of protein misfolding via protein disulfide isomerases and/or changes in the lipid composition of the ER leading to ER dysfunction. In addition, increased CYP activity, located in the ER, may also result in increased ROS generation in the ER. Finally, both compartments, mitochondria and ER, communicate via

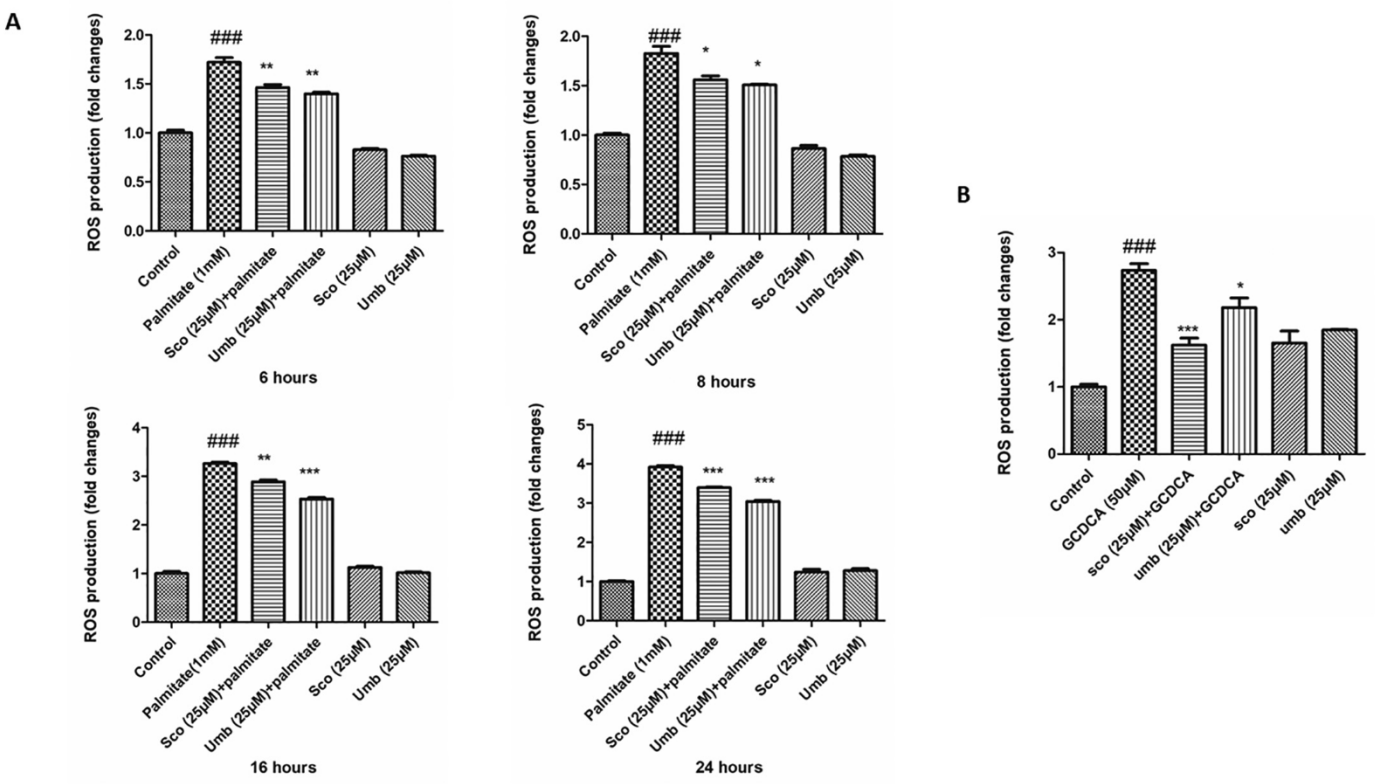

Fig. 5. GCDCA and palmitate increase generation of reactive oxygen species (ROS) in primary rat hepatocytes. (A) Palmitate induced ROS generation at 6 h, 8 h, 16 h and 24 h which was reduced by both SCO and UMB. Data are mean ± SD. (B) GCDCA induced ROS generation at 4 h which was reduced by both SCO and UMB. Data shown are mean ± S.D.
Data are shown as mean ± S.D. ($n = 3$). ### indicates $P < 0.001$ vs the control group, ***indicates $P < 0.001$ vs the positive control group.

mitochondria-associated membranes and Ca^{2+} fluxes between these compartments (Geng et al., 2021). In the present study we did not investigate in detail the order of events leading to ER stress and ROS generation.

Coumarin is a natural compound present in extracts from food products and may lead to liver and lung damage in humans (Lake, 1999). One study showed that coumarin-induced hepatocellular necrosis is related to disturbed mitochondrial function and changes in the expression and localization of CYP2E1 in rats (Tanaka et al., 2016). Another study showed the presence of both apoptosis as well as necrosis in liver tissue of coumarin-treated rats (Lake, 1999). Indeed, it is known that coumarins can be hepatotoxic and that the metabolism and toxicity of coumarins is species-dependent. On the other hand, coumarins are taken up by humans via the diet and the use of fragrances, indicating that there is a safe range of coumarin exposure. Defining this range will be important when translating our findings into clinical practice (Tanaka et al., 2016). Umbelliferone and scopoletin are coumarin-like compounds. In our in vitro studies we used non-toxic doses (6.25–50 μmol/L) that are protective against palmitate-induced necrosis. In contrast, higher concentrations are hepatotoxic. One study indicated that scopoletin inhibited hepatic lipid peroxidation in rats without hepatotoxicity when administered daily for 7 days at doses below 1 mg/kg (Panda and Kar, 2006). It is difficult to compare toxic doses in vitro to toxic doses in vivo, but it can be argued that our protective concentrations in vitro are below the toxic concentrations in vivo. Nevertheless, a thorough determination of non-toxic, protective dosages in rats and its extrapolation to humans is necessary before coumarin-like compounds like umbelliferone and scopoletin can be considered for therapeutic purposes in NAFLD.

Scopoletin and umbelliferone have been reported to be potent oxygen radical scavengers, contributing to its antioxidant properties (Quek et al., 2021). Several additional mechanisms have been proposed to explain the protective effect of coumarins like scopoletin and umbelliferone against oxidative stress, notably the Nrf2 pathway. Nrf2 is the transcription factor that is considered to be the master regulator of the antioxidant response. Umbelliferone has been reported to reduce the toxicity in HepG2 cells of methylglyoxal, a precursor of advanced glycation end-products, by reducing ROS generation and apoptosis (Li et al., 2017) via an Nrf2-dependent mechanism. On the other hand, scopoletin has also been reported to reduce ethanol-induced liver damage in the liver by reducing the expression of CYP2E1, the main ethanol-metabolizing enzyme in the liver and by increasing the expression of the Nrf2 target genes like catalase and superoxide dismutase (Noh et al., 2011). These studies suggest that the hepatoprotective effect of scopoletin and umbelliferone may be pleiotropic. However, we did not observe the induction by umbelliferone and scopoletin of the expression of the known Nrf2 target gene HO-1 (data not shown). Since umbelliferone and scopoletin did reduce ROS generation, it could be that umbelliferone and scopoletin act as direct scavengers of ROS.

Glucose-regulated protein 78 kDa (GRP78) is a chaperone protein that is involved in attenuating ER stress. Overexpression of GRP78 is protective by alleviating ER stress (Kim et al., 2008). The transcription factor C/EBP Homologous Protein (CHOP) is the pro-apoptotic UPR effector has been shown to be the main effector for ER stress induced cell death, e.g. in diabetic db/db mice (Chan et al., 2015). We have previously shown that hesperetin increases the expression of the chaperone protein GRP78 and that this induction is protective in palmitate-induced hepatocyte death (Geng et al., 2020b). In the present study we show that both UMB and SCO increase the expression of GRP78, both in the absence as well as the presence of palmitate or GCDCA. Increased CHOP expression usually correlates with cell death. Indeed, in our study we

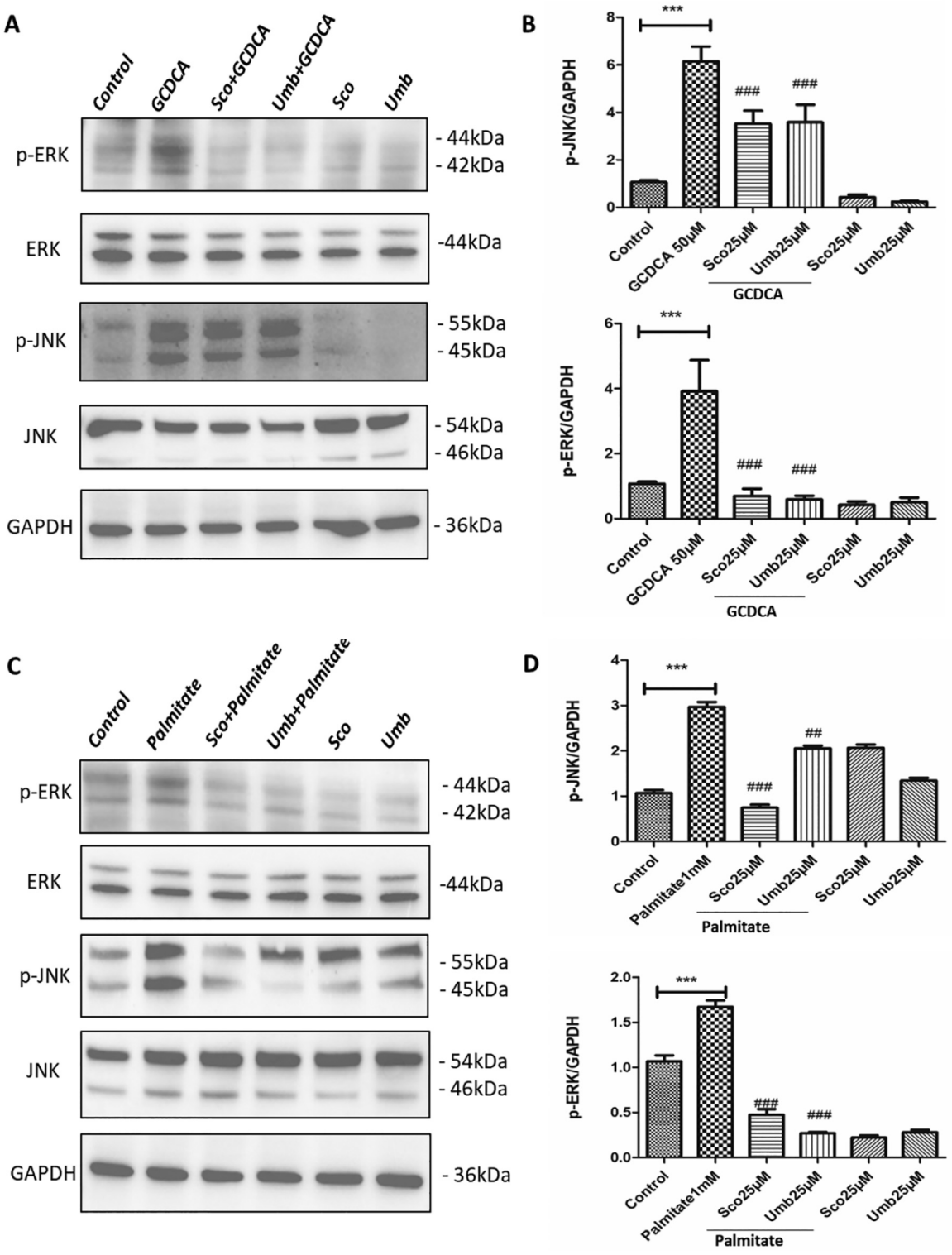

Fig. 6. GCDCA (panel A-B) and palmitate (panel C—D) increase the phosphorylation (activation) of JNK and ERK, which is attenuated by both UMB and SCO. U0126 (panel *E*-H) decreased palmitate- and GCDCA-induced phosphorylation of ERK. U0126 (panel I) tended to decrease GCDCA-induced caspase-3 activity but this decrease did not reach statistical significance. Treatment with palmitate was for 8 h, treatment with GCDCA was for 4 h. Expression levels of the phosphorylated and total forms of ERK and JNK protein and their relative expression levels were quantified by Image J analysis. Caspase-3 activity is expressed relative to control (non-treated) values.

Data are shown as mean ± S.D. (n = 3). *** indicates $P < 0.001$ vs the control group, ### indicates $P < 0.001$ vs the positive control group.

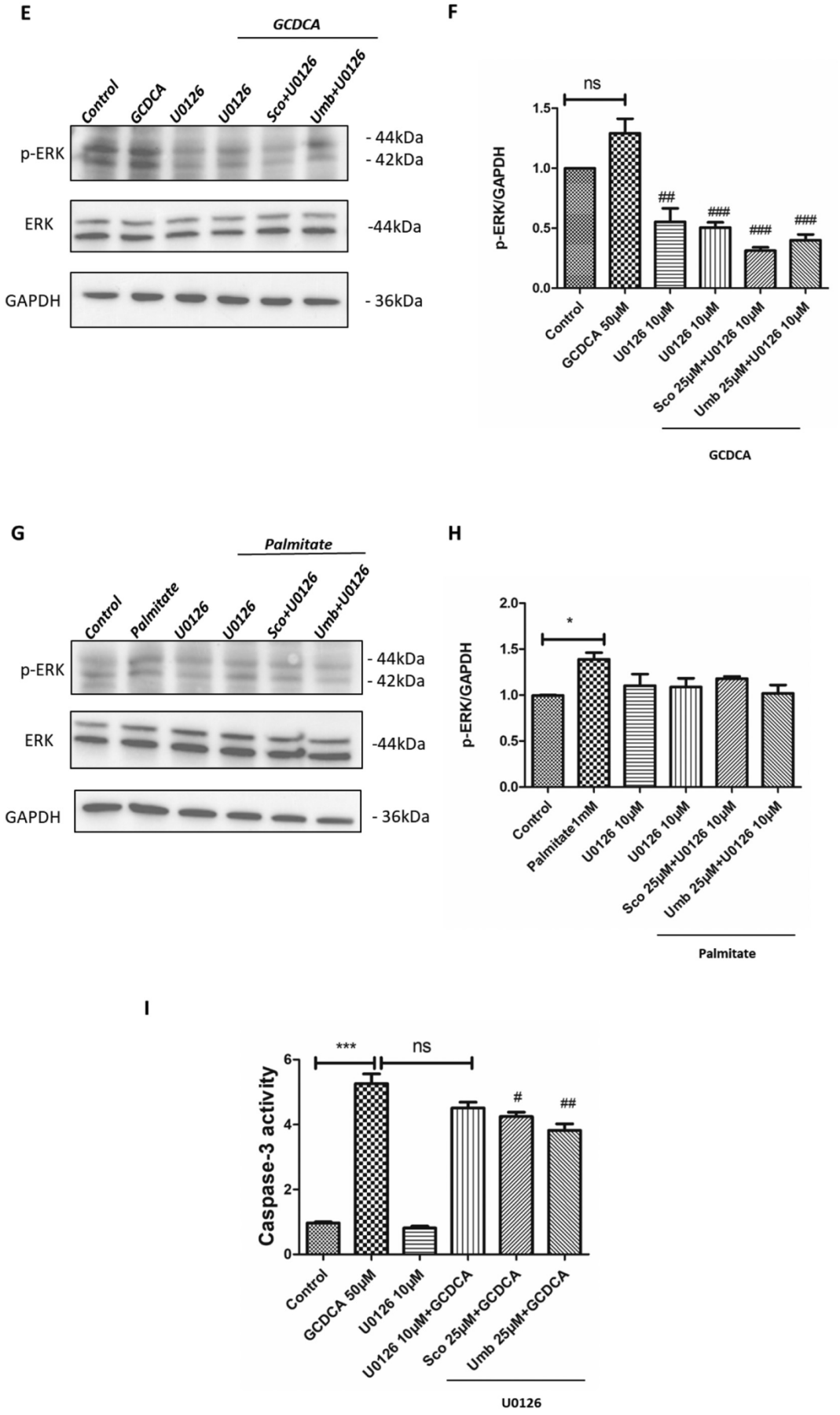

Fig. 6. (*continued*).

demonstrate that both palmitate and GCDCA increase CHOP expression. The increased expression of CHOP expression is reduced by both scopoletin and umbelliferone and may be related to the effect of umbelliferone and scopoletin on GRP78 expression: increased GRP78 expression lowers ER stress and hence the expression of ER stress markers like CHOP and ATF4. Thus, we postulate an additional protective effect of the coumarins scopoletin and umbelliferone: reducing ER stress by increasing the expression of the protective chaperone GRP78.

Reactive oxygen species can induce hepatic cell death. The generation of ROS is not only increased in conditions of mitochondrial dysfunction, but is also regulated by reduced hepatic ATP synthesis and caspase-induced apoptosis. In our study, we show that both palmitate and GCDCA increase ROS generation and both UMB and SCO reduce ROS generation induced by palmitate and GCDCA. Mitogen-activated protein kinases (MAPKs) play an important role in relaying extracellular stimuli into a variety of cellular processes. MAPKs are intimately involved in cell proliferation and apoptosis (Chen, 2012). The results of the present study show that both palmitate and GCDCA activate (phosphorylate) JNK. Phosphorylated JNK is pro-apoptotic and inhibition of JNK activity has been shown to be protective against a wide variety of toxic stimuli. In our study, both UMB and SCO reduced JNK phosphorylation. This is in line with a previous report, in which the coumarin derivative daphnetin was shown to reduce JNK activation in a model of oxidative stress and acetaminophen induced liver damage (Lv et al., 2020). However, we like to point out that the reduced phosphorylation of JNK does not prove that the protective effect of UMB and SCO is exclusively mediated by reduced JNK activation. Additional studies, using specific inhibitors of JNK are necessary to demonstrate a causal relationship. Furthermore, we also tried to investigate the role of ASK1 in the phosphorylation and activation of JNK, but due to the lack of suitable antibodies against rat phospho-ASK1, we were not able to demonstrate a causal relationship between ASK1 activation and JNK phosphorylation. JNK has been shown to be phosphorylated by both reactive oxygen species and ER stress. JNK activation has also been described in conditions of NAFLD: in a high-fat model of obesity and NASH, JNK was found to be activated (Dong et al., 2020). Inhibition of ROS accumulation and JNK activation prevent cytochrome *c* leakage and caspase 3 activation (Kamata et al., 2005). The same study also suggested that ERK1 promotes hepatic steatosis in this diet-induced NASH model, indicating that the ERK1 pathway is associated with hepatic dysfunction. On the other hand, ERK2 has been implicated in the maintenance of lipid metabolism and glucose homeostasis (Lawan and Bennett, 2017). Both SCO and UMB are coumarin-like phenolic compounds found in many plants. They have been shown to have beneficial effects like suppression of insulin resistance and they have anti-inflammatory and antioxidant properties (Subramaniam and Ellis, 2016; Kalpana et al., 2019). SCO has also been shown to prevent lipid accumulation in obese mice by inhibiting CYP2E1 activity (Lee et al., 2014). Amelioration of insulin resistance by SCO may also be the result of induction of PPAR-γ expression and correcting disturbed lipid metabolism (Zhang et al., 2010b). UMB has a beneficial effect on fatty acid composition of the liver via its anti-oxidant activity. UMB (30 mg/kg/day) also reverses the weight loss due to increased secretion of insulin in streptozotocin-induced diabetes (Ramesh et al., 2007). Moreover, the acetylation of UMB to 7-acetoxycoumarin inhibited the phosphorylation of ERK, p38 and JNK in LPS-treated RAW 264.7 macrophages (Park et al., 2020). Our present study is the first to show that SCO and UMB can regulate activity of the MAPKs ERK and JNK in primary rat hepatocytes. Previously, we have shown that inhibition of ERK signaling with U0126 protects does not change GCDCA-induced caspase-3 activity in primary rat hepatocytes (Schoemaker et al., 2004). However, inhibition of ERK did reduce the protective effect of the anti-apoptotic bile acid tauroursodeoxycholic acid (TUDCA) in GCDCA-induced apoptosis, suggesting that the inhibition of ERK signaling per se does not change stressor-induced apoptosis, but might abolish the beneficial effects of protective reagents. In the present study, we did not observe an effect of ERK inhibition on the protective effect of both scopoletin and umbelliferone, suggesting that the protective effect of scopoletin and umbelliferone is independent of ERK-signaling, in contrast to the protective effect of the hydrophilic bile acid TUDCA, which is ERK-dependent (Schoemaker et al., 2004). A pro-apoptotic role of ERK has been demonstrated in a model of cisplatin-induced apoptosis (Wang et al., 2020; Potočnjak et al., 2019). These data suggest that the exact role of ERK in cell death signaling pathways is context-dependent and may vary with cell type, stressor and protective agent. This will complicate the use of ERK as a target of intervention to prevent hepatocyte cell death.

In conclusion, we show that scopoletin and umbelliferone suppress palmitate and bile acid induced cell death in primary rat hepatocytes via reducing endoplasmic reticulum and oxidative stress. The beneficial effects of scopoletin and umbelliferone on ER stress are accompanied by reduced generation of cellular ROS. The reduced generation of ROS attenuates the activation of pro-apoptotic JNK and ERK. Our study suggests that umbelliferone and/or scopoletin may hold promise in the therapy of NAFLD.

Author contribution

Zongmei Wu Contribution: The author contributed to the conception, design, analysis, execution, writing, project administration, and interpretation of the reported study.

Yana Geng Contribution: The author contributed with the execution, analysis and reviewing of the reported study.

Manon Buist-Homan Contribution: The author contributed to the instruction for the execution and reviewing of the reported study.

Han Moshage Contribution: The authors contributed equally to the conception, design, execution, writing, funding acquisition, validation, reviewing, supervision and interpretation of the reported study.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2021.115858.

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