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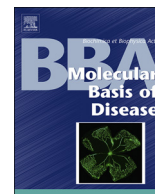
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Protective effect of metformin against palmitate-induced hepatic cell death

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ABSTRACT

Lipotoxicity causes hepatic cell death and therefore plays an important role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metformin, a first-line anti-diabetic drug, has shown a potential protective effect against NAFLD. However, the underlying mechanism is still not clear. In this study, we aim to understand the molecular mechanism of the protective effect of metformin in NAFLD, focusing on lipotoxicity. Cell death was studied in HepG2 cells and primary rat hepatocytes exposed to palmitate and metformin. Metformin ameliorated palmitate-induced necrosis and apoptosis (decreased caspase-3/7 activity by 52% and 57% respectively) in HepG2 cells. Metformin also reduced palmitate-induced necrosis in primary rat hepatocytes ($P < 0.05$). The protective effect of metformin is not due to reducing intracellular lipid content or activation of AMPK signaling pathways. Metformin and a low concentration (0.1 $\mu\text{mol/L}$) of rotenone showed moderate inhibition on mitochondrial respiration indicated by reduced basal and maximal mitochondrial respiration and proton leak in HepG2 cells. Moreover, metformin and rotenone (0.1 $\mu\text{mol/L}$) preserved mitochondrial membrane potential in both HepG2 cells and primary rat hepatocytes. In addition, metformin and rotenone (0.1 $\mu\text{mol/L}$) also reduces reactive oxygen species (ROS) production and increase superoxide dismutase 2 (SOD2) expression. Our results establish that metformin AMPK-independently protects against palmitate-induced hepatic cell death by moderate inhibition of the mitochondrial respiratory chain, recovering mitochondrial function, decreasing cellular ROS production, and inducing SOD2 expression, indicating that metformin may have beneficial actions beyond its glucose-lowering effect and also suggests that mitochondrial complex I may be a therapeutic target in NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as one of the major health problems around the world and its prevalence coincides with the unprecedented increase of obesity in recent decades. NAFLD is characterized as increased accumulation of lipids in the liver and its severity ranges from simple steatosis to non-alcoholic steatohepatitis (NASH). In the last few decades, our knowledge about the role of lipids in the pathogenesis of NAFLD has increased substantially. It has been shown that elevated plasma levels of non-esterified fatty acids (NEFA) are closely associated with hepatic dysfunction and consequently, the detrimental effect of surplus lipids, especially saturated fatty acids, has been termed lipotoxicity [1,2]. Both *in vivo* experiments and clinical studies indicated that the saturated fatty acids are more toxic compared to unsaturated fatty acids and therefore saturated fatty acids may be

one of the factors promoting the progression of steatosis to steatohepatitis [3,4]. Thus, counteracting lipotoxicity has become one of the therapeutic targets in the treatment of NAFLD.

Studies have shown that hepatic cell death represents the major consequence of lipotoxicity in the liver. Hepatic cell death is preceded or associated with a broad spectrum of subcellular dysfunctions, including endoplasmic reticulum (ER) stress, mitochondrial dysfunction, oxidative stress, autophagy, disturbed signaling pathways, etc. [1,2,5] Several studies highlighted the increased mitochondrial activity of NAFLD patients and hypothesized that the accelerated mitochondrial metabolism could be responsible for the elevated generation of ROS [6–9]. However, it is still not completely clear what the role of mitochondria is in lipotoxicity and the pathogenesis of NAFLD.

Metformin, as one of the first-line anti-diabetic medicines, showed beneficial effects against hepatic cell death. Our group has previously

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reported that metformin prevents both bile acid and oxidative stress-induced hepatic injury [10,11]. Accumulating evidence indicates that the activation of AMP-dependent protein kinase (AMPK) plays a central role in the actions of metformin. For instance, metformin-induced improvement in insulin action is AMPK dependent [12]. On the other hand, some of the effects of metformin are AMPK independent. For example, our previous studies showed that the protective effect of metformin against bile-acid induced hepatic injury does not require AMPK/mTOR-signaling [10]. Furthermore, the actions of metformin could also be closely related to its inhibition of the mitochondrial respiration chain complex I [13,14]. Taken together, the exact molecular mechanisms of metformin are still not clear.

In the present study, we aim to evaluate the protective effect of metformin against lipotoxicity and investigate the underlying mechanisms.

2. Materials and methods

2.1. Cell culture and infection

HepG2 cells were obtained from American Type Culture Collection and maintained with Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calf serum (Invitrogen, Breda, The Netherlands), 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL fungizone (1% PSF, Lonza, Verviers, Belgium) at 37 °C in a 5% (v/v) CO₂ condition. During experiments, cells were cultured in serum-free medium. Before infection, HepG2 cells were seeded on plates and grown to 60% confluence and then infected with dominant negative AMPK-adenovirus (ad-DN-AMPK) or control β-galactosidase-adenovirus (ad-LacZ). Ad-DN-AMPK and ad-LacZ were kindly provided by Dr. Birnbaum [15]. After 24 h of infection, cells were treated with or without metformin (1,1-dimethyl biguanide hydrochloride, Sigma-Aldrich) for 60 min, and harvested to determine p-AMPK expression; or cells were pretreated with metformin for 30 min and exposed to palmitate and then harvested for caspase-3/7 assay.

2.2. Animals and primary hepatocyte isolation

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 from male Wistar rats by two-step collagenase perfusion method as described previously [16] and cultured in William's E medium (Invitrogen, Breda, the Netherlands) supplemented with 50 µg/mL gentamicin (Invitrogen) at 37 °C in a 5% (v/v) CO₂ condition. Experiments were started after an attachment period of 4 h.

2.3. Preparation of palmitate-BSA and stearate-BSA

BSA-conjugated palmitate or stearate solution (palmitate/stearate) was prepared by modification of the seahorse protocol (Seahorse Bioscience). A 40 mmol/L palmitate/stearate (Sigma-Aldrich) solution was prepared at 70 °C. After dissolving, the solution was complexed with 10% fatty acid-free BSA (Sigma-Aldrich) at an 8:1 palmitate/stearate to BSA molar ratio. Then the BSA-conjugated palmitate/stearate solution was added to serum-free cell culture medium to achieve a final palmitate/stearate concentration of 0.5 mmol/L or 1.0 mmol/L. The pH was adjusted to 7.4 by addition of 1 M NaOH.

2.4. Sytox green nuclear staining

After exposure to palmitate in the presence and absence of metformin or rotenone, HepG2 cells were incubated with Sytox green nucleic acid dye (Invitrogen) for 15 min as described before to determine necrotic cell death [10]. Fluorescent nuclei were visualized using a Leica DMI6000 at 450–490 nm.

2.5. Determination of mitochondrial membrane potential

JC-10 staining: mitochondrial membrane potential ($\Delta\psi_m$) was assessed via JC-10 (Sigma-Aldrich) staining. In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates. However, when the mitochondrial membrane becomes polarized, JC-10 diffuses out of mitochondria and remains monomer which has a green to greenish orange emission. The mitochondrial membrane potential was shown as the ratio of JC-10 aggregates over monomers. Fluorescence was visualized using a Leica DMI6000 at 570 nm and 490 nm. Images were analyzed by ImageJ (ImageJ; National Institutes of Health, Bethesda, MD, USA, <http://rsbweb.nih.gov/ij/>).

TMRE staining: mitochondrial membrane potential ($\Delta\psi_m$) was assessed by flow cytometry using TMRE (Tetramethylrhodamine ethyl ester perchlorate, T669, Invitrogen) fluorescent dye. Briefly, HepG2 cells and primary rat hepatocytes were harvested and incubated with 200 nmol/L TMRE or PBS for 20 min at 37 °C (the latter allows for autofluorescence determination). Then, the cells were centrifuged and resuspended in PBS. Cells treated with FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, 50 µmol/L) for 30 min were used as a positive control. Samples were analyzed via CytoFLEX S benchtop flow cytometer (Beckman Coulter Life Sciences). TMRE fluorescence was excited at 488 nm and detected at 585/42 nm. Data were processed using Flowjo software (Becton, Dickinson & Co).

2.6. Lactate dehydrogenase (LDH) release assay

Necrotic cell death was quantified by LDH measurement, described previously by Woudenberg-Vrenken et al. [17]. Briefly, the LDH release was calculated as the percentage of the activity of LDH released in the medium vs. the total LDH activity (in both the medium and cell lysates). The LDH activity was measured spectrophotometrically at 340 nm using a Bio-Tek Epoch2 microplate reader (Bio-Tek Instruments, Inc., Bad Friedrichshall, Germany).

2.7. Caspase-3/7 enzyme activity assay

Cells were harvested at indicated time points and washed twice with ice-cold phosphate buffered saline (PBS, Life technologies) before addition of caspase cell lysis buffer. Total cell lysates were prepared and caspase-3/7 activity was determined as described previously [10].

2.8. ROS measurement

HepG2 cells were seeded on 96-well plates. After 24 h of culture, the medium was replaced with medium containing palmitate and cells were treated with metformin or rotenone. Dihydroethidium (DHE, Life technology) fluorescence method was used for determination of ROS levels. After treatment, cells were incubated with 5 µmol/L DHE for 30 min in dark. Fluorescence was recorded at Ex/Em 518/605 using a Bio-Tek FL600 microplate fluorescent reader (Bio-Tek, Germany).

2.9. Oil Red O staining and triglyceride determination

Lipid droplets were visualized by Oil Red O staining. Cells were seeded on coverslips. After culturing, cells were rinsed with PBS and fixed with 4% (v/v) formaldehyde for 20 min at room temperature.

After washing with distilled water, cells were incubated with 60% (v/v) isopropyl alcohol and then mounted with oil red O solution (Sigma-Aldrich, dissolved in isopropyl alcohol). Then cells were rinsed in distilled water, 60% (v/v) isopropyl alcohol, counterstained with hematoxylin, rinsed with tap water, and sealed with glycerin-gelatin. The coverslips were scanned and the lipid droplets identified by their red staining.

To quantify the lipid accumulation, triglyceride levels in cells were measured using a triglyceride measurement kit (Abcam, UK) following the guideline provided by the company. The amount of triglyceride was normalized to the number of cells.

2.10. Western blot analysis and antibodies

To evaluate the expression of specific proteins, the total cell lysate was subjected to Western blot as described by Woudenberg-Vrenken *et al.* [10]. Proteins were detected using the following antibodies: p-AMPK α (Thr172) (Cell Signaling, Danvers, MA, USA), p-eIF2 α (Cell Signaling Technology, Danvers, MA, USA) eIF2 α (Cell Signaling Technology, Danvers, MA, USA) and PARP (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000. Monoclonal mouse antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Calbiochem, San Diego, CA, USA) at a dilution of 1:50,000 were used to ensure equal protein loading.

2.11. Monitoring of mitochondrial metabolism by Seahorse

The oxygen consumption rate (OCR) was measured by Seahorse system. HepG2 cells were initially seeded in XF24 cell culture plates at 1×10^5 cells/well and primary hepatocytes at 5×10^4 cells/well. After challenging with palmitate in the presence and absence of metformin or rotenone, cells were washed with PBS and XF assay medium supplemented with 1 mmol/L sodium pyruvate and incubated in a non-CO₂ incubator at 37 °C for 60 min. OCR was measured with XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Inc., Billerica, MA, USA) using manufacturer recommended protocols. After normalization to protein concentration, basal respiration, proton leak and maximal respiration were calculated. Specifically, the basal respiration is calculated without glucose and all rates were corrected for non-mitochondrial OCR. An illustration graph is shown in Fig. 5A.

2.12. RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instruction. The RNA quantity and quality were measured with the Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). 2.5 μ g RNA was used for reverse transcription. Quantitative real-time PCR was performed with the 7900HT Fast Real-time System (Applied Biosystems) 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.

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

Gene	Sense 5'-3'	Antisense 5'-3'	Probe 5'-3'
18S	CGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	CGCGCAAATTACCCACTCCCGA
SOD1	CTCACTTTAATCCTCTATCCAGAAAACA	ATCTTTGTGACAGTCACATTGC	CAACATGCCTCTCTCATCCTTTGGCC
SOD2	TaqMan® Gene Expression Assay: Hs01553554-m1		
CHOP	GGAAATGAAGAGGAAGAATCAAAAAT	GTTCCTGGCTCCTCCTCAGTCA	TTCACCACTCTTGACCCCTGCTTCTCTGG
GRP78	TGGTGATCAAGATACAGGTGACCT	GTGTTCCCTTGGAAATCAGTTTGGT	TCCCTTACACTTGGTATTGAAACTGTGGG
Gadd34	GGCTCAAGGCCCAGAAACC	AATGGACAGTGACCTTCTCG	

2.13. Luciferase-based ATP measurement

Luminescent ATP detection assay kit (Promega, USA) was used to measure ATP production in HepG2 cells. In brief, 1×10^4 cells/well were seeded in 96 well plates following the instructions provided by the company. Luminescence from luciferase activity was measured and recorded using a Bio-Tek FL600 microplate fluorescent reader (Bio-Tek, Germany).

2.14. Immunofluorescence microscopy

HepG2 cells were seeded on coverslips. At the end of the experiments, cells were fixed with 4% paraformaldehyde (Merck Millipore) and permeabilized with 1% Triton X-100 followed by blocking with 1% BSA. Then cells were incubated with the primary antibody anti-SOD2 (Enzo Life Sciences, Brussels, Belgium) and labeled with secondary antibodies Alexa Fluor 488 (Invitrogen). The coverslips were mounted in fluorescence mounting medium supplemented with anti-fading agent DAPI (Vector Laboratories, Inc., Peterborough, UK). Images were captured using a Leica DMI6000 microscope and analyzed by ImageJ.

2.15. Statistical analysis

All results are presented as a mean of at least 3 independent experiments \pm S.D. For each experiment, statistical analyses were performed using the one-way ANOVA test, followed by Tukey's multiple comparison tests or Mann Whitney test; $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Metformin protects HepG2 cells from palmitate and stearate-induced cell death in both HepG2 cells and primary rat hepatocytes

Palmitate dose-dependently induced caspase-3/7 activity in HepG2 cells (Supplemental Fig. 1A) and a pathophysiologically relevant concentration of palmitate (0.5 mmol/L) was used for subsequent experiments [18]. Palmitate time-dependently induced caspase-3/7 activity peaking at 16 h after exposure (Supplemental Fig. 1B). Therefore, the effect of metformin and rotenone on palmitate-induced caspase-3/7 activity was measured at 16 h, whereas staining for necrosis using Sytox green (an endpoint assay) was determined at 24 h. Gene expression analysis and mitochondria-related experiments were performed after 12 h or 16 h of palmitate exposure.

Metformin (1.0 mmol/L) inhibited palmitate (0.5 mmol/L) induced necrotic cell death in HepG2 cells, as shown by the reduction of Sytox Green staining (Fig. 1A). The inhibition of necrosis was quantified by LDH release measurement. Palmitate increased LDH release and metformin inhibited it by 52% ($P < 0.01$) (Fig. 1B). Moreover, metformin reduced palmitate-induced apoptotic cell death as shown by a reduction of caspase-3/7 activity (-57% , $P < 0.001$) (Fig. 1C) and a decreased level of cleaved PARP ($P < 0.001$) (Fig. 1D). In addition, metformin also inhibited palmitate-induced ROS generation in HepG2 cells ($P < 0.05$) (Fig. 1E). These results imply that metformin protects against both palmitate-induced necrotic and apoptotic cell death.

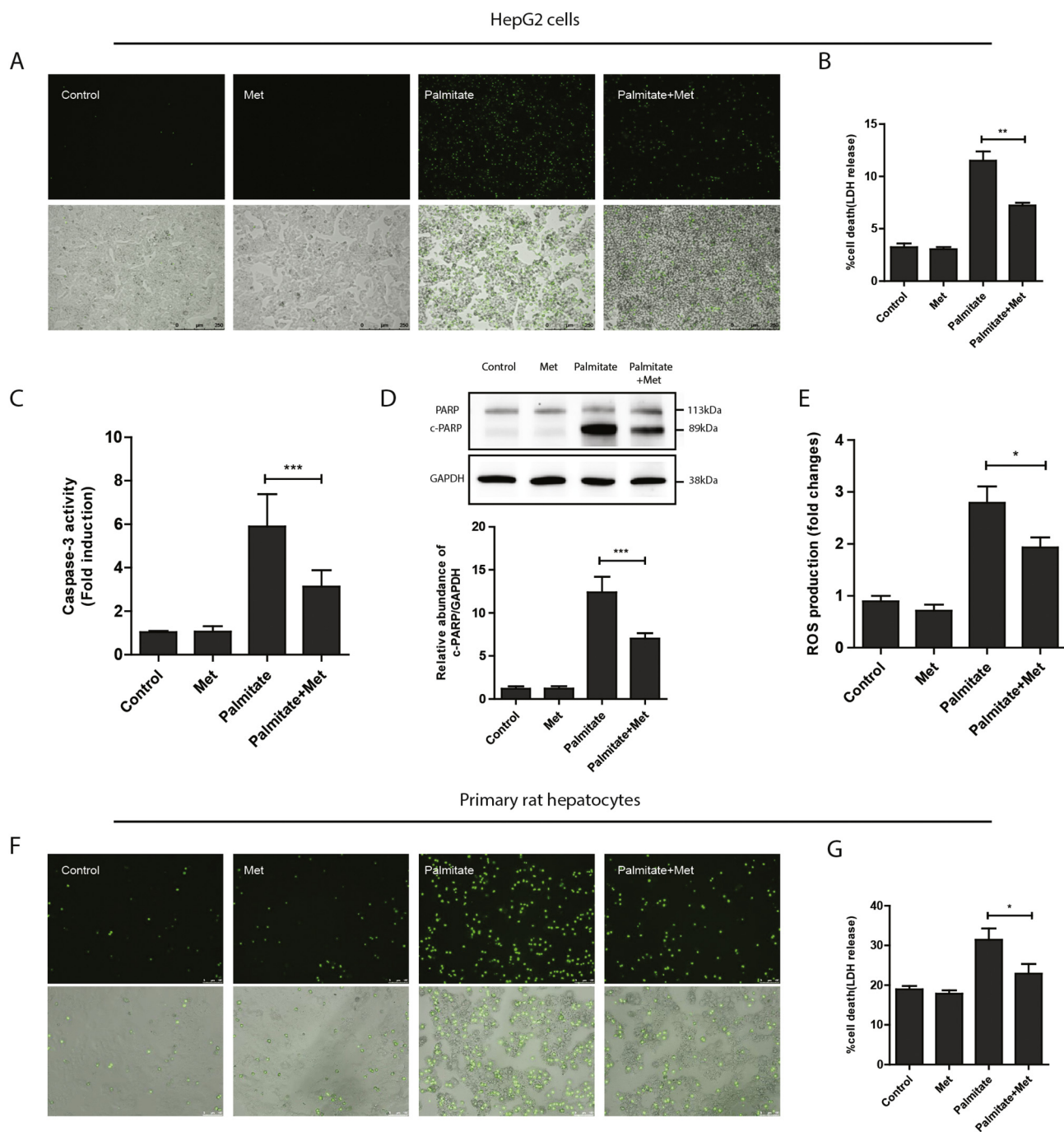


Fig. 1. Metformin (Met) inhibits palmitate-induced hepatic cell death and reduces intracellular ROS production.

(A) HepG2 cells were pretreated with metformin (1.0 mmol/L, -30 min), then exposed to palmitate (0.5 mmol/L, 24 h). Plasma membrane rupture indicative of necrotic cell death is demonstrated by Sytox Green staining. Magnification 10 \times , scale bars 250 μ m. (B and G) Necrosis is quantified by LDH release shown as a percentage of total LDH release in the medium. (C) HepG2 cells were pretreated with metformin then exposed to palmitate (0.5 mmol/L, 16 h). Apoptosis is presented as caspase-3/7 activity shown as fold induction compared to control values. Control values were set at 1. (D) After exposure to palmitate, protein levels of PARP, c-PARP and GAPDH were measured by Western-blot and quantified from the immunoblots by densitometry from three independent experiments with ImageJ. (E) Intracellular ROS production was measured 12 h after exposing to palmitate with or without pretreatment with metformin. ROS was measured by the intensity of DHE emission. (F) Primary rat hepatocytes were pretreated with metformin (1.0 mmol/L, 30 min), then exposed to palmitate (1.0 mmol/L, 24 h). Necrotic cell death is demonstrated by Sytox Green staining. Magnification 10 \times , scale bars 250 μ m. * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$.

Additionally, we also examined the effect the metformin on stearate-induced lipotoxicity. Metformin also significantly reduced stearate-induced caspase 3/7 activity ($P < 0.05$) (Supplemental Fig. 3).

In primary rat hepatocytes, metformin also protected against palmitate (1.0 mmol/L) induced necrotic cell death, as shown by the reduced Sytox Green staining (Fig. 1F) and reduced LDH release (-68% , $P < 0.05$) (Fig. 1G).

3.2. Metformin does not reduce palmitate-induced lipid accumulation

Considering that metformin may affect lipid accumulation, we checked the intracellular lipid content. As shown by Oil Red O staining, metformin does not influence palmitate-induced lipid droplet formation in both HepG2 cells and primary rat hepatocytes (Fig. 2A and C). The lipid droplets were quantified by intracellular TG measurement, which

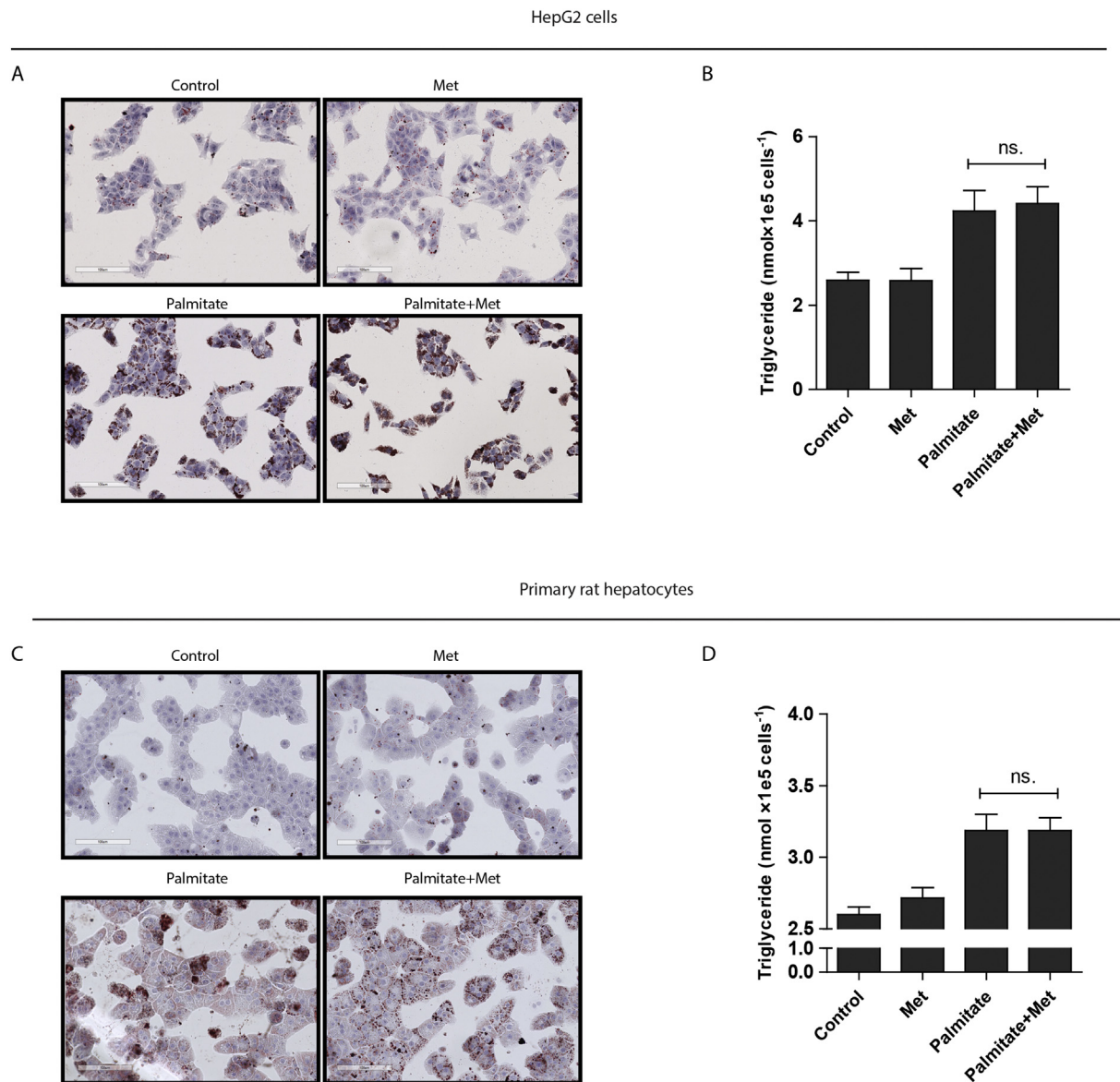


Fig. 2. Metformin does not reduce intracellular lipid content.

Cells were pretreated with metformin (1.0 mmol/L) for 30 min, then exposed to palmitate (HepG2 cells: 0.5 mmol/L; primary rat hepatocytes: 1.0 mmol/L) for 12 h. (A and C) Lipid droplets were stained by Oil Red O staining. Magnification 20 \times , scale bars 100 μ m. (B and D) intracellular triglyceride content was quantified. ns indicates $P > 0.05$.

shows that metformin also does not reduce the amount of TG in both HepG2 cells and primary rat hepatocytes (Fig. 2B and D). Thus, the results indicate that the protective effect of metformin against palmitate is not by reducing intracellular lipid content.

3.3. Metformin inhibits palmitate-induced eIF2 α phosphorylation but does not reduce CHOP, Gadd34 and GRP78 mRNA expression

It has been reported that palmitate induces ER stress and that metformin may inhibit lipotoxicity by reducing ER stress [19,20]. Indeed, we confirm that palmitate induced ER stress demonstrated by increased p-eIF2 α level and increased mRNA expression of CHOP, GRP78 and Gadd34 (Fig. 3A and B). However, although metformin significantly inhibited p-eIF2 α (Fig. 3A), it did not reduce the mRNA expression of CHOP, Gadd34 and GRP78, which are the downstream target genes of p-eIF2 α at both 12 and 16 h (Fig. 3B). Thus, the results demonstrate that the dephosphorylation of p-eIF2 α did not lead to deactivation of the downstream UPR signaling pathways, indicating

that metformin does not directly reduce ER stress.

3.4. The protective effect of metformin is independent of AMPK activation

Metformin has been shown to activate AMPK signaling to promote cell survival [21]. Thus, in order to examine whether the protective effect of metformin is AMPK related, HepG2 cells were infected with dominant negative AMPK adenovirus. The expression of p-AMPK was significantly decreased at 24 h after infection and metformin was not able to activate AMPK (Fig. 3C). Importantly, metformin could still inhibit palmitate-induced caspase-3/7 activation to the same extent compared to cells with an intact AMPK environment (-52% , $P < 0.001$) (Fig. 3C and D). Therefore, we conclude that the protective effect of metformin is independent of AMPK signaling.

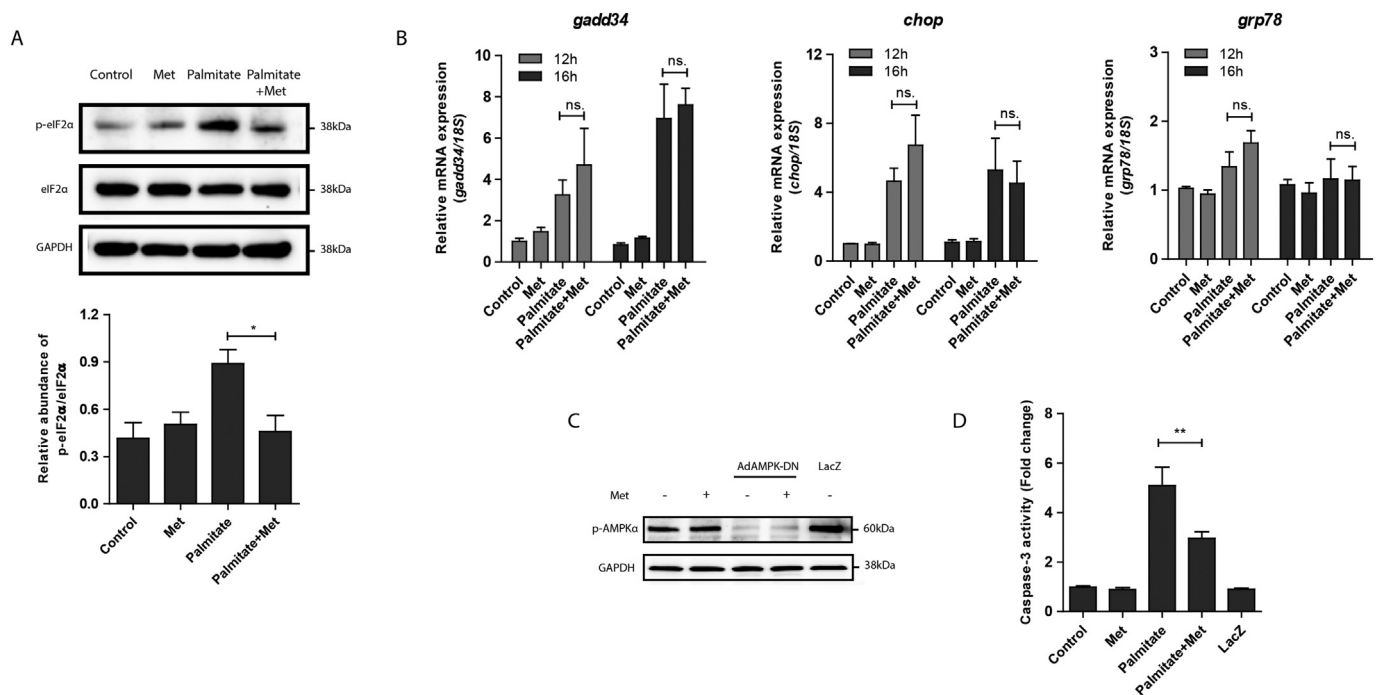


Fig. 3. Metformin inhibits the phosphorylation of eIF2α but does not reduce CHOP, GRP78 and Gadd45 mRNA expression and its protective effect is not dependent on the activation of AMPK signaling.

(A) HepG2 cells were pretreated with metformin (1.0 mmol/L) for 30 min, then exposed to palmitate (0.5 mmol/L) for 12 h. The level of p-eIF2α, eIF2α and GAPDH were measured by Western-blot. Levels of eIF2α/ p-eIF2α were quantified from the immunoblots by densitometry from three independent experiments with ImageJ. (B) After 12 and 16 h of exposure to palmitate, the mRNA levels of CHOP, GRP78 and Gadd34 were evaluated by real-time quantitative PCR. (C) HepG2 cells were infected with either Ad-DN-AMPK or Ad-LacZ for 24 h, then treated with or without metformin for 1 h. p-AMPK and GAPDH protein were detected by Western-blot. (D) After infection with the adenovirus, HepG2 cells were exposed to palmitate (16 h post-infection) in the presence and absence of metformin. Caspase-3/7 activity was determined. Data were shown as mean \pm S.D. ($n \geq 3$). * Indicates $P < 0.05$, ns indicates $P > 0.05$.

3.5. A low, but not high, concentration of rotenone protects against palmitate-induced cell death and reduces ROS production

Metformin has been suggested to mediate its effects in part by inhibiting the mitochondrial respiratory chain complex I [13,14,22]. Therefore, we also investigated the effect of rotenone, a known inhibitor of mitochondrial respiratory chain complex I. Interestingly, our results showed that a low concentration of rotenone (0.1 $\mu\text{mol/L}$) inhibited palmitate-induced necrosis and apoptosis, whereas a high concentration of rotenone (1.0 $\mu\text{mol/L}$) failed to inhibit palmitate-induced necrosis and apoptosis (Fig. 4A and B). In addition, the intracellular ROS generation correlated with necrosis and apoptosis: rotenone reduced ROS production at 0.1 $\mu\text{mol/L}$ but failed to reduce ROS generation at 1.0 $\mu\text{mol/L}$ (Fig. 4C). These results demonstrated that the inhibition of mitochondrial complex I has critical effects on palmitate-induced toxicity.

3.6. Metformin protects against palmitate-induced cell death by partial inhibition of mitochondrial respiratory chain complex I

Given the divergent effects of low and high concentrations of rotenone on palmitate-induced cell death, the Seahorse Analyzer was used to explore the effects of metformin and rotenone on mitochondrial respiration. In HepG2 cells, metformin partially inhibited mitochondrial respiration and decreased both basal and maximal mitochondrial respiration (-36% ($P < 0.05$) and -43% respectively) (Fig. 5B). The low, protective concentration of rotenone (0.1 $\mu\text{mol/L}$) has a similar effect on mitochondrial respiration, indicated by similar reductions of basal and maximal mitochondrial respiration (-41% ($P < 0.05$) and -46% respectively) (Fig. 5C). However, the high, toxic concentration of rotenone (1.0 $\mu\text{mol/L}$) strongly blocked the mitochondrial respiratory chain, as shown by the sharp reduction of both basal and

maximal mitochondrial respiration (-86% ($P < 0.001$) and -88% ($P < 0.0001$) respectively) (Fig. 5C). In addition, as shown in Fig. 6C, metformin and the low dose of rotenone slightly but non-significantly reduced cellular ATP production ($P > 0.05$), however, the high dose of rotenone significantly impaired the cellular ATP production ($P < 0.05$).

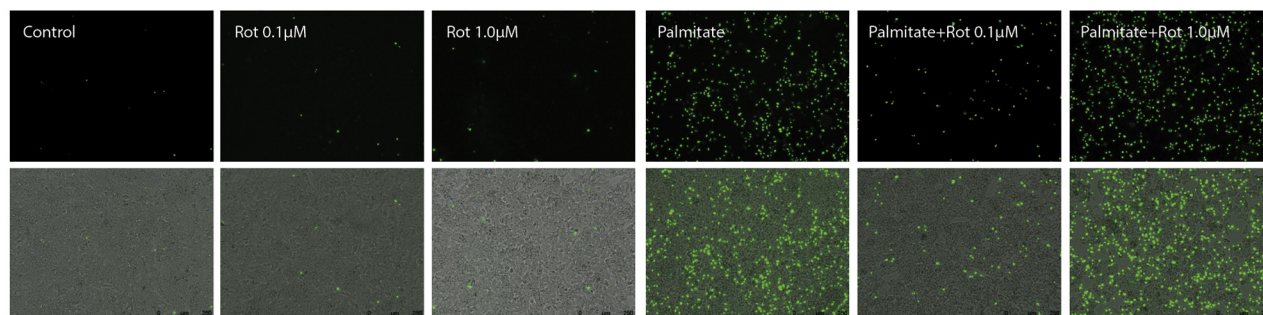
In primary rat hepatocytes, palmitate severely impaired mitochondrial respiration and metformin was shown to restore the impaired mitochondrial respiration, demonstrated by improved responsiveness to oligomycin, FCCP and Rot/AA and recovery of maximal respiration ($P > 0.05$) (Fig. 5E and F).

These results indicate that metformin and rotenone (0.1 $\mu\text{mol/L}$) may protect against palmitate-induced hepatic cell death by partially inhibiting mitochondrial respiratory chain complex I without significantly impairing cellular ATP production. On the other hand, a complete blockage of the mitochondrial respiratory chain has no protective effect and in fact, appears to aggravate palmitate-induced cellular damage.

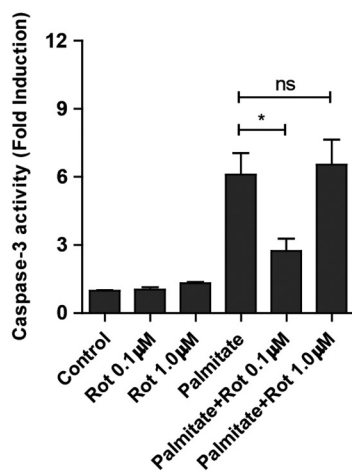
3.7. Metformin and rotenone (0.1 $\mu\text{mol/L}$) restore mitochondrial function and induce mitochondrial SOD2 expression

Mitochondria play a key role in regulating cellular energetics and activating the intrinsic pathway of cell death signaling. Thus, we determined the mitochondrial function by measuring the mitochondrial membrane potential, a marker of mitochondrial integrity. In HepG2 cells, palmitate caused mitochondrial depolarization as indicated by the decrease of the JC-10 aggregates/monomers ratio, while metformin and rotenone (0.1 $\mu\text{mol/L}$) significantly restored mitochondrial function as shown by the recovery of the JC-10 aggregates/monomer ratio ($P < 0.05$, Fig. 6A and B). However, the high concentration of rotenone (1.0 $\mu\text{mol/L}$) did not reverse mitochondrial membrane

A



B



C

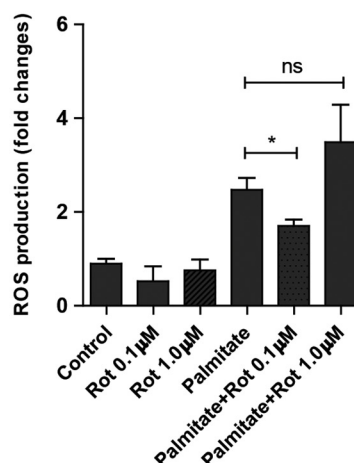


Fig. 4. Rotenone at 0.1 $\mu\text{mol/L}$ protected against palmitate-induced cell death and reduced ROS production, but not at 1.0 $\mu\text{mol/L}$.

(A) HepG2 cells were pretreated with rotenone (0.1 $\mu\text{mol/L}$ or 1.0 $\mu\text{mol/L}$, -30 min), then exposed to palmitate. Necrosis is demonstrated by Sytox Green staining. Magnification $10\times$, scale bars 250 μm . (B) HepG2 cells were pretreated with rotenone (0.1 $\mu\text{mol/L}$ or 1.0 $\mu\text{mol/L}$, -30 min) then exposed to palmitate. Apoptosis is presented as caspase-3/7 activity. (C) Intracellular ROS production was measured after 12 h exposure to palmitate with or without pretreatment of rotenone. ROS was measured by the intensity of DHE emission. * indicates $P < 0.05$; ns indicates $P > 0.05$.

depolarization ($P > 0.05$, Fig. 6B). In addition, we also used the TMRE method with FACS to confirm the effect of metformin and rotenone on mitochondrial membrane potential. Using TMRE FACS analysis, palmitate decreased mitochondrial membrane potential as shown by a decreased percentage of TRME positive cells, while metformin (non-significantly, $P = 0.07$) and rotenone (0.1 $\mu\text{mol/L}$) (significantly, $P < 0.05$) restored mitochondrial membrane potential (Supplemental Fig. 4A and B). In contrast, rotenone at 1.0 $\mu\text{mol/L}$ failed to restore mitochondrial polarization ($P > 0.05$, Supplemental Fig. 4B). Moreover, in primary rat hepatocytes, palmitate also caused mitochondrial depolarization and metformin significantly restored the mitochondrial depolarization ($P = 0.05$, Supplemental Fig. 4C). These results indicate that metformin and rotenone (0.1 $\mu\text{mol/L}$) recover the mitochondrial function in hepatocytes.

As shown in Figs. 1E and 4C, palmitate increased intracellular ROS generation, while both metformin and rotenone (0.1 $\mu\text{mol/L}$) reduced proton leak and decreased ROS generation. Therefore, the effects of metformin and rotenone (0.1 $\mu\text{mol/L}$) on antioxidant enzymes were investigated. We found that both metformin and rotenone induced SOD2 mRNA expression but did not induce SOD1 mRNA expression (Fig. 7A). Moreover, the protein level of SOD2 was also increased in mitochondria by metformin and rotenone (0.1 $\mu\text{mol/L}$) in HepG2 cells as shown by immunofluorescence (Fig. 7B). Furthermore, exogenous cell-permeable SOD (PEG-SOD, 100 U/mL) significantly reduced palmitate-induced caspase 3/7 activity ($P < 0.05$) (Fig. 7C). These results

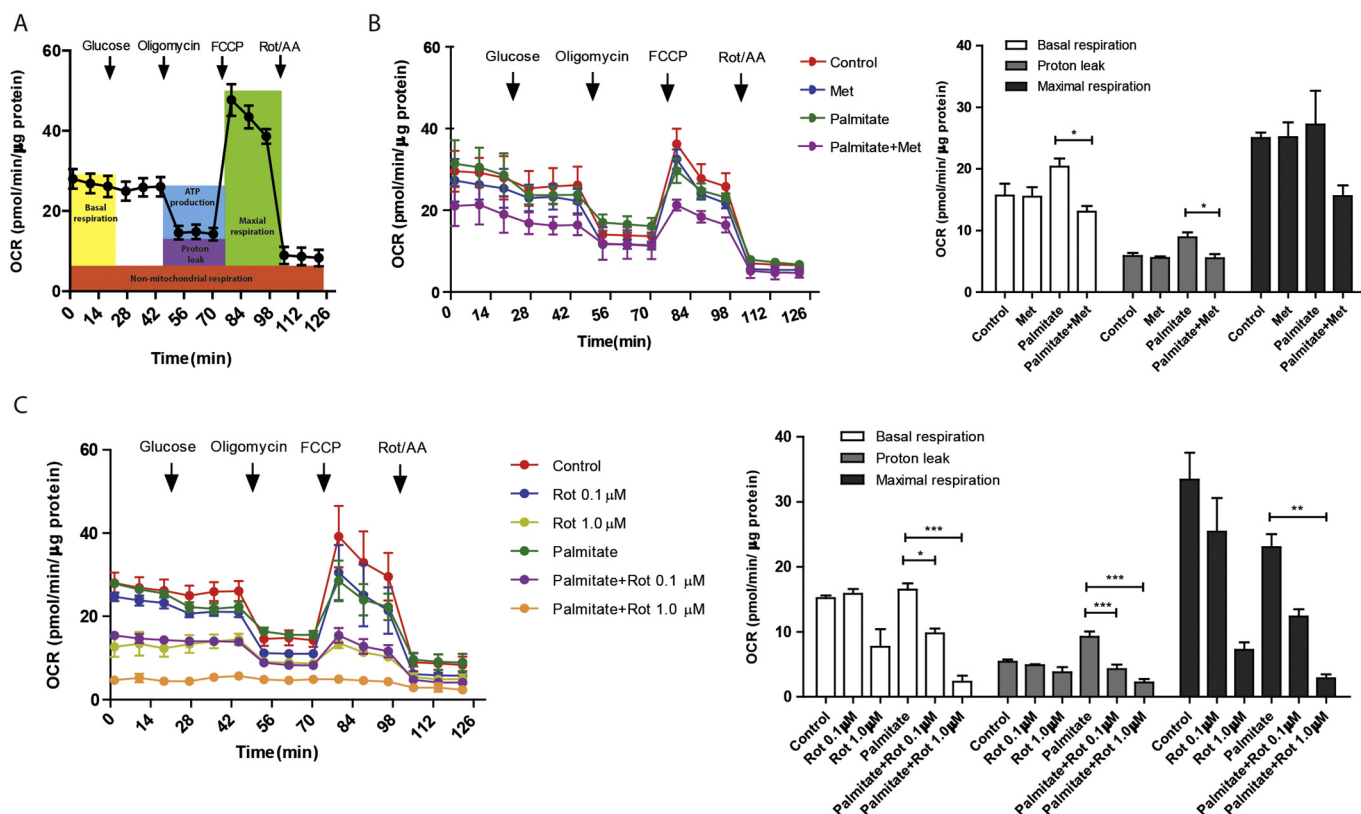
confirmed that metformin and rotenone (0.1 $\mu\text{mol/L}$) restored mitochondrial function and might prevent ROS accumulation by inducing SOD2 expression.

4. Discussion

In the present study, we showed that metformin protected hepatocytes from palmitate-induced cell death. The protective effect of metformin was mimicked by a low dose of the mitochondrial complex I inhibitor rotenone (0.1 $\mu\text{mol/L}$). Moreover, metformin and rotenone represented similar protective effects: both metformin and rotenone (0.1 $\mu\text{mol/L}$) partially inhibited mitochondrial respiration, restored mitochondrial membrane potential, reduced ROS production and induced SOD2 expression. Therefore, it is likely that metformin protects against palmitate-induced cell death by partially inhibiting mitochondrial complex I, thus restoring mitochondrial function. Of note, the protective effect of metformin was independent of AMPK signaling or reduction of lipid accumulation. The latter result differs from Woo *et al.* who reported a reduction of lipid accumulation in a hepatoma cell line by metformin [23]. However, in that study, lipid accumulation was assessed by Oil Red O staining only and not quantitated or statistically analyzed. We did quantitate lipid accumulation in HepG2 cells by measuring triglyceride content as well.

The protective effects of metformin was observed in both the hepatoma cell line HepG2 as well as in primary rat hepatocytes. HepG2

HepG2 cells



Primary rat hepatocytes

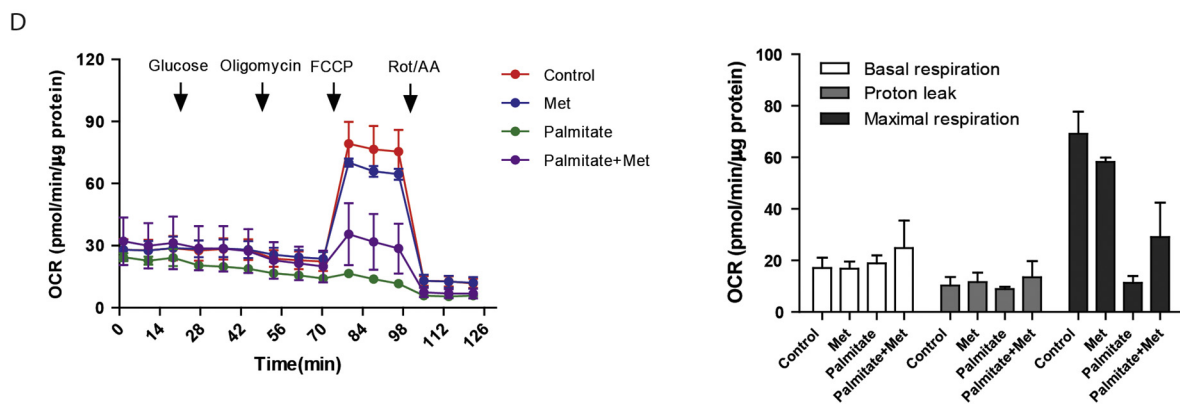


Fig. 5. Effect of metformin and rotenone on the mitochondrial respiratory chain.

(A) An illustration graph of the calculation of basal respiration, proton leak and maximal respiration. (B, C) HepG2 cells were pretreated with metformin or rotenone, then exposed to palmitate for 12 h. Real-time OCR was measured using XF24 analyzer. Injections include glucose (5 mmol/L), oligomycin (1 μmol/L), FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine, 0.25 μmol/L) and rotenone (1 μmol/L)/AA (antimycin, 1 μmol/L) sequentially. Basal respiration, proton leak and maximal respiration were calculated to illustrate the difference in metabolic profile. (D) Primary rat hepatocytes were pretreated with metformin, then exposed to palmitate (1.0 mmol/L) for 12 h. Real-time OCR was measured using XF24 analyzer. Data were shown as mean ± S.D. (n ≥ 3) * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001.

cells are one of the most commonly used substitutes for primary human hepatocytes, especially for studies on hepatotoxicity *in vitro*. It is one of the reasons that we choose HepG2 cells in this study. Besides, HepG2 cells also exhibit some metabolic features that are similar to in primary hepatocytes. For example, the HepG2 cell line is one of the few cell lines that demonstrate an insulin-stimulated increase of fatty acid uptake and decrease of lipid secretion and is therefore frequently used to investigate insulin-dependent pathways [24]. However, because of its

tumorigenic origin, HepG2 cells show some differences compared to primary hepatocytes with regard to metabolic pathways. One of the differences is the utilization of glucose. HepG2 cells exhibit a greater glucose incorporation rate [25], but impaired gluconeogenesis [24], as well as a lower *de novo* lipogenesis ability to convert glucose to lipids [25,26]. Besides, HepG2 cells have a lower fatty acid oxidation rate and accumulate more lipids upon fatty acid treatment compared to primary hepatocytes [25,26]. Upon palmitate challenge, we did observe some

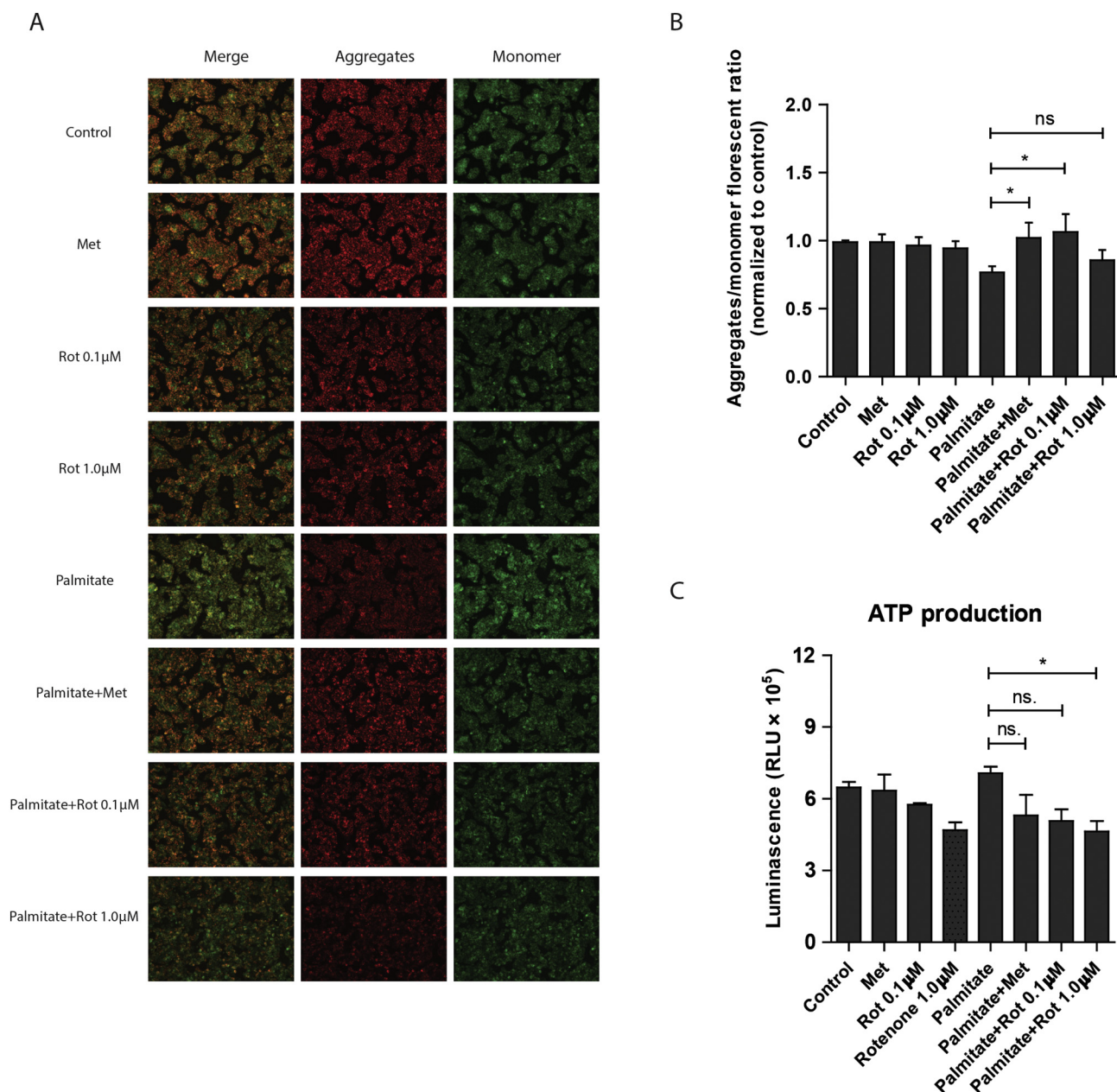


Fig. 6. Effect of Metformin and rotenone on mitochondrial membrane potential and ATP production.

HepG2 cells were pretreated with metformin or rotenone, then exposed to palmitate for 12 h. (A) HepG2 cells were stained by JC-10. Red emission indicates mitochondrial membrane depolarization, green emission indicates mitochondrial membrane polarization. Magnification 10 \times . (B) Statistical analysis of the aggregates/monomer fluorescence ratio. (C) ATP production was measured after 12 h of palmitate treatment in the presence and absence of metformin or rotenone. Data were shown as mean \pm S.D. (n \geq 3) * indicates $P < 0.05$; 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.)

differences between these two cell types: palmitate induced both apoptosis and necrosis in HepG2 cells, whereas it only induced necrosis in primary rat hepatocytes. Moreover, primary rat hepatocytes exhibited more severe damage of mitochondrial respiration (Fig. 5) and depolarization of the mitochondrial membrane potential (Fig. 6) compared to HepG2 cells, which might be explained by the higher fatty acid oxidation rate of primary hepatocytes compared to hepatoma cell lines [25,26].

Lipotoxicity is an important trigger of ER stress [27,28]. Saturated fatty acids or their toxic metabolites can disrupt protein folding and ER-to-Golgi trafficking resulting in activation of the unfolded protein response (UPR) [27]. There are three major signaling arms in UPR signaling that are initiated via three ER membrane associated proteins:

protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6). The PERK/p-eIF2 α arm is supposed to be the main signaling pathway related to lipoapoptosis. Once activated, the PERK/p-eIF2 α can transcriptionally induce the expression of its downstream targets, including Gadd34, GRP78 and CHOP, which also serve as markers to assess ER stress [27,28]. A protective effect of metformin against palmitate-induced ER stress has been reported in rat insulinoma cells [19]. Similarly, another study showed that metformin inhibited lipotoxicity in hepatocytes by reducing ER stress [20]. In our study, we also observed a reduction of p-eIF2 α upon treatment with metformin at 12 h. However, metformin did not subsequently reduce CHOP, GRP78 and Gadd34 mRNA expression at both 12 h and 16 h. The ER stress results reported in both papers

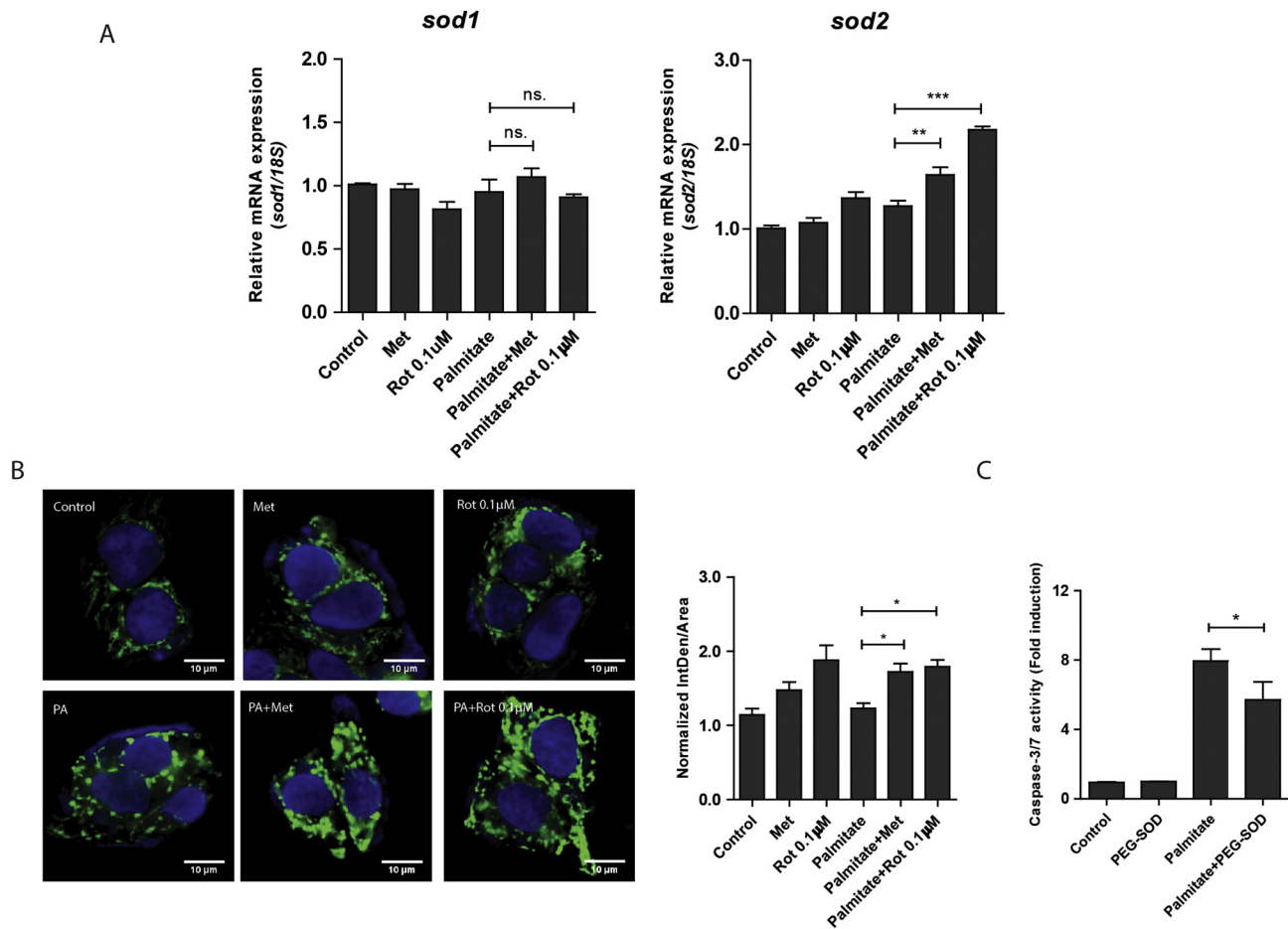


Fig. 7. Metformin and rotenone (0.1 $\mu\text{mol/L}$) induced SOD2 mRNA and protein expression but did not induce SOD1 mRNA expression. HepG2 cells were pretreated with metformin or rotenone, then exposed to palmitate for 12 h. (A) mRNA levels of SOD2 and SOD1 were measured and normalized with 18S. (B) The immunofluorescence signals of SOD2 were quantified by fluorescence intensity. (C) HepG2 cells were pretreated with PEG-SOD (100 U/mL) for 30 min, then exposed to palmitate for 16 h. Caspase 3/7 activity was measured. Magnification $40\times$, scale bars 10 μm . Data were shown as mean \pm S.D. ($n \geq 3$). * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; ns indicates $P > 0.05$.

mentioned above were at the protein level. Therefore, our results indicate that the reduced activation of p-eIF2 α did not lead to a reduction of its downstream UPR signaling, suggesting that metformin does not directly reduce ER stress and that the decreased phosphorylation of eIF2 α may be due to other mechanisms.

Another important target in lipotoxicity are mitochondria. Mitochondria play a key role in the intrinsic pathway of apoptosis and are one of the most important sources of ROS generation. Both clinical studies and animal experiments have shown that there is a significant increase in mitochondrial oxidative metabolism in NAFLD patients and animal models [29,30]. The elevated TCA cycle flux and impaired mitochondrial respiration chain could explain the common characteristic of increased oxidative stress in NAFLD. Furthermore, we showed that lowering the content of the intracellular antioxidant glutathione (GSH), using the GSH synthesis inhibitor buthionine-sulfoximine (BSO), increased palmitate-induced caspase-3/7 activity (Supplemental Fig. 2), indicating an important role of ROS in promoting lipoapoptosis. On the other hand, in our *in vitro* lipotoxicity model, the mitochondria showed more pronounced malfunctioning, indicated by the depolarization of the mitochondrial membrane potential and impaired mitochondrial respiration. In addition, palmitate can initiate apoptotic cell death by increasing cytochrome *c* release from mitochondria [31,32]. However, whether targeting of mitochondria will be a therapeutic option is still under investigation. Several studies showed that targeting different mitochondrial components could be either protective or detrimental [33–36]. In our present study, we found that moderate inhibition of

mitochondrial complex I (by metformin and rotenone at 0.1 $\mu\text{mol/L}$) can protect against palmitate-induced cell death. However, a severe inhibition (by rotenone at 1.0 $\mu\text{mol/L}$) was lethal. Although both moderate and severe inhibition of mitochondrial complex I induce SOD2 expression (part of the data unpublished), only moderate inhibition inhibits ROS production. Therefore, we hypothesize that moderate inhibition of mitochondrial complex I preserves mitochondrial function which leads to a recovery of mitochondrial potential and might relate to the increased expression of SOD2. In contrast, complete inhibition of mitochondrial complex I leads to severe dysfunction of mitochondria and aggravates cell death. This hypothesis is supported by the fact that moderate inhibition of complex I resulted in a slight, but not significant inhibition of ATP production, whereas, severe inhibition of complex I caused a significant depletion of ATP, which could also explain the different outcomes of two concentrations of rotenone. Of note, metformin and low dose rotenone only induced expression of SOD2, the mitochondrial isoform of SOD, but not the expression of SOD1, the cytoplasmic isoform. These results further support the notion that the mitochondria could be the first target of metformin in antagonizing lipotoxicity. We used two ROS scavengers (PEG-SOD and mito-TEMPO) to mimic the protective effects of metformin. The mitochondrial specific ROS scavenger (mito-TEMPO) tended to reduce palmitate-induced toxicity (data not shown) but only the general ROS scavenger (PEG-SOD) significantly reduced palmitate-induced caspase 3/7 activity, indicating that reduction of mitochondrial ROS production may not be the (sole) mechanism for the protective effect of metformin.

Metformin, as an inexpensive and widely accessible medicine, has shown a broad application in diseases. In the treatment of NAFLD, metformin presents beneficial effects in ameliorating hepatic steatosis and inflammation [37,38]. Both animal experiments and clinical trials have reported liver and body weight loss, histological improvement and decrease in aminotransferases levels after metformin treatment [23,37–40]. On the other hand, there are also clinical studies showing that metformin demonstrated limited improvements in NAFLD patients [41,42]. Given the dose-dependent results of rotenone in our present study, we hypothesize that the dosage might play a critical role in the actions of metformin. Since a proper inhibition of mitochondrial complex I was vital to counter lipotoxicity, either too low or too high doses of metformin may fail to show beneficial actions. Additionally, so far, there is no study testing the inhibitory effect of metformin on mitochondrial respiration in the context of NAFLD. Therefore, further studies are needed to assess metformin as an option for the treatment of NAFLD.

In conclusion, this study revealed that metformin protected against palmitate-induced cell death in hepatocytes, in an AMPK-independent manner, by partially inhibiting mitochondrial complex I, restoring mitochondrial function and increasing SOD2 expression and ROS scavenging. Furthermore, a low concentration of rotenone (0.1 $\mu\text{mol/L}$) showed identical results, whereas a high concentration of rotenone (1.0 $\mu\text{mol/L}$) exaggerated palmitate-induced cell death and failed to restore mitochondrial function and reduce ROS. We conclude that moderate inhibition of mitochondrial complex I promotes cell survival, which may relate to the recovery of mitochondrial function and induction of SOD2 expression. Taken together, metformin could be an attractive candidate for the treatment of NASH/NAFLD. Furthermore, mitochondrial complex I could be a potential therapeutic target in the treatment of NAFLD.

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Transparency document

The [Transparency document](#) associated with this article can be found, in the online version.

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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.

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