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Protective effects of caffeine against palmitate-induced lipid toxicity in primary rat hepatocytes is associated with modulation of adenosine receptor A1 signaling

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ABSTRACT

Background: Epidemiological evidence has shown an association between coffee consumption and reduced risk for chronic liver diseases, including metabolic-dysfunction-associated liver disease (MALFD). Lipotoxicity is a key cause of hepatocyte injury during MAFLD. The coffee component caffeine is known to modulate adenosine receptor signaling via the antagonism of adenosine receptors. The involvement of these receptors in the prevention of hepatic lipotoxicity has not yet been explored. The aim of this study was to explore whether caffeine protects against palmitate-induced lipotoxicity by modulating adenosine receptor signaling.

Methods: Primary hepatocytes were isolated from male rats. Hepatocytes were treated with palmitate with or without caffeine or 1,7DMX. Lipotoxicity was verified using Sytox viability staining and mitochondrial JC-10 staining. PKA activation was verified by Western blotting. Selective (ant)agonists of A1AR (DPCPX and CPA, respectively) and A2AR (istradefyline and regadenoson, respectively), the AMPK inhibitor compound C, and the Protein Kinase A (PKA) inhibitor Rp8CTP were used. Lipid accumulation was verified by ORO and BODIPY 453/50 staining.

Results: Caffeine and its metabolite 1,7DMX prevented palmitate-induced toxicity in hepatocytes. The A1AR antagonist DPCPX also prevented lipotoxicity, whereas both the inhibition of PKA and the A1AR agonist CPA (partially) abolished the protective effect. Caffeine and DPCPX increased lipid droplet formation only in palmitate-treated hepatocytes and decreased mitochondrial ROS production.

Conclusions: The protective effect of caffeine against palmitate lipotoxicity was shown to be dependent on A1AR receptor and PKA activation. Antagonism of A1AR also protects against lipotoxicity. Targeting A1AR receptor may be a potential therapeutic intervention with which to treat MAFLD.

1. Introduction

Coffee is one of the most widely consumed beverages around the world. Recent studies have shown that coffee consumption is associated with health benefits, including a decreased risk for developing chronic diseases such as metabolic syndrome, diabetes and metabolic-dysfunction-associated liver disease (MAFLD), formerly known as non-alcoholic liver disease (NAFLD) [1]. The beneficial effects of coffee are proposed to be linked to its bioactive components, such as caffeine, chlorogenic acids, trigonelline, and melanoidins, among others [2]. Caffeine is a trimethylxantine; its main physiological effects are derived from its antagonism of the adenosine receptors A1, A2A, A2B, and A3. The most prominent antagonistic effects of caffeine are against adenosine receptors A1 and A2A, due to the high affinity of these receptors for caffeine [3]. Adenosine receptor signaling has been implicated in hepatic steatosis, liver inflammation, and fibrosis [4]. The beneficial effects of caffeine in liver diseases have not yet been experimentally linked to its effects on adenosine receptor signaling.

Metabolic-dysfunction-associated liver disease (MAFLD) is a global public health problem, which includes a wide range of pathologies. Risk factors for MAFLD include obesity and diabetes type II, leading to metabolic alterations which favor steatosis (fat accumulation >5%) in

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the liver parenchyma [5]. Steatosis is linked to disturbed lipid metabolism, resulting in increased levels of free fatty acids (FFAs) in the liver. These disturbances are a result of: (1) the increased release of FFAs as a result of increased lipolysis in adipose tissue or from dietary sources; (2) enhanced de novo lipogenesis; and (3) the impaired secretion of very-low-density lipoprotein (VLDL) [6]. Lipotoxicity is considered a pivotal mechanism in the pathogenesis of MAFLD, and is closely related to oxidative stress, ER stress, and mitochondrial dysfunction, eventually inducing the activation of inflammatory and cell death responses [7]. The accumulation of FFAs, especially saturated fatty acids (SFAs), causes toxic effects and triggers various cellular stress responses. These detrimental effects are collectively known as lipotoxicity, which lead to the activation of inflammatory responses and the activation of non-parenchymal liver cells, further contributing to the progression of MAFLD to the inflammatory stage, non-alcoholic steatohepatitis (NASH), and eventually, to the development of liver fibrosis [8]. Therefore, the prevention of lipotoxicity may be an attractive target for MAFLD therapy.

The consumption of coffee is associated with a lower risk of development of MAFLD [9,10]. In particular, caffeine appears to be able to prevent inflammation and oxidative stress [11]. It has been demonstrated that caffeine regulates metabolic capacity and lipid metabolism and improves mitochondrial function and metabolic capacity in vitro and in vivo [12–14]. These effects of caffeine can be partially attributed to its effects on cAMP signaling [15]. Caffeine has also been demonstrated to inhibit liver fibrosis in different models. These anti-fibrotic effects of caffeine can also be partially attributed to cAMP-mediated pathways, i.e., caffeine inhibits rat hepatic stellate cell activation via antagonistic effects on adenosine receptors [16,17]. Although beneficial effects of caffeine have been demonstrated in experimental models of liver diseases, its effects on MAFLD, and in particular, on hepatocytes in the context of MAFLD, have not been fully explored. Specifically, whether the molecular mechanisms of the hepatoprotective effects of caffeine are related to adenosine receptor signaling has not yet been investigated. Therefore, the aim of this study was to explore the effects of caffeine on free-fatty-acid-induced lipotoxicity in primary rat hepatocytes and to investigate the involvement of adenosine receptor signaling in the protective effect of caffeine.

2. Materials and methods

2.1. Chemicals, stock solutions, and treatments

Caffeine (C0750 Sigma Aldrich Saint Louis, USA) and 1,7-Dimethylxanthine (D5385 Sigma Aldrich) were dissolved in deionized water to prepare stock solutions, and then sterilized by filtration using nitrocellulose membrane (0.25 μm). Palmitate stock solution (20 mmol/L) was prepared using sodium palmitate (Sigma Aldrich P9767) in an aqueous solution of bovine serum albumin, free-fatty-acid-free (BSA FFA-free), as previously described [18]. Compound C was used as an AMPK inhibitor (10 μmol/L) (Sigma Aldrich, #171260). RP-8CPT-cAMPS (8-[(4-chlorophenyl)thio]-adenosine cyclic 39,59-[hydrogen (R)-phosphorothioate]) (Cayman Chemicals 17142, Ann Arbor, USA) was used as a selective PKA inhibitor (100 μmol/L) and selective EPAC1 inhibitor CE3F4 ((5,7-dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2 H)-quinoline carboxaldehyde) and EPAC2 inhibitor ESI05 (1,3,5-trimethyl-2-[(4-methylphenyl)sulfonyl]-benzene, mesityl(4-methylphenyl) sulfon) were used at a final concentration of 10 μmol/L, as previously described [19]. Regadenoson (Sigma Aldrich SML2506) and N6-Cyclopentyladenosine CPA (Sigma Aldrich C8031) were used as selective agonists of A2A and A1 receptors, respectively; Istradefylline (Sigma Aldrich SML0422) and 8-Cyclopentyl-1,3-dipropylxanthine DPCPX (Sigma Aldrich C101) were used as selective antagonists of A2A and A1 receptors at final concentration of 10 μmol/L, respectively [20,21]. The compounds were dissolved according to the manufacturer's instructions in DMSO to obtain stock solutions and were stored at − 20 ºC protected from light.

2.2. Animals and compliance with requirements for studies using experimental animals

Experiments were performed according to Dutch laws on the welfare of laboratory animals (The Animal act 2011) and Permission No 16778–01–002 of the Committee for the care and use of laboratory animals of the University of Groningen. Specified pathogen-free male Wistar rats (220–250 g) aged 5–8 weeks old and Zucker rats (12–14 weeks) were purchased from Charles River Laboratories Inc (Wilmington, MA, USA). Animals were housed in polypropylene cages at room temperature (25 ± 2º C) with standard bedding, regular (12 h light/12-hour dark) day cycle, and free access to standard laboratory chow and water at the animal facility center of the University Medical Center of Groningen. During isolation, anesthesia was first induced through the use of an isofluorane (5%) chamber for 5 min. For anesthesia, a combination of ketamine (100 mg/mL, 60 mg/kg bodyweight) and medetomidine hydrochloride (1 mg/mL, 0.5 mg/kg bodyweight) was used.

2.3. Isolation of primary rat hepatocytes and cell culture

Primary rat hepatocytes were isolated from male pathogen-free Wistar rats (180–250 g; Charles River Laboratories Inc. (Wilmington, MA, USA)), using two-step collagenase perfusion method according to the protocol previously described [22]. All experiments were performed according to Dutch laws on the welfare of laboratory animals (The Animal act 2011) and Permission No 16778–01–002 of the committee for the care and use of laboratory animals of the University of Groningen.

Cell viability was measured by the trypan blue exclusion method; only cell isolations with a viability above 80% were used for experiments. Hepatocytes were cultured in William's E medium (Invitrogen, Breda, the Netherlands), supplemented with 5% fetal bovine serum, dexamethasone, 50 μg/mL gentamicin (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 an atmosphere containing 5% (v/v) CO_2. Experiments were started after an attachment period of 4 h. Hepatocytes were treated with palmitate (1.0 mmol/L) with or without caffeine (1.0 mmol/L) or 1,7DMX paraxanthine (0.05 mmol/L) for 16–24 h, in serum-free and dexamethasone-free medium supplemented as described above.

2.4. Cell death assessment

To determine cell death, SYTOX® Green nucleic acid stain (S7020 Invitrogen, Carlsbad, CA) and propidium iodide (PI) staining were used. Hepatocytes were treated with test substances for the indicated time periods. Sytox Green solution (125 nmol/L) or PI solution (1 mg/mL) were added to the cells after the treatment for 15 min at 37 °C in an atmosphere containing 5% (v/v) CO_2. Necrotic cells with disrupted membrane integrity were stained green (SYTOX) or red (PI) and visualized using a Leica DMI6000 fluorescence microscope (Leica, Amsterdam, the Netherlands) at 512–542 nm and 585–624 nm, respectively. Caspase-3 activity was assessed as previously described [23].

2.5. ATP measurement

The cellular ATP level was determined using ATP quantitative assay (Promega, Leiden, The Netherlands). Briefly, hepatocytes were seeded in 96-well plates (50×10^4 cells/well) and treated for 8–24 h. The ATP level was then measured according to the manufacturer's instructions and luminescence was detected using a Bio-Tek FL600 microplate fluorescent reader (Bio-Tek, Winooski, VT, USA).

2.6. Mitochondrial membrane potential assessment, JC-10

To assess mitochondrial membrane potential, after 2 h treatment with test substances, hepatocytes were stained with a JC-10 probe (100 μmol/L; Enzo Life Sciences®, Farmingdale, NY, USA ENZ-52305) for 15–30 min. JC-10 accumulates in the mitochondria and generates a characteristic orange J-aggregate emission profile (590 nm) in healthy cells, while green monomer emission (520 nm) indicates depolarization. The visualization of JC-10 aggregates was performed using a Leica DMI6000 fluorescence microscope (Leica, Amsterdam, the Netherlands) at 512–542 nm and 590 nm.

2.7. Mitochondrial ROS measurement

Mitochondrial ROS production was evaluated using MitoSOX™ Red superoxide indicator (Molecular Probes Inc, Eugene, USA M36008). Cells were seeded on coverslips and treated with test substances for 2 h. Cells were subsequently washed with warm HBSS buffer and stained with 2.5 μmol/L MitoSOX™ Red superoxide indicator for 15 min. Cells were then washed three times with buffer and mounted on cover glasses with DAPI antifade mounting medium (Vectashield). Cells were visualized using a Leica DMI6000 fluorescence microscope (Leica, Amsterdam, the Netherlands) at 590 nm. Quantitative analysis of the fluorescent images was performed using ImageJ.

2.8. Oil Red Staining

Intracellular neutral lipids were determined using Oil Red staining. Cells were seeded on coverslips and treated with test substances for 24 h. Free fatty acid treatment (2:1 oleate 500 μmol/L, palmitate 250 μmol/L) was used as a positive control for lipid accumulation. After treatment, cells were washed twice with buffer and twice with isopropanol–water solution (60%), then stained for 10 min with ORO solution (dissolved in 60% isopropanol). After staining, cells were washed again with isopropanol–water solution and then counter-stained with hematoxylin. Coverslips were mounted on cover glasses with glycerin–gelatin and visualized using a digital slide scanner (Hamamatsu, Japan). Image analysis was performed using NDPview2 viewing software. The Oil Red area was further quantified using ImageJ.

2.9. BODYPY staining

To visualize lipid droplets, a BODIPY 493/50 probe (Molecular probes) was used, according to the protocol previously described (Qiu and Simon et al., 2016). Briefly, cells were seeded on coverslips and treated with test substances for 24 h. Cells were subsequently washed with warm HBSS buffer. Subsequently, cells were stained with BODIPY solution (2 μmol/L) for 15–20 min and then washed and fixed with paraformaldehyde (3.7%). Coverslips were mounted on cover glasses with DAPI antifade mounting medium (Vectashield). Images were visualized using a Leica DMI6000 fluorescence microscope (Leica, Amsterdam, the Netherlands) at 512–524 nm.

2.10. Triglyceride quantification

The intracellular triglyceride content was quantified in hepatocytes after 24 h of treatment. Cells were seeded on 12-well plates and lysed with 5% NP-40 solution. Cell lysates were used to extract lipids via at least 3 cycles of heating at 90–100 ºC and cooling or until precipitation was observed. Samples were then centrifuged, and the supernatant was used to quantify total intracellular triglycerides using a Triglyceride Assay Kit (Abcam), following the manufacturer's protocol. Samples were measured using fluorometric quantification at 535/587 nm using a Bio-Tek FL600 microplate fluorescent reader (Bio-Tek). The triglyceride content was calculated based on a standard curve and total amount of TG was calculated relative to the total amount of protein per sample (μg TG/mg protein).

2.11. RNA isolation and quantitative RT-PCR (qRT-PCR)

For RNA analysis, cells were harvested 8 h or 16 h after treatment for lipid-metabolism-related genes, ER stress markers, and adenosine receptors A1, A2A, and A2B. The basal expression of adenosine receptors A1, A2A, and A2B was determined in different populations of primary liver cells: hepatocytes, Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells. First, cells were washed twice with cold buffer, and total RNA was isolated using TRI reagent (Sigma-Aldrich), according to the manufacturer's instructions. Reverse transcription (RT) was performed using 2.5 μg of total RNA, 1X RT buffer (500 mmol/l Tris-HCl [pH 8.3]; 500 mmol/l KCl; 30 mmol/l $MgCl_2$; 50 mmol/l DTT), 1 mmol/l deoxynucleotides triphosphate (dNTPs, Sigma-Aldrich), 10 ng/μL random nanomers (Sigma-Aldrich), 0.6 U/μL RNaseOUT™ (Invitrogen, Carlsbad, CA), and 4 U/μL M-MLV reverse transcriptase (Invitrogen) at a final volume of 50 μL. The cDNA synthesis program was 25 °C/10 min, 37 °C/60 min, and 95 °C/5 min. Complementary DNA (cDNA) was diluted 20 × in nuclease-free water. Real-time qPCR was carried out using a StepOnePlus™ (96-well) PCR System (Applied Biosystems, Thermo Fisher, Wilmington, DE) with TaqMan probes. For qPCR, 2 × reaction buffer (dNTPs, HotGoldStar DNA polymerase, 5 mmol/l $MgCl_2$) (Eurogentec, Belgium, Seraing), a 5 μmol/l fluorogenic probe, and 50 μmol/l of sense and antisense primers (Invitrogen) were used. mRNA levels were normalized to the 18 S housekeeping gene and further normalized to the mean expression level of the control, as shown in Table 1.

2.12. Western Blot analysis

Total protein lysates were prepared using lysis buffer (50 mmol/l Tris-base, pH 7.4, 0.2% Triton X-100, 0.25% Na-deoxycholate, 150 mmol/l NaCl, 1 mmol/EDTA) supplemented with protease inhibitors. Cell lysates were resolved on Mini-PROTEAN® TGX Stain-Free™ Precast Gels (BioRad, Oxford, UK). Semi-dry blotting was performed using a Trans-Blot Turbo Midi Nitrocellulose Membrane with Trans-Blot Turbo System Transfer (BioRad). Ponceau S 0.1% w/v (Sigma-Aldrich) staining was used to confirm the protein transfer of anti-phospho-PKA Substrate (RRXS*/T *) (Cell Signaling #9624), phospho-PKA C (Thr197) (Cell Signaling #5661), anti-phospho-AMPK-α (Thr172) (Cell Signaling #2531), anti-phospho-HSL (Ser660) (Cell Signaling #4126), anti-GRP78 (Cell Signaling #3177), anti-phospho-eIF2α (Ser51) (Cell Signaling #9721), and anti-eIF2α (Cell Signaling #9722). Anti-GAPDH (Calbiochem #CB1001) or anti-α-tubulin (Sigma Aldrich T9026) were used as loading controls. The appropriate anti-rabbit or anti-mouse peroxidase-conjugated secondary antibodies were used. Blots were analyzed using a ChemiDoc XRS system (Bio-Rad). Protein band intensities were quantified using ImageLab 6.1 software (BioRad).

2.13. Statistical analysis

All the experiments were performed using hepatocytes from at least three independent isolations ($n = 3$) and with two replicates for each experimental condition. Normality was assessed using Kolmogorov–Smirnov or Shapiro–Wilk tests. The statistical significance of the differences between the means of the experimental groups was evaluated using one-way and two-way analyses of variance (ANOVAs), and Student's t-test as a post-test analysis. The results are presented as the mean ± standard error (mean ± SD) ns $p > 0.05$, * $p \leq 0.05$, * *, $p \leq 0.01$, * ** : $p \leq 0.001$, * ** *: $p \leq 0.0001$. Analyses were performed using GraphPad Prism software 9 (Version 9.4.1, GraphPad Software Inc., CA, USA).

Table 1
List of primers and probes sequences used in this study.

Gene	Forward 5′– 3′	Reverse 5′– 3′	Probe 5′– 3′
18 S (human, rat)	CGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	CGCGCAAATTACCCACTCCCGA
DGAT1	GGTGCCCTGACAGAGCAGAT	CAGTAAGGCCACAGCTGCTG	CTGCTGCTACATGTGGTTAACCTGGCCA
DGAT2	GGGTCCAGAAGAAGTTCCAGAAG	CCCAGGTGTCAGAGGAGAAGAG	CCCCTGCATCTTCCATGGCCG
SRBPE1c	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA	CAGCTCATCAACAACCAAGACAGTGACTTCC
PPARa	CACCCTCTCTCCAGCTTCCA	GCCTTGTCCCCACATATTCG	TCCCCACCAGTACAGATGAGTCCCCTG
CD36	GATCGGAACTGTGGGCTCAT	GGTTCCTTCTTCAAGGACAACTTC	AGAATGCCTCCAAACACAGCCAGGAC
PNPLA3	GTAGCCACTGGATATCTTCATGGA	TCTTGCTGCCCTGCACTCT	CACCAGCCTGTGGACTGCAGCG
ATF4	CGGCAAGGAGGATGCCTTT	ACAGAGCATCGAAGTCAAACTCTTT	CCATTTTCTCCAACATCCAATCTGTCCC
CHOP	TCCTGTCCTCAGATGAAATTGG	TCAAGAGTAGTGAAGGTTTTTGATTCT	CACCTATATCTCATCCCCAGGAAACGAAGA
GRP78	AAAGAAGGTCACCCATGCAGTT	CAATAGTGCCAGCATCCTTGT	ACTTCAATGATGCACAGCGGCAAGC
A1AR	GAGAATCCAGCAGCCAGCTATG	GATCGATACCTCCGAGTCAAGATC	CTCTCCGGTACAAGACAGTGGTGACCCA
A2AR	CGCCATCGACCGCTACAT	TCGCCCTCACACCTGTCA	CCATCCGAATTCCACTCCGGTACAATG
A2BR	GTGGCTGTCGACCGGTATCT	CCCTCTTGCTCGTGTTCCA	CCATTCGCGTCCCGCTCAGGTATAAA

3. Results

3.1. Caffeine protects hepatocytes against palmitate-induced lipotoxicity

In this study, the hepatoprotective effect of caffeine and its main metabolite, 1,7DMX (Fig. 1), was tested in primary rat hepatocytes. A range of concentrations was tested between 0.05 and 1.5 mmol/L for both treatments, as previously reported [24–26]. We selected the lowest concentration which still showed maximal protection: 1.0 mmol/L for caffeine and 0.5 mmol/L for 1,7DMX (data not shown). These concentrations were used in all subsequent experiments.

Palmitate treatment at 1 mmol/L induced significant hepatocyte cell death after 24 h, which was significantly reduced in cells co-treated with caffeine at 1.0 mmol/L (89% reduction) and 1,7DMX at 0.5 mmol/L (70% reduction) (Fig. 2 A, C,D). The protective effect of caffeine, but not that of 1,7DMX, was correlated with the preservation of mitochondrial membrane potential: JC-10 staining revealed that caffeine, but not 1,7DMX, prevented the loss of mitochondrial membrane potential in palmitate-treated hepatocytes (Fig. 2B). Caffeine did not prevent the palmitate-induced loss of cellular ATP, despite its protection against cell death (Fig. 2D). In addition, we tested the effect of caffeine on HepG2 cells to evaluate cell death and caspase-3 activation, because palmitate induces apoptotic cell death in HepG2 cells. Caffeine protected HepG2 cells from palmitate-induced cell death and decreased caspase-3 activation in a concentration-dependent manner (Fig. S1). Effects on ER stress markers were assessed by determining the mRNA levels of CHOP, ATF4, and GRP78. Palmitate induced a slight increase in ATF4 and CHOP expression after 8 h of treatment, and co-treatment with caffeine (PA+CF) tended to reduce the expression of CHOP; however, these differences did not reach statistical significance. No significant effect of caffeine was found on the mRNA levels of ATF4 or GRP78 (Fig. S2). Protein levels of the ER stress markers GRP78 and p-eIF2α were also checked by Western blot. Neither palmitate alone, nor co-treatment with caffeine or 1,7DMX, induced any changes in the protein expression of

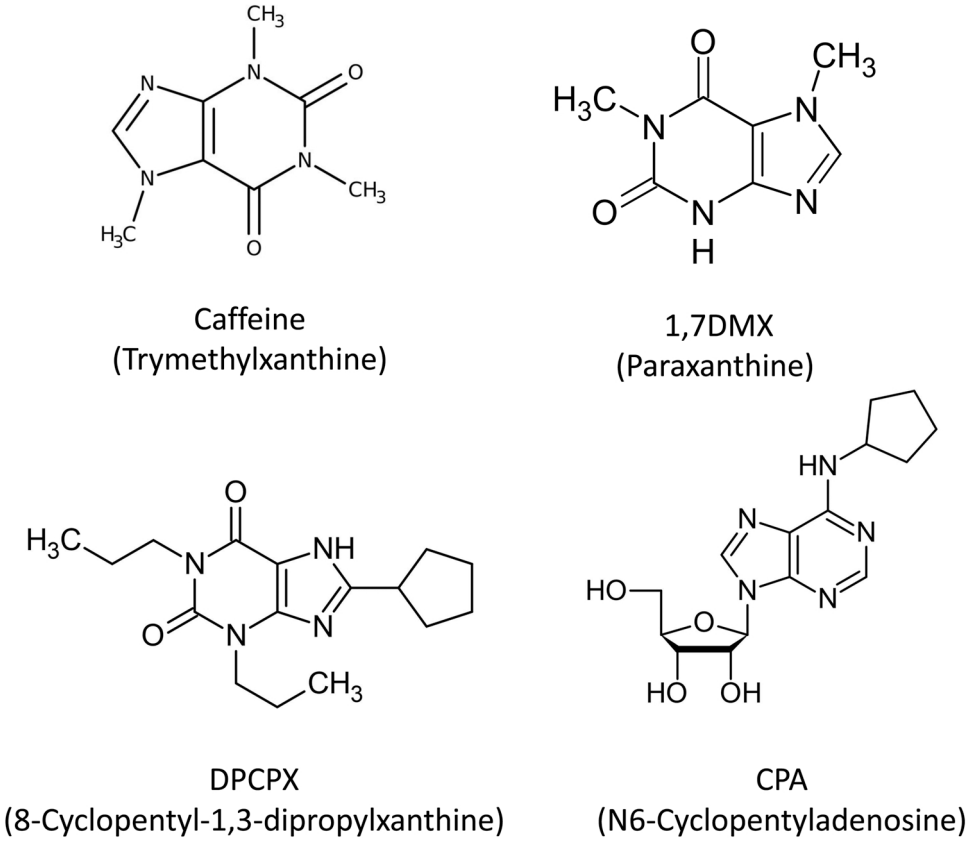

Fig. 1. A. Chemical structure of compounds used in this study: caffeine, 1,7-DMX, DPCPX, and CPA.

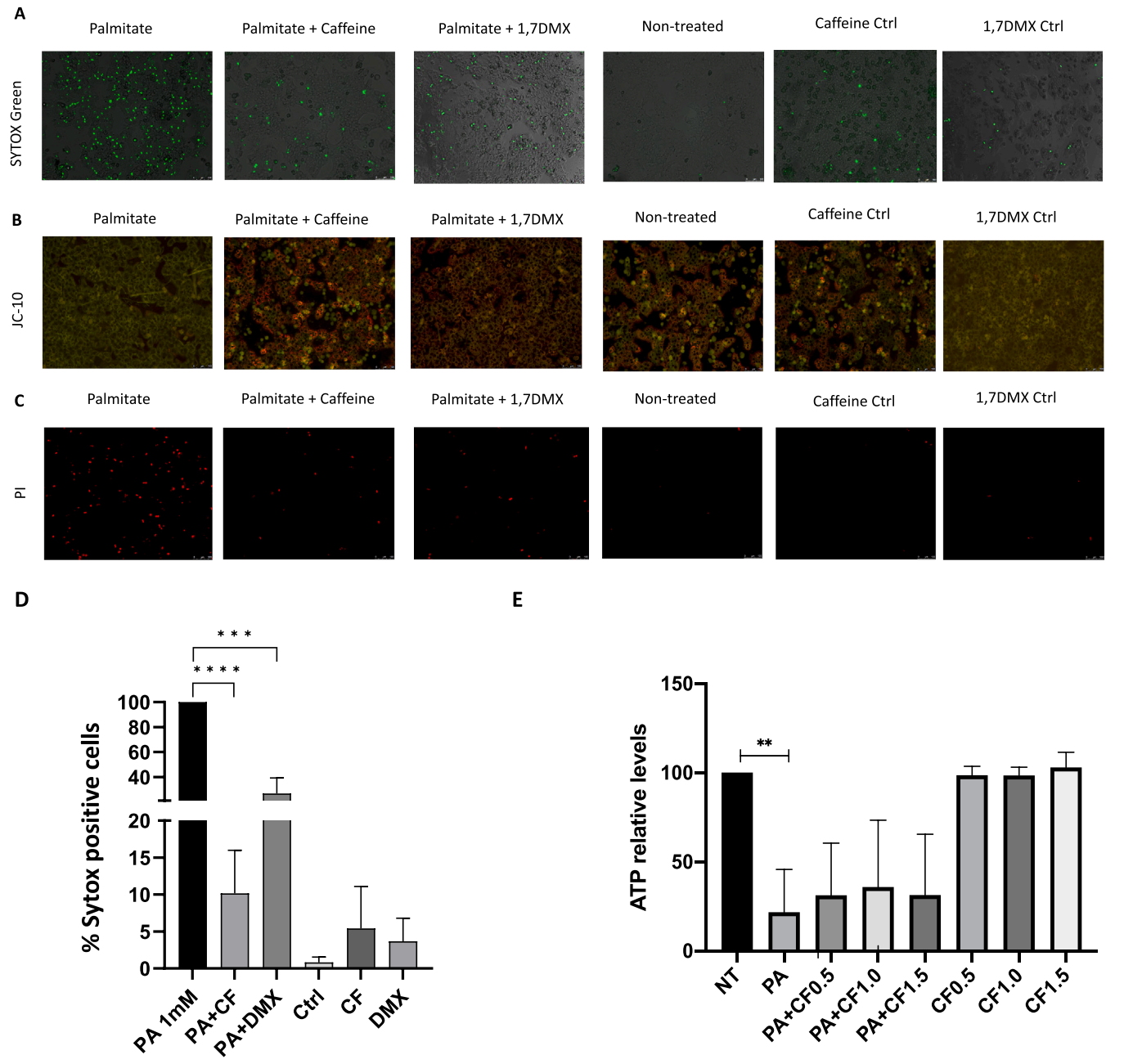

Fig. 2. Caffeine and 1,7DMX protect against palmitate-induced hepatocyte toxicity. A. SYTOX Green staining. Green nuclei correspond to SYTOX-positive necrotic cells. B. JC-10 staining. The presence of orange aggregates (590 nm) indicates intact mitochondrial membrane polarization. C. Propidium iodide (PI) staining. Red nuclei correspond to necrotic cells. D. Quantitative analysis of SYTOX positive cells. E. ATP levels in hepatocytes after 24 h treatment. Scale bar: 100 μm. PA, palmitate 1 mmol/L; CF, caffeine 1 mmol/L; DMX, 1,7DMX 0.5 mmol/L; Ctrl, control, non-treated cells (BSA FFA-free 0.25%).

GRP78 and p-eIF2α (data not shown).

3.2. *Caffeine increases lipid accumulation in the presence of palmitate*

Lipid accumulation in hepatocytes was assessed by ORO and BODIPY lipid droplet staining in palmitate-treated hepatocytes, with and without co-treatment with caffeine. Control and palmitate-treated hepatocytes did not show significant lipid droplet accumulation in hepatocytes. However, an increase in lipid droplet quantity was observed when caffeine was added in combination with palmitate (Fig. 3 A-C). Caffeine alone did not have a significant effect on lipid accumulation, suggesting that the modulation of lipid droplet formation is involved in the protective mechanism of caffeine. Treatment with FFAs served as a positive control for lipid accumulation in hepatocytes. The amount of lipid accumulation observed in palmitate- and caffeine-treated hepatocytes was comparable to FFA-treated hepatocytes, but the morphology and distribution patterns of lipid droplets visualized by BODIPY staining showed different patterns. Large lipid droplets were mainly observed in FFA-treated cells, and were distributed evenly in the cell. In contrast, lipid droplets induced by caffeine treatment were smaller in size and they appeared to have a more peripheral distribution in the cell (Fig. 3B).

Increased lipid droplet formation observed with ORO and BODIPY was correlated with the total intracellular TG content: hepatocytes treated with palmitate in combination with caffeine showed higher TG levels in comparison to palmitate- and non-treated cells, but caffeine alone did not have an effect on the intracellular TG levels (Fig. 3D). Next, the mRNA expression levels of different lipid-metabolism-related

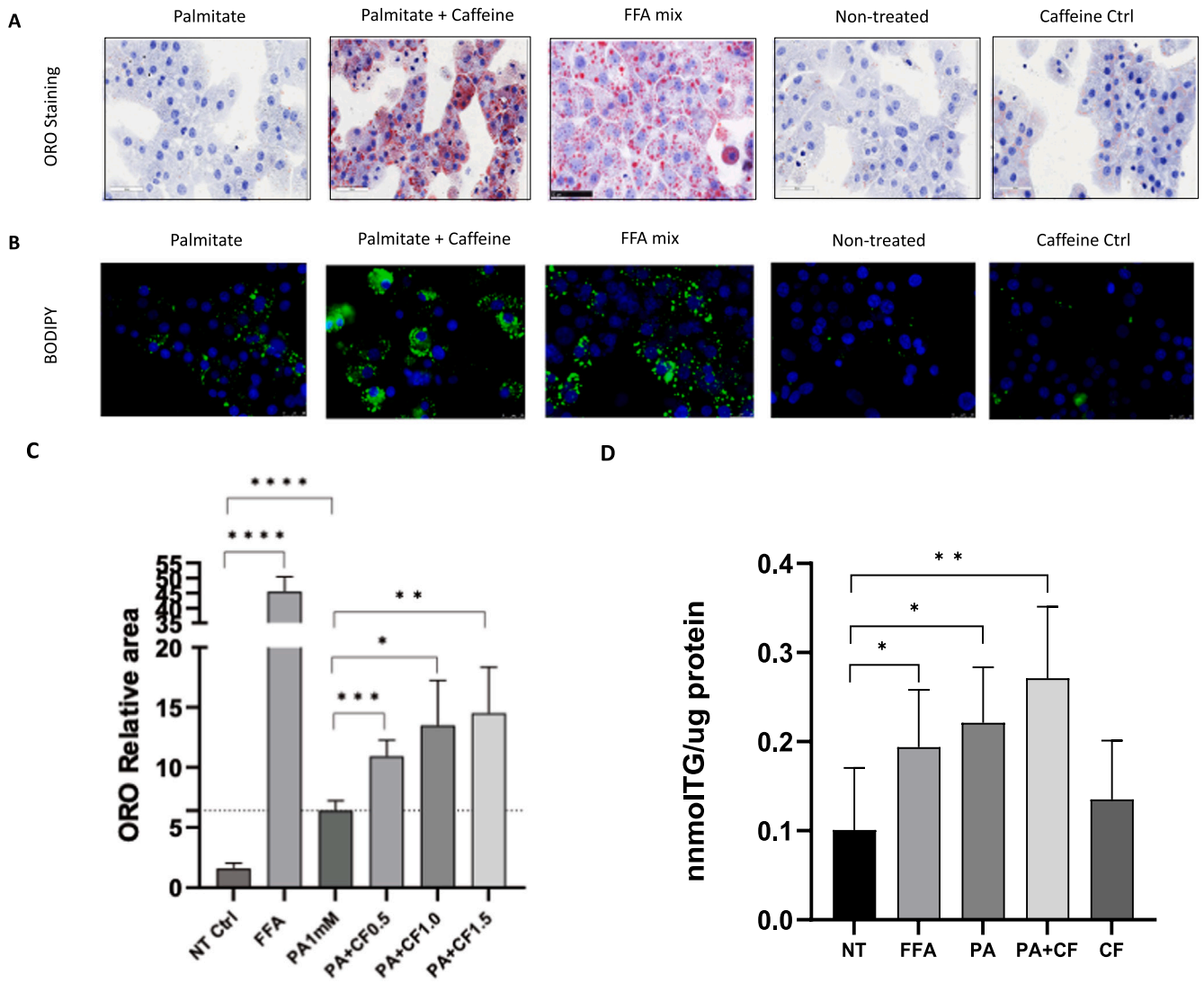

Fig. 3. The protective effect of caffeine correlates with increased lipid droplet accumulation in palmitate-treated hepatocytes. A. ORO staining and B. BODIPY 593/50 staining were used to detect lipid droplets (green); nuclei were visualized with DAPI (blue). Scale bar: 25 μm. C. Quantitative analysis of ORO staining and D. total intracellular triglyceride quantification. PA, palmitate 1 mmol/L; CF: caffeine 1 mmol/L; DMX: 1,7DMX 0.5 mmol/L; FFA: free fatty acid mix: OA 0.5 mmol/L:PA 0.25 mmol/L; NT Ctrl: non-treated (control) cells (BSA FFA-free 0.25%). Scale bars: 50 μm (ORO) and 25 μm (BODIPY).

genes were measured. No significant differences between the experimental conditions were observed in the evaluated genes; however, caffeine treatment showed an upward trend in PPARα and CD36 mRNA levels compared with non-treated cells (Fig. S3), although this trend did not reach statistical significance. Additionally, the expression of SREBP1c, the transcription factor that regulates de novo lipogenesis, was similar in the palmitate+caffeine condition compared with palmitate-treated cells. Moreover, caffeine did not show any effects on lipid accumulation in FFA-treated hepatocytes, supporting the notion that lipid droplet regulation as a mechanism of the response induced by caffeine only occurs in lipotoxic conditions (Fig. S4).

3.3. The protective effect of caffeine is dependent on PKA and AMPK activation but independent of EPACs

Caffeine is known to regulate cAMP levels in different experimental models [16,27,28]. In order to determine whether the protective effect of caffeine against lipotoxicity is related to the modulation of cAMP levels and the subsequent activation of downstream pathways, we explored the involvement of the cAMP downstream targets PKA and AMPK, using the selective inhibitors RP-8CPT-cAMPS and compound C, respectively. The protective effect of caffeine against palmitate-induced cell death was strongly diminished by both inhibitors (Fig. 4A-C), but statistical significance was only observed for compound C (Fig. 4C). In the palmitate+caffeine condition, cell death was approximately 11%, whereas cell death increased to approximately to 88% and 79% in the presence of inhibitors of PKA and AMPK, respectively.

In addition, the activation of PKA and AMPK was confirmed by identifying the active phosphorylated isoforms, p-PKA C and p-AMPK, by Western blot. Interestingly, caffeine and 1,7DMX appeared to increase AMPK activation by itself; however, no significant differences were observed in p-PKA or p-AMPK levels among the treatments (Figs. 5A, B, S5), probably due to the high basal level of activation. Moreover, no concentration-dependent effects were observed when different concentrations of caffeine or 1,7DMX were used (data not shown).

To confirm PKA activation, we used a pan-PKA antibody to detect all phosphorylated target substrates of PKA in different experimental conditions. Increased PKA activation was confirmed by the increased phosphorylation of specific proteins in caffeine and 1,7DMX-treated cells, compared with palmitate-treated cells after 8 h of treatment (Fig. 6A). Although after 16 h PKA phosphorylated targets were still

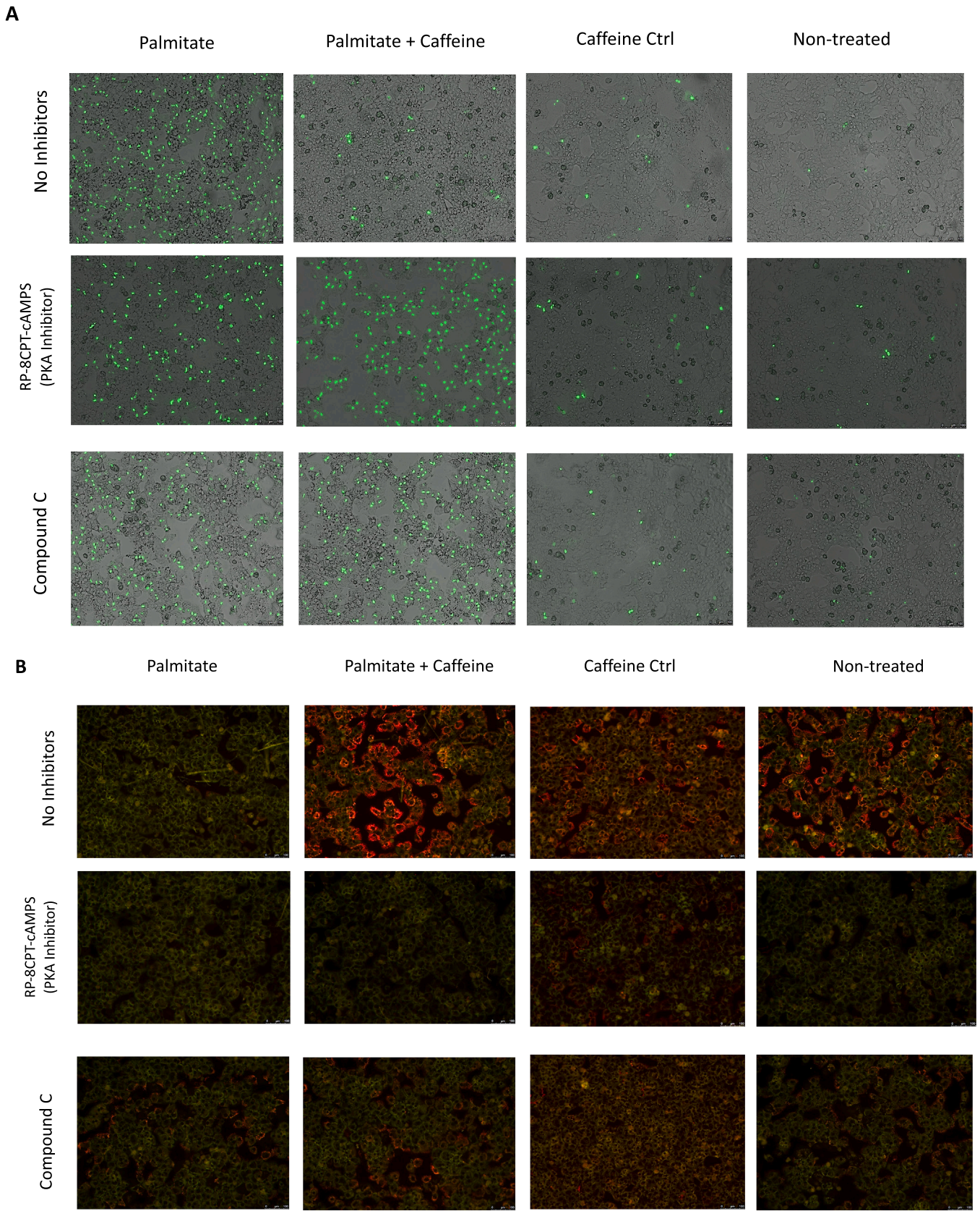

Fig. 4. The protective effect of caffeine is dependent on AMPK and PKA activation and independent of EPACs. A. SYTOX Green staining. Green nuclei correspond to SYTOX-positive, necrotic cells. B. JC-10 staining. The presence of orange aggregates (590 nm) indicates intact mitochondrial membrane polarization. C. Quantitative analysis of SYTOX-positive cells. PA, Palmitate 1 mmol/L; CF, caffeine 1 mmol/L; PKA Inh, RP-8CPT-cAMPS; Comp C, compound C. Scale bar: 100 μm.,.

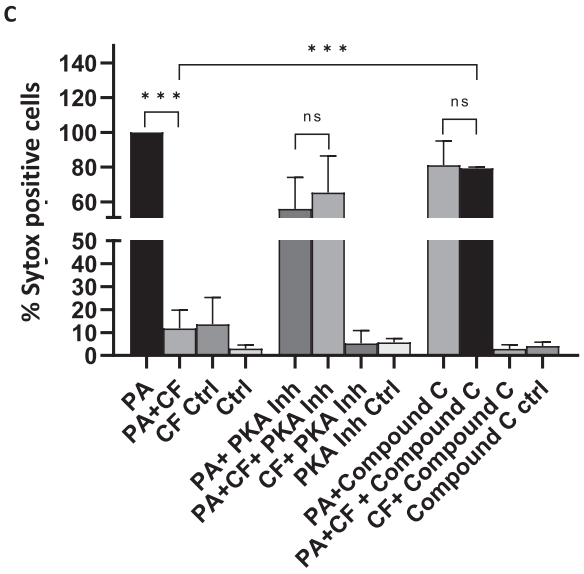

Fig. 4. (*continued*).

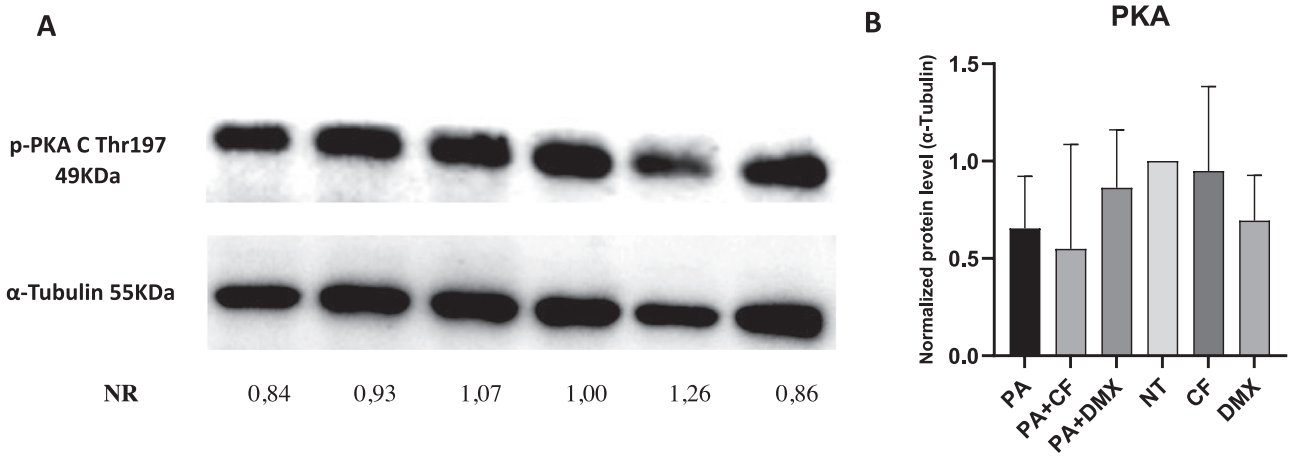

Fig. 5. The effect of caffeine and DMX on PKA activation. A. Detection of phosphorylated PKA after 8 h of treatment. B. Quantitative analysis of PKA protein expression. NR, normalized ratio; PA, palmitate 1 mmol/L; CF, caffeine 1 mmol/L; DMX, 1,7DMX 0.5 mmol/L; NT Ctrl, non-treated (control) cells (BSA FFA-free 0.25%).

detectable, the expression was significantly lower compared with 8 h detection (data not shown). These results confirm that PKA is critical in mediating the protective effects of caffeine and 1,7DMX against lipotoxicity. In addition to PKA, cAMP elevation activates alternative pathways mediated by EPAC (exchange protein activated by cAMP) enzymes [29,30]. To investigate whether EPAC activation is involved in the downstream effects of cAMP, selective EPAC inhibitors CE3F4 (EPAC1) and ESI05 (EPAC2) were used. The EPAC inhibitors did not affect the protective effect of caffeine against palmitate toxicity, suggesting that the protective effect is EPAC-independent (Fig. S6).

3.4. Expression of adenosine receptors A1, A2A, and A2B in different rat liver cells

Caffeine and its main metabolite, 1,7DMX, are antagonists of adenosine receptor signaling; however, there are differences in the affinity for these receptors, with a higher affinity reported for the A2A and A1 receptor. Therefore, we investigated the mRNA expression of A1, A2A, and A2B receptors in different rat liver cell populations, including hepatocytes, Kupffer cells (KC), liver sinusoidal endothelial cells (LSECs), and quiescent and activated stellate cells (qHSCs and aHSCs, respectively). A1 and A2B receptors were highly expressed in hepatocytes compared with other liver cell types, except for aHSCs, where A2B expression was higher than in any other liver cell type (Fig. 7 A). In the case of A2A, expression was higher (100–200 fold) in non-parenchymal cells compared with hepatocytes, whereas the expression of A1 was much higher in hepatocytes compared with non-parenchymal cells. Interestingly, a shift in the expression of A2AB was observed in aHSCs compared with qHSCs, suggesting the potential importance of this receptor in HSC activation. Furthermore, adenosine receptor A1 expression in primary rat hepatocytes was significantly higher than A2A and A2B receptor expression (Fig. 7B). These results suggest that A1 receptor effects in the liver are mainly mediated by hepatocytes. A2A expression was high in all populations of non-parenchymal cells, particularly in LSECs and qHSCs. A2B expression was low in non-parenchymal cells compared with hepatocytes. To investigate whether lipid accumulation regulates A1AR expression in MAFLD, we also determined the A1AR mRNA expression in rat hepatocytes isolated from Zucker obese diabetic rats (ZDF Fat) and lean controls, respectively. Fat accumulation in Zucker rat liver increases A1AR mRNA expression in ZDF hepatocytes which showed a higher expression of A1AR mRNA (Fig. 7 C). These results confirm our hypothesis and are in line with the in vitro results.

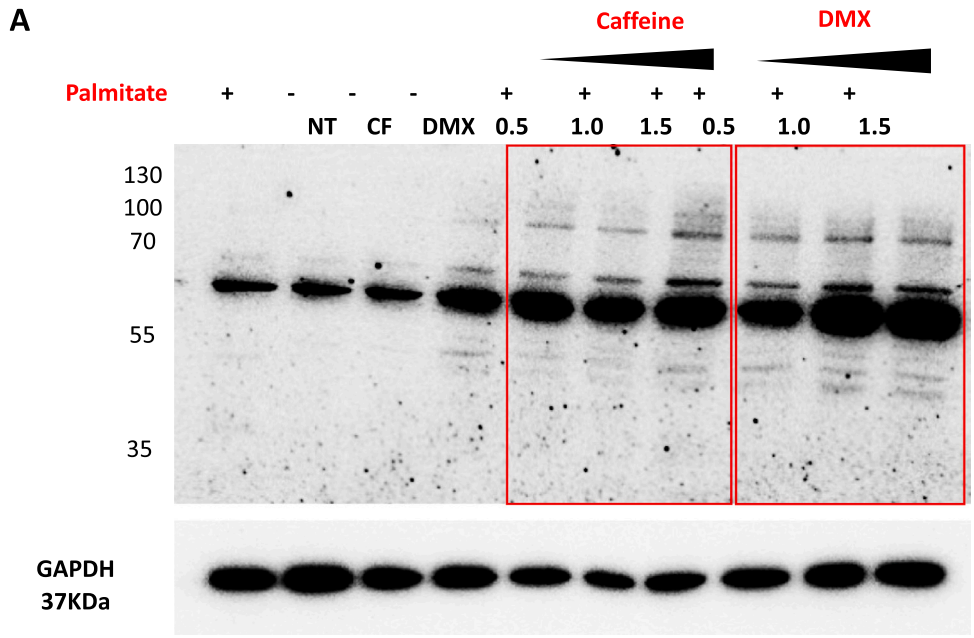

Fig. 6. The detection of PKA phosphorylated targets. A. Pan-PKA blot to demonstrate PKA activation after 8 h. PA, palmitate 1 mmol/L; CF, caffeine 1 mmol/L; DMX, 1,7DMX 0.5 mmol/L; NT Ctrl, non-treated (control) cells (BSA FFA-free 0.25%).

Palmitate treatment for 8 and 16 h reduced A1AR mRNA expression, but statistical significance was only observed after 8 h treatment (Fig. 7D, Fig. S7). Caffeine treatment at 0.5–1.5 mmol/L for 8 h significantly increased the expression of A1AR mRNA in palmitate-treated cells (PA+CF 0.5–1.5) (Fig. 7D). Palmitate treatment also significantly increased A2AR mRNA expression compared with non-treated hepatocytes. Caffeine and 1,7DMX also appeared to increase the expression of A2AR, but this increase did not reach statistical significance (Fig. 7E). Finally, neither palmitate, caffeine, nor 1,7DMX had any significant effect on the expression levels of A2BR (data not shown).

3.5. *Antagonism of the A1 receptor prevents lipotoxicity in primary rat hepatocytes*

To explore whether adenosine receptor signaling is involved in the protective effect of caffeine against lipotoxicity, selective agonists and antagonists for the A1 and A2A receptors were used. A1 and A2A (ant) agonists were selected, because caffeine has higher affinity for these two receptors and because A1 expression was high and predominantly expressed in rat hepatocytes. For the A1 receptor, the selective antagonist DPCPX and the selective agonist CPA were used; for the A2A receptor, the selective antagonist regadenoson and the selective agonist istradefyline were used. Antagonism of the A1 receptor with DPCPX significantly eradicated palmitate toxicity in rat hepatocytes and did not affect the protective effects of caffeine or 1,7DMX. Likewise, the A1 agonist CPA reversed the protective effect of both caffeine and 1,7DMX; however, this difference was not significant (Fig. 8A-B). These results demonstrate that the protective effects of caffeine and 1,7DMX against palmitate lipotoxicity in rat hepatocytes are dependent on A1 receptor antagonism. In addition, this effect is selective for the A1 receptor, because the A2A receptor antagonist regadenoson and the A2A agonist istradefyline did not affect the protective effect of caffeine. Interestingly, istradefyline prevented palmitate lipotoxicity without affecting lipid accumulation (Fig. S8).

As shown in Fig. 3, the protective effect of caffeine against lipotoxicity was correlated with an increase in lipid droplets. Therefore, we performed ORO and BODIPY staining in the presence of the A1 selective agonist and antagonist to assess whether this receptor can affect lipid accumulation. Similarly to caffeine, DPCPX increased the lipid droplet content in palmitate-treated hepatocytes and did not significantly change the effect of caffeine on lipid accumulation. CPA did not change the lipid droplet content in palmitate-treated hepatocytes; however, CPA did reduce the lipid-droplet-increasing effect of caffeine in palmitate-treated hepatocytes (CPA+caffeine+palmitate vs. caffeine+palmitate). (Fig. 9A-C). Thus, A1 receptor antagonism, either by caffeine or DPCPX, prevents lipotoxicity; this effect was associated with significantly increased lipid droplet accumulation in hepatocytes compared with palmitate-treated hepatocytes, similar to the effect observed for caffeine+palmitate (Fig. 9A-C). However, there was no significant difference between the caffeine+palmitate+DPCPX group and the caffeine+palmitate group.

We also assessed the effect of A1 receptor antagonism on PKA activation. DPCPX did not significantly increase PKA activation in combination with caffeine, showing that the addition of another antagonist of the A1 receptor does not further increase PKA activity (Fig. S9).

Finally, since it has been reported that ERK1/2 is a downstream target of PKA/cAMP, we determined the phosphorylation of ERK1/2 in response to caffeine after 6 h or 24 h treatment. We detected increased levels of phospho-ERK1/2 in caffeine-treated hepatocytes compared to non-treated hepatocytes, suggesting that this pathway may be involved in the protective effect of caffeine, although, this result needs to be further explored to confirm the exact role of ERK 1/2 pathway in the protective effects of caffeine (Fig. S10). We did not observe any involvement of the NF-κB pathway in the protective effect of caffeine, as caffeine did neither increase the expression of the NF-κB-inducible gene iNOS nor the phosphorylation of the NF-κB subunit p65 (data not shown).

3.6. *Caffeine inhibits palmitate-induced mitochondrial ROS production*

Palmitate induces ROS production in hepatocytes; it has been proposed that caffeine can have an antioxidant effect [31–33], and caffeine has been shown to reduce oxidative stress markers in HFD mice [34]. Therefore, we evaluated mitochondrial ROS production in hepatocytes in the presence of caffeine or the selective A1 (ant)agonists. Palmitate induced moderate ROS production in primary rat hepatocytes, which was effectively prevented by treatment with caffeine. The effect of caffeine was not affected by the A1 agonist CPA, suggesting that the

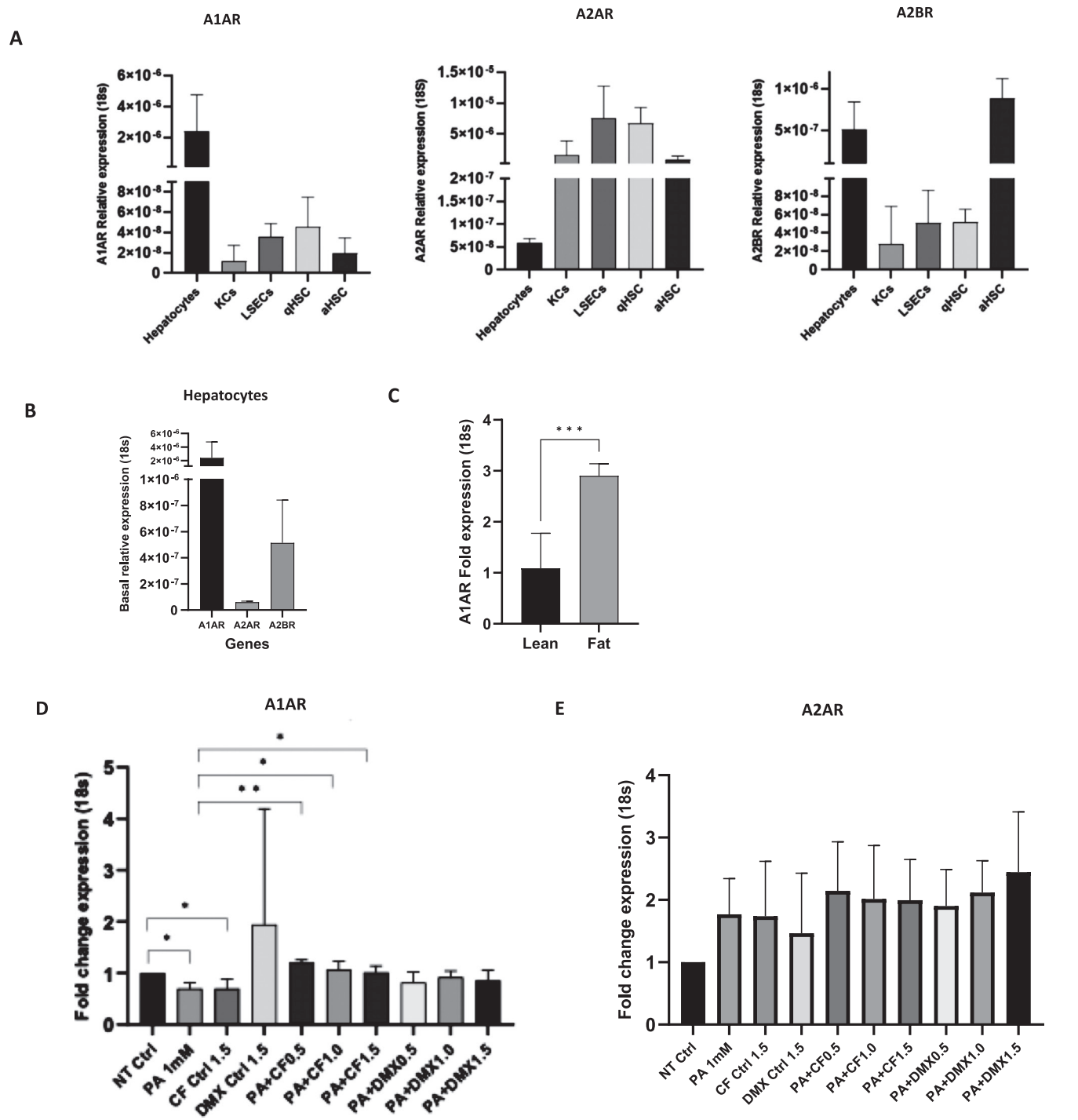

Fig. 7. Adenosine receptor mRNA expression in rat liver cells. A. Basal expression of adenosine receptors A1, A2A, and A2B in different rat liver cell types relative to 18 S expression. B. Comparison of the basal expression of adenosine receptors A1A, A2A, and A2B in hepatocytes. C. Basal expression of adenosine receptor A1A mRNA in hepatocytes isolated from obese Zucker diabetic rats and lean control rats. Regulation of D. A1AR and E. A2AR mRNA expression in palmitate-, caffeine-, and 1,7DMX-treated cells after 8 h treatments. PA, palmitate 1 mmol/L; CF, caffeine 0.5–1.5 mmol/L; DMX, 1,7DMX 0.5–1.5 mmoL/L.

antioxidant effect of caffeine is independent of A1 receptor signaling (Fig. 10). In contrast, the A1 antagonist DPCPX did not protect against mitochondrial ROS production, whereas both DPCPX and CPA increased mitochondrial ROS in palmitate-treated hepatocytes. The selective PKA inhibitor Rp8CTP increased mitochondrial ROS production in all experimental conditions, including the palmitate+caffeine condition, demonstrating that the ROS-lowering effect of caffeine was dependent on PKA activation. These results suggest that caffeine can have antioxidant effects independent of A1 receptor signaling.

4. Discussion

Coffee intake decreases the progression of MAFLD and is inversely correlated with the degree of hepatic fibrosis [35]. Coffee comprises many bioactive compounds and beneficial effects of some of these compounds on human health have been reported, particularly in different experimental models of MAFLD [36]. Caffeine is considered a key compound with pharmacological properties due to its effects on adenosine receptor signaling [37]. Indeed, caffeine intake has been

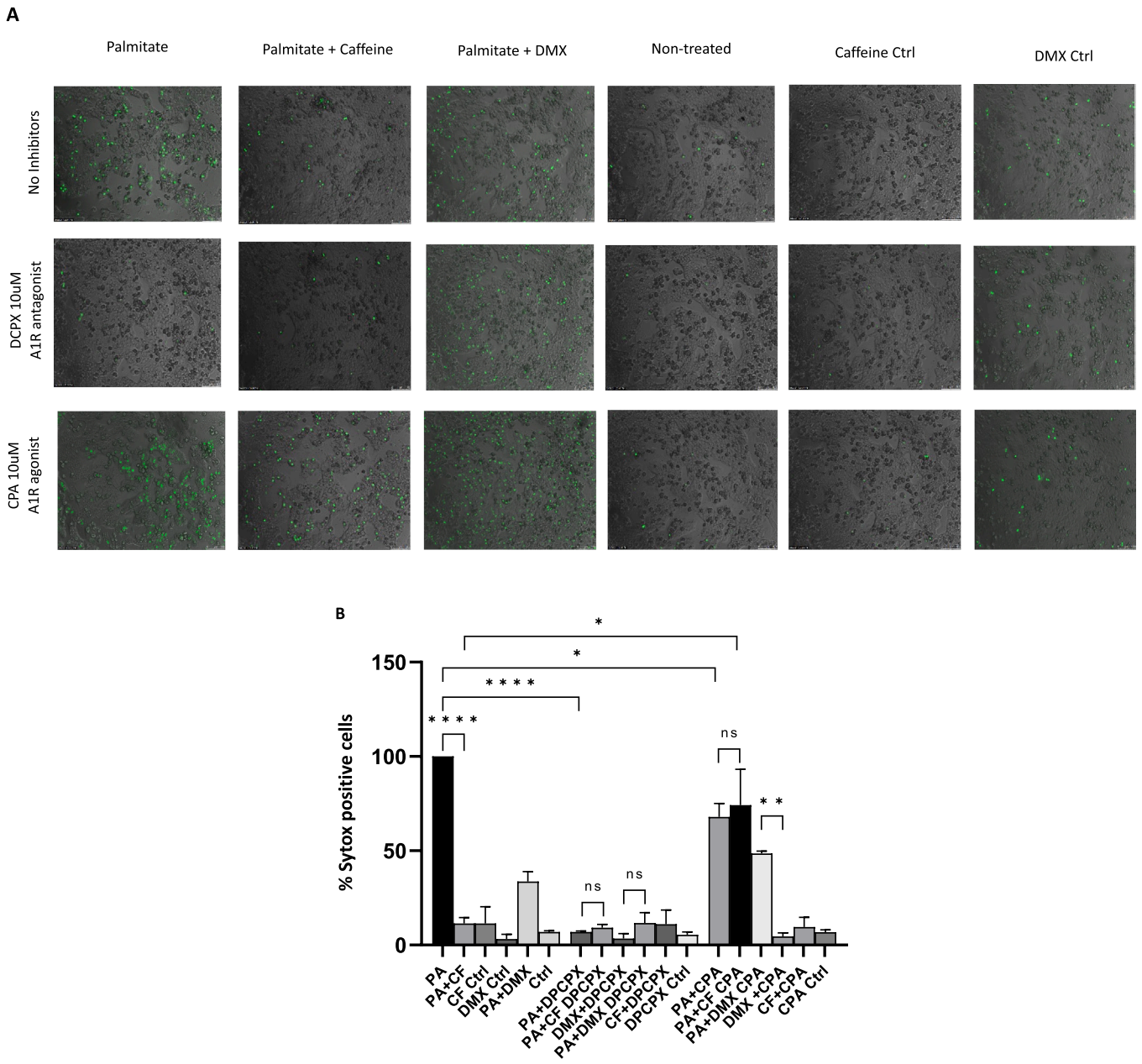

Fig. 8. The antagonism of A1 receptor signaling prevents palmitate-induced cell death in primary rat hepatocytes. A. SYTOX Green staining. B. Quantification of SYTOX-positive cells in caffeine- and 1,7DMX-treated hepatocytes. PA, palmitate 1 mmol/L; CF, caffeine 1 mmol/L; DMX, 1,7DMX; DPCPX, A1AR antagonist 10 μmol/L; CPA, A1AR agonist 10 μmol/L. Scale bar: 100 μm.

reported as an independent factor associated with a lower risk of MAFLD [38]. The effects of caffeine on lipid metabolism have been explored in several in vivo and in vitro models [32,39–41], and caffeine has also been shown to mitigate experimental non-alcoholic steatohepatitis [36,42]. However, little is known about the mechanism(s) of the protective effects of coffee and caffeine in relation to MAFLD and lipotoxicity. Lipotoxicity is a central mechanism in the progression of MAFLD; therefore, the present study was designed to elucidate the molecular mechanisms related to the protective effects of caffeine in MALFD with special emphasis on the role of adenosine receptors.

Caffeine is a non-selective adenosine receptor antagonist and acts on all adenosine receptor subtypes, A1, A2A, A2BA, and A3, with major affinity for A2A and A1 (K_D 2.4 μmol/L and 12 μmol/L, respectively) [20,21,43]. Adenosine receptors are differentially expressed in several tissues, and all are present in liver tissue. The distribution of adenosine receptors determines the effects and sensitivity to adenosine and caffeine [44]. First, we confirmed that all receptor subtypes were present in liver tissue and differentially expressed in different liver cell types. The A1 receptor was the main subtype expressed in hepatocytes. The A2A receptor was expressed at similar levels in hepatocytes and non-parenchymal cells, and increased expression of the A2B receptor was observed in activated HSCs compared with quiescent HSCs. The exact role of adenosine receptors in liver diseases is still unclear; however, adenosine signaling is known to be involved in liver injury and inflammation [4,45], suggesting that these receptors can be exploited as therapeutic targets [46,47]. Recently, the A2A receptor has been identified as relevant in the pathophysiology of liver diseases and hepatic fibrosis, and it has been reported that A2A agonism can attenuate hepatocyte lipotoxicity in a rat model of NASH [31].

Coffee consumption has been associated with improvements in redox status and activation of the Nrf2 antioxidant response [48]. Many coffee constituents contribute to this antioxidant effect [49,50], and caffeine

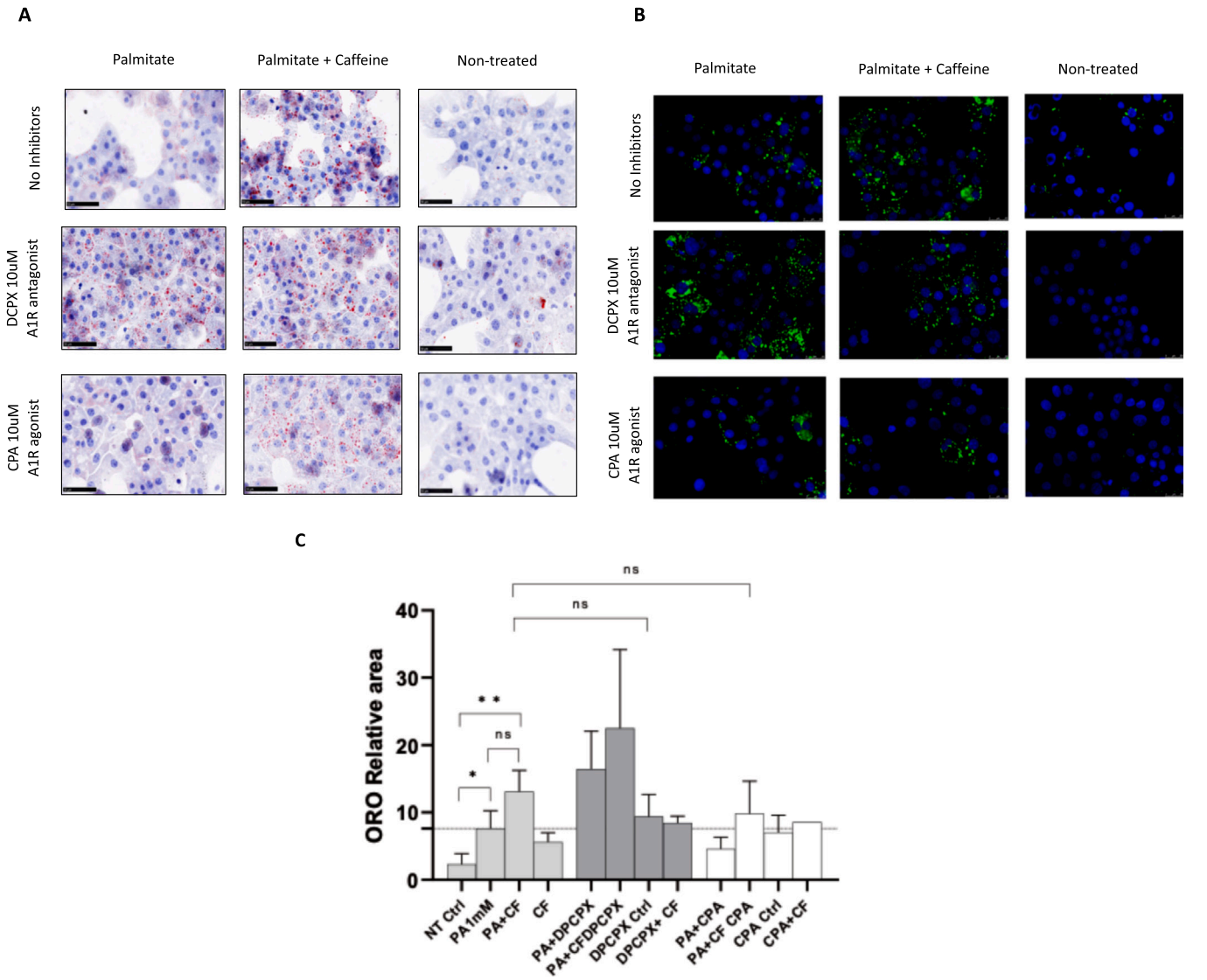

Fig. 9. The effect of A1A receptor antagonism on lipid accumulation in palmitate-treated hepatocytes. A. ORO staining and B. BODIPY 593/50 staining were used to detect lipid droplets (green); nuclei were visualized with DAPI (blue). C. Quantitative analysis of ORO staining. PA, palmitate 1 mmol/L; CF, caffeine 1 mmol/L; DMX, 1,7DMX; DPCPX, A1AR antagonist 10 μmol/L; CPA, A1AR agonist 10 μmol/L. Scale bars: 50 μm (ORO) and 25 μm (BODIPY).

has also been shown to have antioxidant properties and to mitigate oxidative stress [33]. Whether this antioxidant effect is the mechanism related to the protection against lipotoxicity is still unclear. In our study, we provide preliminary evidence that caffeine decreases palmitate-induced mitochondrial ROS production; however, this effect appears to be independent of A1AR receptor. The long-term intake of caffeine has been found to improve the antioxidant status in rat brains, resulting in the increased expression of antioxidant enzymes such as glutathione reductase (GR), glutathione peroxidase (GPx), and superoxide dismutase (SOD) [31]. Further studies to clarify the effects of caffeine on the modulation of antioxidant-related pathways, such as the Nrf2 pathway, are necessary. Moreover, in HFD-induced hepatic steatosis rat models, caffeine has been shown to improve oxidative stress markers in liver tissue, reducing oxidative stress and lipid peroxidation [34].

Caffeine effects have been explored before in in vivo models of MAFLD, demonstrating that caffeine prevents inflammation and oxidative stress and delayed progression of experimental MAFLD [17,51]. Beneficial effects were also observed in other models of liver damage, such as thioacetamide- [52] or alcohol-induced liver disease [28]. Moreover, it has been shown that caffeine improves hepatic lipid metabolism and hepatic steatosis in mice models of hepatic steatosis [34,53]. However, additional preclinical and clinical studies are necessary to establish the value of caffeine as a potential therapeutic agent for MAFLD.

Caffeine has been shown to have antifibrotic effects, mediated by antagonism of the A2A receptor and downstream cAMP/PKA signaling [16,54]. In our study, we demonstrate that PKA is also an important target of caffeine in palmitate-treated rat hepatocytes. This suggests that caffeine is a strong PKA activator and that PKA activity is essential in the protection against lipotoxicity. Taken together, our data indicate that mechanisms independent of A1R signaling are involved in the activation of PKA by caffeine, e.g., PDE inhibition. We also observed that the protective effect of caffeine is dependent on AMPK activity, most likely via preventing the palmitate-induced downregulation of AMPK. The activation of AMPK has been demonstrated to be an effective way to prevent hepatic lipotoxicity in MAFLD and to improve hepatic lipid metabolism [55,56]. Furthermore, caffeine has been shown to be an activator of AMPK and to regulate lipid metabolism in hepatocytes [25]. In addition, caffeine-induced AMPK activation has been demonstrated to prevent the accumulation of toxic lipid metabolites derived from palmitate, such as DAG and ceramides [57]. However, despite the

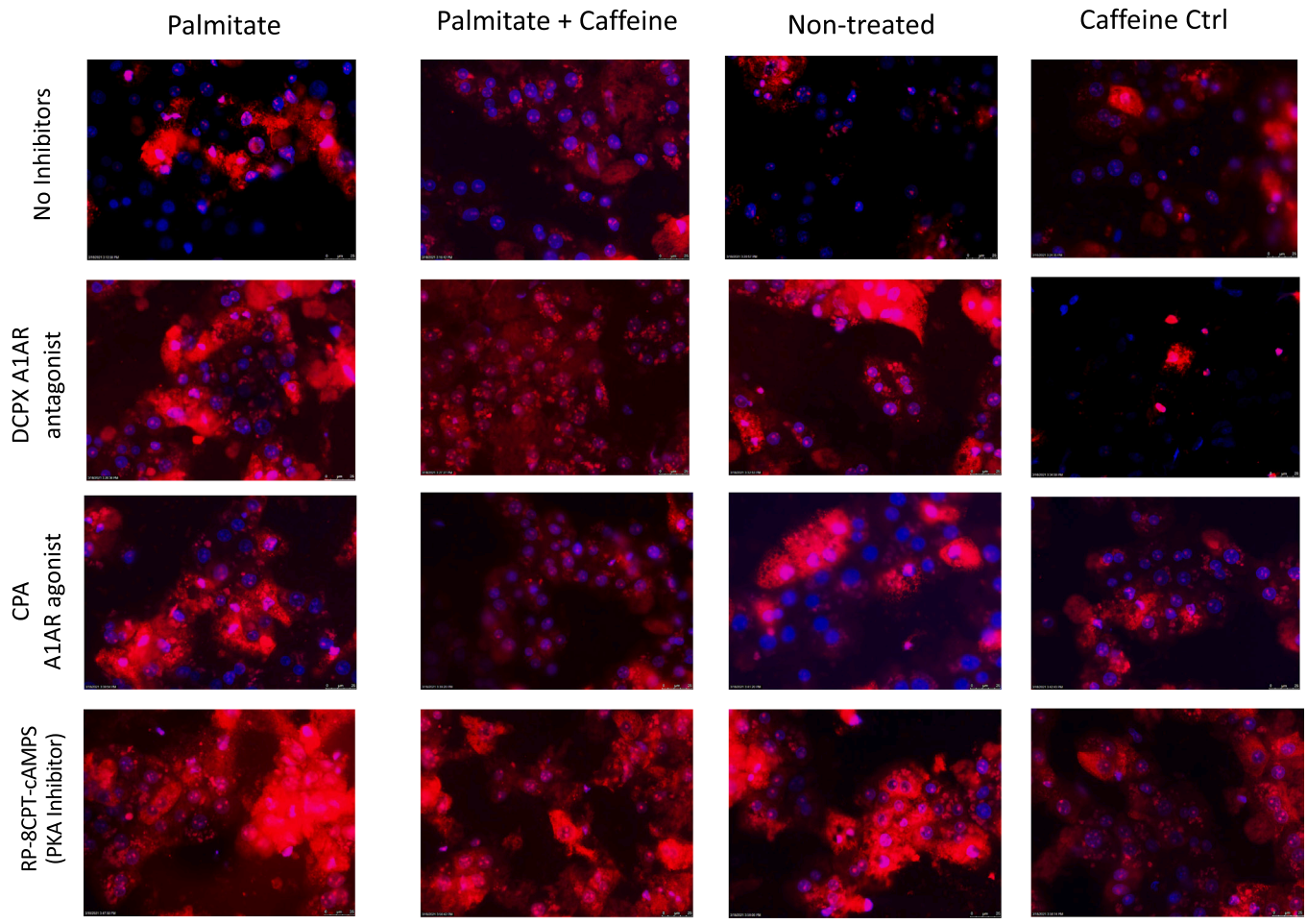

Fig. 10. The protective effect of caffeine is associated with reduced mitochondrial ROS production in palmitate-treated hepatocytes in an A1R-independent mechanism. MitoSOX staining (red) was used to measure mitochondrial ROS production. Nuclei were visualized with DAPI (blue). PA, palmitate 1 mmol/L; CF, caffeine 1 mmol/L; NT Ctrl, non-treated (control) cells (BSA FFA-free 0.25%); DPCPX, A1AR antagonist 10 μmol/L; CPA, A1AR agonist 10 μmol/L. Scale bar: 25 μm.

dependency on PKA and AMPK activity, caffeine did not induce significant changes in mRNA levels of various lipid-metabolism-related genes when hepatocytes were treated with both palmitate and caffeine, suggesting that the protective effects of caffeine do not correlate with transcriptional regulation in the experimental model we used. The present study shows that the protective effects of caffeine are dependent on A1AR receptor antagonism, with subsequent PKA activation. These results support a role for A1AR in the regulation of downstream molecular targets in hepatocytes, like PKA, which should be explored in further studies to determine the effect of A1AR antagonism on MAFLD progression. Caffeine antagonism by direct blockade of A1AR has been demonstrated in brain tissue using in vivo radio-labeling experiments [58,59]. In this regard, we showed that A1AR expression is abundant in rat hepatocytes. Therefore, direct antagonism of A1AR by caffeine is also plausible in the liver, although in vivo studies are necessary to confirm this hypothesis.

Lipotoxicity refers to the toxic effects caused by excessive levels of FFAs [7,60]. Lipotoxic cellular effects include oxidative stress, ER stress, and mitochondrial dysfunction. Recent evidence suggest that the storage of excess lipids as lipid droplets may be a protective mechanism against lipotoxicity [6,61,62]. Lipid droplets containing triglycerides might contribute to lower levels of saturated fatty acids inside the cells, contributing to reduced organellar stress [63]. Indeed lipid droplet formation can increase the ratio of non-toxic unsaturated fatty acids to toxic saturated fatty acids (SFAs), because desaturases can convert SFAs into unsaturated FAs which are more readily incorporated into lipid droplets [64,65].

Caffeine was found to decrease hepatic lipid accumulation, body weight, and lipid content in an over-feeding zebrafish model by decreasing fatty acid uptake and lipogenesis and attenuating ER stress and the inflammatory response [66]. We observed that adenosine A1 receptor (A1AR) antagonism caused by caffeine or the selective A1AR antagonist DPCPX protects against palmitate-induced toxicity in hepatocytes, with a parallel increase in lipid droplet content in hepatocytes. Particularly, caffeine has been shown to promote the conversion of SFAs into monounsaturated fatty acids (MUFAs) in mice and *C. elegans* through the upregulation of stearoyl-coenzyme A desaturase (SCD-1) expression and its *C. elegans* homologue *fat-5*, respectively, resulting in higher ratios of palmitoleic acid (16:1) and palmitic acid (16:0). Caffeine dose-dependently increases SCD-1 and PGC-1α mRNA and protein levels, key proteins involved in fatty acid synthesis. Likewise, monounsaturated fatty acid formation increased after caffeine treatment [14]. These findings are in line with the present study, supporting the hypothesis that caffeine promotes lipid droplet formation in hepatocytes as part of its protective mechanisms against lipotoxicity, and that this mechanism might be dependent on A1AR receptor.

A1AR is considered an inhibitory receptor and is associated with the inhibition of adenyl cyclase. The activation of A1AR reduces lipid availability due to the inhibition of lipolysis in adipose tissue [67]. Similarly, lipolysis is enhanced by the A1AR selective antagonist DPCPX in rat adipocytes. This effect appears to be dependent on increased cAMP levels and PKA activity, but without changes in lipogenesis [68]. Similar results have been observed in mouse adipose tissue treated with the A1AR selective antagonist DPCPX and A1AR [69]. The present study

shows that A1AR antagonism might be involved in the protection of hepatocytes against lipotoxicity by stimulating lipid accumulation in lipid droplets. Concordantly, the antagonism of A1AR has been reported to prevent ethanol-induced fatty livers in mice [70], whereas its activation induces fatty acid synthesis via the stimulation of SREBP1 [4]. These findings might seem contradictory to those observed in adipocyte tissue; however, the effects of A1AR on lipid metabolism have not been fully explored in pathological conditions or in hepatocytes undergoing lipotoxicity, where regulatory molecular mechanisms can differ from adipocytes. Furthermore, palmitate may affect other adenosine receptors expressed in hepatocytes, which might explain the observed results. We also evaluated the involvement of A2AR using the selective agonist istradefyline and selective antagonist regadenoson (Supplementary data). Although the protective effect of caffeine is mainly mediated by A1AR (because the A2AR selective agonist or antagonist did not reduce the effect of caffeine), the A2AR agonist regadenoson did reduce palmitate toxicity and enhance the protective effect of caffeine, probably by increasing cAMP levels and PKA activation via this receptor. Exploring the role of other adenosine receptors such as A2BR and A3R is, therefore, important to understand adenosine-dependent effects during lipotoxicity in hepatocytes. However, our results demonstrate that A1R is the most abundant adenosine receptor in hepatocytes, suggesting that this receptor contributes considerably to the observed protective effects of caffeine.

Interestingly, caffeine treatment induces a differential pattern of lipid droplets, characterized by a smaller size and peripheral distribution compared with FFA-treated cells. This finding might be associated with effects on lipid droplet metabolism, because it has been reported that caffeine is able to modulate lipid droplet content and structure in mouse and human mesenchymal-derived adipocytes. In this study, caffeine treatment increased lipid content, accompanied by an enhanced abundance of PGC-1α with nuclear localization, in most adipocytes, resulting in enhanced mitochondrial biogenesis and an increased number of structural changes in mitochondria, which was correlated with improvements in the metabolic capacity [12]. Moreover, these changes were accompanied by enhanced contact between lipid droplets, mitochondria, and the endoplasmic reticulum. Lipid droplet contact with organelles such as mitochondria has been proposed as a mechanism that potentiates fatty acid metabolism during metabolic stress conditions, including lipotoxicity [71]. Additional studies on the effects of caffeine on lipid droplet metabolism and morphology are needed in order to understand these mechanisms underlying the protective effects of caffeine. These studies include detailed analyses of fatty acid composition and lipidomic analyses of lipid droplets.

5. Conclusions

In this study, we demonstrated a protective effect of caffeine against palmitate lipotoxicity in primary rat hepatocytes, most likely mediated by A1AR receptor. We show that caffeine modulates A1AR mRNA expression and activates PKA and ERK1/2 signaling and regulates cAMP levels in lipotoxic conditions. Moreover, we show that A1AR antagonism protects against palmitate-induced lipotoxicity and seems to play an important role in the regulation of lipid metabolism in hepatocytes. The specificmolecular mechanisms involved in the protective effects of caffeine still needs to be explored in more detail in order to identify novel therapeutic targets. Further studies are needed to confirm the antioxidant effects of caffeine, as well as its effects on lipid droplet composition and morphology. Our results support the use of A1AR modulation as a target for intervention in the treatment of MAFLD.

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CRediT authorship contribution statement

Johanna Carolina Arroyave Ospina: Conceptualization, Investigation, Formal analysis, Writing – original draft/review, Funding acquisition. **Manon Buist-Homan**: Investigation, Resources, Project management, Methodology. **Martina Schmidt**: Conceptualization, Writing – review & editing. **Han Moshage**: Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

All authors have approved the manuscript and agree with this submission. All the authors confirm that the manuscript nor any parts of its content are currently under consideration or published in another journal.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114884.

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