The specialized pro-resolving lipid mediator maresin 1 protects hepatocytes from lipotoxic and hypoxia-induced endoplasmic reticulum stress

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Short Title: Protective actions of SPMs on liver cells

Nonstandard abbreviations: ATF: activation transcription factor, BiP: binding immunoglobulin protein, CHOP: CCAAT/-enhancer-binding protein homologous protein, ER: endoplasmic reticulum, HFD: high-fat diet, IL: interleukin, MaR1: maresin 1, miRNA: microRNA, NAFLD: non-alcoholic fatty liver disease, PCLS: precision-cut liver slices, SPMs: specialized pro-resolving mediators, UPR: unfolded protein response.

ABSTRACT

Endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) are hallmarks of non-alcoholic fatty liver disease (NAFLD), which is the hepatic manifestation of the metabolic syndrome associated with obesity. The specialized pro-resolving lipid mediator maresin 1 (MaR1) preserves tissue homeostasis by exerting cytoprotective actions, dampening inflammation and expediting its timely resolution. Here, we explored whether MaR1 protects liver cells from lipotoxic and hypoxia-induced ER stress. Mice were rendered obese by high-fat diet feeding and experiments were performed in primary hepatocytes, Kupffer cells and precision-cut liver slices (PCLS). Palmitate-induced lipotoxicity increased ER stress and altered autophagy in hepatocytes, effects that were prevented by MaR1. MaR1 protected hepatocytes against lipotoxicity-induced apoptosis by activating the UPR pro-survival mechanisms and preventing the excessive up-regulation of pro-apoptotic pathways. Protective MaR1 effects were also seen in hepatocytes challenged with hypoxia and TNFα-induced cell death. High-throughput miRNA sequencing revealed that MaR1 actions were associated with specific miRNA signatures targeting both protein folding and apoptosis. MaR1 also prevented lipotoxic-triggered ER stress and hypoxia-induced inflammation in PCLS. Of interest, MaR1 enhanced Kupffer cell phagocytic capacity. Together, these findings describe the ability of MaR1 to oppose ER stress in liver cells under conditions frequently encountered in NAFLD.

Keywords: specialized pro-resolving mediators (SPMs), non-alcoholic fatty liver disease (NAFLD), unfolded protein response (UPR), miRNA, liver cells.

INTRODUCTION

Endoplasmic reticulum (ER) is the intracellular organelle responsible for lipid biosynthesis, storage of Ca^{2+} and the trafficking, maturation and folding of proteins (1-3). Adverse and stressful conditions such as nutrient excess, elevated levels of free fatty acids (lipotoxicity), hypoxia and high or low glucose levels can pathologically alter ER homeostasis leading to the accumulation of unfolded or misfolded proteins, which is the most relevant feature of ER stress (4, 5). Eukaryote cells have developed an evolutionary conserved cell response to mitigate ER stress and restore homeostasis, a process known as unfolded protein response (UPR) (6-8). The UPR involves three transmembrane sensors/transducers: inositol-requiring kinase 1α (IRE1α), protein kinase R (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6), which culminate in the transcriptional regulation of a large subset of genes aimed at reestablishing ER homeostasis (7, 9). These pathways are maintained in an inactive state through their association with the ER chaperone binding immunoglobulin protein (BiP or GRP78) the master regulator of UPR (10). Failure of UPR to decrease ER stress leads to the activation of apoptotic pathways via ATF3 and CCAAT/-enhancer-binding protein homologous protein (CHOP), which operate downstream of ATF4 (11). Failure of the ER adaptive capacity and UPR is also linked to the activation of inflammatory signaling pathways (12).

ER stress is a hallmark of the metabolic syndrome including obesity and non-alcoholic fatty liver disease (NAFLD) (13). NAFLD is a condition that encompasses a wide spectrum of hepatic disorders ranging from the simple accumulation of lipids in the cytoplasm of hepatocytes (steatosis) to the combination of steatosis with inflammation, a stage known as steatohepatitis or NASH (14). NASH is the aggressive form of NAFLD and increases the risk for advanced liver disease culminating in hepatic fibrosis, cirrhosis and hepatocarcinoma (14).

The mechanisms underlying the progression of NAFLD have not been completely delineated, but stressful conditions present in the liver of these patients, including an excess of free fatty acids such as palmitic acid (lipotoxicity) as well as low oxygen diffusion (hypoxia) have been proposed to induce ER stress in the liver (4, 15-18). This is important because unresolved ER stress leads to hepatocyte apoptosis, which is also a hallmark of NAFLD (2, 13). Therefore, any intervention able to restore ER homeostasis prior to ER-stress-induced cell death may be useful for prevention and treatment of NAFLD.

A new family of endogenous bioactive lipid mediators has recently been identified to promote the return of the injured tissue to homeostasis (19). This new genus of specialized proresolving mediators (SPMs) includes essential fatty acid–derived lipoxins, resolvins, protectins, and, more recently, maresins. Maresins are produced by macrophages from docosahexaenoic acid (DHA) and were first described by Serhan et al. (20) using lipidomics on self-resolving inflammatory exudates. The complete stereochemistry of maresin 1 (MaR1; 7*R*,14*S*-dihydroxydocosa-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-hexaenoic acid) and its biosynthesis from DHA via 12-lipoxygenase (12-LOX) were recently established (21-23). MaR1 exerts organ-protective actions, promotes enhanced tissue regeneration and possesses the characteristic pro-resolving actions (enhances macrophage phagocytosis and clearance of apoptotic PMN and microbial particles) of the other members of the SPM family (20-24). In view of these properties and considering that MaR1 is a good template for developing potential resolution-based therapies, in the current study we investigated whether MaR1 may protect liver cells from injury by preventing and/or resolving lipotoxic and hypoxia-induced ER stress under conditions commonly encountered in patients with NAFLD.

MATERALS AND METHODS

Experimental model of obesity-induced NAFLD: Male C57BL/6J mice were housed in wood-chip bedding cages with 50–60% humidity and 12-h light/dark cycles. At 6 wk of age, the mice were placed on either a standard rodent chow diet (13% kcal from fat) (Lean group) or a high-fat diet (HFD) (60% kcal from fat; Research Diets) (Obese group) for 16 wk. At the end of the intervention period, the mice were euthanized via ketamine/xylazine injection (intraperitoneally, 4:1), and liver and WAT were excised and rinsed in Dulbeccos`phosphate buffer saline without calcium and magnesium ($DPBS^{-/-}$). Portions of liver and WAT were fixed in 10% formalin and embedded in paraffin, whereas portions of liver were also directly placed in OCT immersed in cold 2-methylbutane on dry ice and kept at -80°C. In addition, portions of liver and WAT were snap-frozen in liquid nitrogen (N_2) for RNA, protein and fatty acid analyses. All animal studies were conducted in accordance with the Investigation and Ethics Committee criteria of the Hospital Clínic and European Union legislation.

Histological analysis: Paraffin-embedded liver and adipose tissue samples were cut into 5-µm sections and stained with H&E. Hepatic steatosis was assessed by Oil Red-O staining in OCT-embedded cryosections. Sections were visualized at x200 magnification in a Nikon Eclipse E600 microscope (Kawasaki, Japan).

Precision-cut liver slices (PCLS)**:** Twenty-week old C57BL/6J mice were anesthetized with ketamine/xylazine. The inferior vena cava was clipped, blood was allowed to drain for 1 min and the liver was excised and quickly placed into ice-cold $DPBS^{-/-}$. Blocks of 0.5 cm^3 from the main lobe were cut with a scalpel and embedded in 4% UltraPure low-melting agarose (Invitrogen, Carlsbad, CA) diluted in Hank's-balanced salt solution without calcium and magnesium ($HBSS^{-/-}$). The agarose cube with the tissue inside was cut into 250 µm-thick

slices in ice-cold $HBSS^{-/-}$ using a Leica VT1000S vibrating-blade microtome (Leica Microsystems, Wetzlar, Germany). PCLS were individually transferred into P-35 Petri dishes containing Millicell cell culture inserts (Millipore), pre-balanced for 20 min with pre-warmed William's E medium containing GlutaMAX-I, 25 mM D-glucose and 50 µg/ml gentamicin. PCLS were maintained at 37°C in a 5% CO_2 incubator for 120 min before being incubated under normoxia (21% O_2) or hypoxia (1% O_2, 94% N_2 and 5% CO_2) conditions for 24 h in the presence of vehicle (≤0.005% EtOH) or MaR1 (10 nM) (Cayman Chemical, Ann Harbor, MI) for the last 6 h. In another set of experiments, PCLS were pre-treated with MaR1 (10 nM) before being exposed to lipotoxic damage induced by palmitate (0.5 mM) for 18 hours. A stock solution of palmitate (8 mM) conjugated to 18.4% free fatty acid (FFA)-bovine serum albumin (BSA) was prepared by gentle overnight agitation at 37ºC. The solutions were filtered and maintained at -20ºC for a maximum of 1 month. At the end of the incubation period, PCLS were snap-frozen in N_2 for further analysis.

Isolation of hepatocytes: Hepatocytes were isolated from C57BL/6J mice by a three-step *in situ* perfusion procedure using 0.04% collagenase IV through the portal vein, as previously described (25). The cell suspension was passed through a 100-µm cell strainer (Beckton Dickinson, Franklin Lakes, NJ) and subsequently washed by centrifugation at 50 *g* for 2 min at 4ºC with cold William´s E medium. Pelleted hepatocytes were characterized by phase-contrast microscopy and viability was determined by Trypan blue exclusion. Isolated hepatocytes were seeded on collagen I-coated 12-well plates (4×10^5 cells/well) with William's E medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, 1 µM insulin, 15 mM Hepes, and 50 µM β-mercaptoethanol. After 4 hours, the hepatocytes were washed three times with $DPBS^{-/-}$ and

maintained overnight in a humidified 5% CO_2 incubator at 37°C in 0.1% FBS-William's E medium.

Isolation of Kupffer cells: Kupffer cells were isolated by collagenase IV perfusion as described for hepatocyte isolation. After pelleting the hepatocytes, the remaining cell suspension was centrifuged at 600 *g* for 10 min at 4ºC. The pellet containing the non-parenchymal cells was resuspended in Gey's Balanced Salt Solution and purified by density gradient centrifugation using 16% Nycodenz (Sigma, St. Louis, MO). Kupffer cells were isolated from the top layer of the gradient and seeded in RPMI with 1% FBS in 96-well plates and allowed to attach for 1 hour.

Differentiation of 3T3-L1 into mature adipocytes: Mouse 3T3-L1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were seeded onto 12-well plates (150.000 cells/well) in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin and 4 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37 °C and allowed to grow to confluence for 2 days, as described previously (25). Confluent 3T3-L1 cells were cultured in adipocyte induction medium containing insulin (5 µg/mL), isobutylmethylxanthine (0.5 mM), dexamethasone (0.25 µM), penicillin/streptomycin (100 U/mL), and L-glutamine (4 mM) in DMEM supplemented with 10% FBS. After 2 days, the cells were cultured in continuation medium (5 µg/mL insulin) for 72 h and then maintained in DMEM supplemented with 10% FBS until exhibiting a mature adipocyte phenotype, as characterized by phase-contrast microscopy. At day 7–8 of differentiation, the cells were placed in 1% serum free/insulin free DMEM overnight before performing the experiments.

Cell incubations: Hepatocytes seeded in 12- or 96-well plates were exposed to lipotoxic damage by incubating for 1 hour with vehicle (<0.05% ethanol) or increasing concentrations of MaR1 (1, 10 and 50 nM) at 37°C before the addition of palmitate (0.5 mM) or vehicle (18.4% FFA-BSA) for 2-24 h as specified in each figure. Hepatocytes were also incubated with vehicle (<0.05% ethanol) or MaR1 (1, 10 and 50 nM) alone for 18 hours. Experiments simulating a therapy treatment was also performed by incubating hepatocytes with palmitate (0.5 mM) for 18 h before the addition of increasing concentrations of MaR1 (1,10 and 50 nM) for the last 6 hours of lipotoxic damage. In another set of experiments, hepatocytes were incubated for 1 hour with vehicle (<0.05% ethanol) or increasing concentrations of MaR1 (1, 10 and 50 nM) at 37°C under normoxia (21% O_2) or hypoxia (1% O_2, 94% N_2 and 5% CO_2) conditions for 2-24 h as specified in each figure legend. Kupffer cells and adipocytes were also pre-treated with MaR1 for 1 h before the addition of palmitate (0.5 mM) for 18 and 20 h, respectively.

Western Blot analysis: Total protein from liver and adipose tissue was extracted using a lysis buffer containing 50 mM Hepes, 20 mM β-glycerophosphate, 2 mM EDTA, 1% Igepal, 10% (vol/vol) glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 150 mM NaCl, supplemented with a mixture of protease (Complete Mini; Roche Diagnostics) and phosphatase inhibitors (PhosSTOP; Roche Diagnostics) mixtures. For protein isolation from hepatocytes and adipocytes, the cells were scraped into ice-cold DPBS and resuspended in 150 μL of lysis buffer. Homogenates were incubated on ice for 10–15 min and centrifuged at either 1,000 × *g* for 2 min (cells) or 9,300 × *g* for 20 min (tissue) at 4°C. Total protein (10-50 μg) from supernatants was placed in SDS-containing Laemmli sample buffer, heated for 5 min at 95 °C, and separated by 10-15 % (vol/vol) SDS/PAGE for 90 min at 120 V. Transfer was performed by the iBlot Dry Blotting System (Invitrogen) onto PVDF membranes at 20 V over

5–7 min, and the efficiency of the transfer was visualized by Ponceau S staining. The membranes were then soaked for 1 h at room temperature in 0.1% T-TBS and 5% (wt/vol) nonfat dry milk. Blots were washed three times for 5 min each with 0.1% T-TBS and subsequently incubated overnight at 4°C with primary rabbit anti-mouse Atg7 (8558; dilution 1/1000; Cell Signaling), rabbit polyclonal phospho-IRE-1α (NB100-2323; dilution 1/1000; Novus Biologicals), rabbit polyclonal XBP1 (ab37152; dilution 1/1000; Abcam), rabbit polyclonal phospho-eIF2α (ab4837; dilution 1/1000; Abcam), rabbit polyclonal HIF-1α (sc-10790; dilution 1/500, Santa Cruz Biotechnology), rabbit polyclonal phospho-SAPK/JNK (Thr183/Tyr185) (9251, dilution 1:250; Cell Signaling), rabbit monoclonal p62 (Ab109012; dil 1/10,000, Abcam), LC3I/II (12741S; dil 1/1000; Cell Signaling) and ATG12 (4180; dil 1/500; Cell Signaling) in 0.1% T-TBS containing 5% BSA. After washing 3 times for 5 min each with 0.1% T-TBS, the blots were incubated with horseradish-peroxidase-linked donkey anti-rabbit antibody (dilution 1:5000) for 1 h at room temperature in 0.1% T-TBS containing 0.5% BSA. Rabbit monoclonal HRP conjugated β-actin (5125; 1/1000; Cell signaling) was used as an endogenous control in all the experiments. Bands were visualized by enhanced chemiluminescence (ECL) detection system (GE Healthcare, Chalfont St. Giles, UK). To assess total JNK and eif2α protein expression, membranes were stripped at 37ºC for 15 min in Restore Western blot stripping buffer (Thermo Fisher, Cambridge, UK) and reblotted overnight at 4°C with rabbit monoclonal SAPK/JNK (56G8) (9258; dil 1/250, Cell Signaling) and mouse monoclonal eIF2α (sc-133132; dilution 1/200; Santa Cruz Biotechnology). Membranes were visualized as described above.

Gene expression analysis by real-time PCR: Isolation of total RNA from liver and adipose tissue, hepatocytes and PCLS, was performed using the TRIzol reagent. RNA concentration was assessed in a NanoDrop-1000 spectrophotometer and its integrity tested in a Bioanalyzer

2100. cDNA synthesis from 0.5-1 µg of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR analysis for IL-10 (Mm00439614_m1), TNFα (Mm00443258_m1), IL-6 (Mm00446190_m1), PAI-1 (Mm 00435860_m1), 12/15-LOX (Mm_00772337_m1), COX-2 (Mm00478374_m1), IL-1β (Mm01336189_m1), CCR7 (Mm01301785_m1), CHOP (Mm01135937_g1), BiP (Mm00517690_g1), ATF3 (Mm00476032_m1), ATF4 (Mm00515325_g1), Erdj4 (Mm01622956_s1), EDEM1 (Mm_00551797_m1), GLUT-1 (Mm00441480_m1), HIF-1α (Mm00468869_m1), CA9 (Mm00519870_m1) and NLRP3 (Mm00840904_m1) was carried out in an Applied Biosystems 7900HT Fast Real Time PCR System using β-actin (Actb; Mm00607939_s1) or 18S ribosomal RNA (Rn18s) (Mm03928990_g1) as endogenous controls. PCR results were analyzed with the Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantification of gene expression was performed using the comparative Ct method. The amount of target gene, normalized to β-actin and relative to a calibrator, was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$ described in the comparative Ct method.

Analysis of 12/15-LOX by end-point reverse transcription-PCR: End-point amplification of 12/15-LOX and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was performed with specific oligonucleotides as previously described (26). PCR products were analyzed by electrophoresis in 2.0% agarose gels and visualized by Sybr safe staining, using a 100-bp DNA ladder (Invitrogen).

miRNA isolation: Total RNA containing miRNA was isolated from hepatocytes using RNeasy Micro Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Total RNA concentration was assessed in a NanoDrop-1000 spectrophotometer.

miRNA sequencing: Libraries for small RNA sequencing were prepared from 1 µg of total RNA using the TruSeq small RNA Sample Prep Kit (Illumina) according to the manufacturer's protocol. After adaptor ligation, reverse transcription, and PCR amplification, libraries were size-selected using 6% Novex® TBE Gels (ThermoFisher Scientific). Fragments with insert sizes between 18 and 40 bp were cut from the gel, and DNA was precipitated and eluted in 10 µl EB. The libraries were analyzed using a DNA High Sensitivity chip in an Agilent Bioanalyzer and then quantified by qPCR using the KAPA Library Quantification Kit (KapaBiosystems). The libraries were pooled and sequenced with 50 bp single reads with TruSeq v3 chemistry in an Illumina's HiSeq 2000 sequencer. Enrichment pathway analysis was performed using the DAVID gene functional classification tool. The raw data from Illumina deep-sequencing were deposited in the NCBI Short Read Archive (SRA) database with Accession numer: PRJNA354839.

miRNA *expression analysis by real-time PCR:* cDNA synthesis and real-time PCR were performed using miR-X miRNA First-Strand Synthesis Kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) following manufacturer's instructions. miRNA levels were determined by real-time PCR on an ABI 7900HT cycler (Applied Biosystems). Individual miRNA expression was normalized to U6 expression. Relative expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Gene-specific primers were produced by Integrated DNA Technologies (Leuven, Belgium). The sequences of the gene-specific forward primers are listed in **Table S1**. A universal reverse primer is included in the miR-X miRNA First-Strand Synthesis Kit.

Caspase 3/7 apoptosis assay: Hepatocytes (40.000 cells/well) were seeded in white-walled 96-well plates. These cells were treated with MaR1 (1-50 nM) or 4-PBA (10 mM) for 1 hour and then incubated for 12 hours with vehicle, TNFα (20 ng/mL) and actinomycin D (250 ng/mL). Following incubation, caspase 3/7 activity was determined using the Caspase-Glo 3/7 assay (Promega, Madison, WI). Briefly, the plates containing cells were removed from the incubator and allowed to equilibrate to room temperature for 30 minutes. Fifty µl of Caspase-Glo reagent was added to each well and gently mixed with a plate shaker at 300–500 rpm for 30 seconds. The luminescence signal was measured in a plate-reading luminometer (Fluostar) with parameters of a 15-minute lag time and 0.5 second/well read time.

TUNEL Assay: Cells were seeded into 8-well culture/chamber slides (50.000 cells/well) and induced to lipotoxic damage by palmitate as described above. At the end of the incubation period, hepatocytes were fixed with fresh prepared paraformaldehyde and apoptotic cells were detected using In Situ Cell Death Detection Kit, TMR red (TUNEL) (Roche, Mannheim, Germany) according to the manufacturer´s specifications . Cell nuclei were counterstained with ProLong® Gold Antifade Reagent with DAPI (Invitrogen) and visualized on an Olympus fluorescence microscope.

Fatty Acid Profiling by Gas Chromatography (GC): Total lipids were extracted from frozen liver and adipose tissues by the Folch method, with modifications (27). Briefly, chloroform/methanol (2:1 v/v) containing 0.005% butylated hydroxytoluene (as antioxidant) was added and mixed vigorously for 30 s before adding 100 µl of 0.25% $MgCl_2$ and 1 ml of 0.01N HCl and mixed again. The chloroform phase containing lipids was collected. The remains were extracted with 3 ml of chloroform. The chloroform phases were pooled and dried under nitrogen and subjected to methylation. Fatty acid methyl esters were prepared by

methods similar to those described previously (28), using methanol containing 14% boron trifluoride (BF_3/MeOH). The extracted lipid samples were mixed with BF_3/MeOH reagent (1 ml), and the mixtures were heated at 100°C in a metal block for 1 h, cooled to room temperature and methyl esters were extracted twice in the upper (hexane) layer after addition of 1 ml H_2O. The samples were centrifuged at 3600 rpm for 10 min and then the upper hexane layer was removed and evaporated under nitrogen. Fatty acid methyl esters were analyzed by flame ionization GC. GC analysis was carried out with an Agilent 7890 Autosampler apparatus (Agilent Technologies) equipped with a capillary column (SupraWAX-280, Teknokroma, Barcelona, Spain), 30 m length, 0.25 mm i.d. and film thickness 0.25 µm. Column conditions were: initial temperature, 120ºC for 1.0 min; ramp 15 ºC/min to 210 ºC hold to 35 min; carrier gas, helium. Data acquisition and processing were performed with Agilent-Chemstation software for GC systems.

Phagocytosis assay: Kupffer cells (35.000-45.000 cells/well) were seeded in black-walled 96-well plates and 50 µl of opsonized zymosan bioparticles (ratio cells:bioparticles = 1/10) were added to each well (final volume = 200 µl) and incubated at 37ºC for 60 min. Cells were then washed with sterile $DPBS^{-/-}$, and 100 µl Trypan Blue Solution (diluted at 1:10 in sterile $DPBS^{-/-}$) was added to quench fluorescence of bioparticles bound to the outside of the cell. The plate was incubated for 2 min at room temperature. The plate was centrifuged for 5 min at 400 *g* at room temperature and excess of Trypan Blue was carefully aspirated. The fluorescent intensity of each sample was read in a microplate reader (FLUOstar OPTIMA).

Statistical analysis: Statistical analysis of the results was performed by analysis of variance (one-way or two-way ANOVA) or unpaired Student's t-test. Results are expressed as mean ± SEM and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Livers from obese mice show increased ER stress. Role of free fatty acids.

Obese mice showed increased body, liver and WAT weight and higher serum insulin, glucose, triglycerides, cholesterol, AST and ALT levels than paired-aged lean mice (**Table S2**). Analysis of H&E stained liver sections revealed signs of necroinflammation in obese mice (**Fig. 1*A***). These mice also showed extensive hepatic steatosis as indicated by the presence of significantly increased area stained with Oil Red-O (**Fig. 1*A***). Consistent with the view that an excessive influx of free fatty acids leading to steatosis is a potent inducer of ER stress and cell injury in obesity-induced NAFLD (13, 29, 30), livers from obese mice displayed a remarkable induction of eif2α and JNK phosphorylation and up-regulation of downstream targets ATF3 and BiP (**Fig. 1*B***). No changes were observed in IRE1α phosphorylation and CHOP expression (**Fig. 1*B***). ATG7, a marker of autophagy, was slightly increased in obese livers (**Fig. 1*B***). To provide further evidence linking hepatic steatosis to ER stress, we next modeled hepatic lipotoxicity *in vitro* by incubating primary hepatocytes with palmitic acid. The levels of this saturated fatty acid, as estimated by the ratio between palmitoleic and palmitic acids (C16:1/C16:0) (28), were remarkably increased in livers from obese mice (**Fig. 1*C***). A schematic diagram of the design of the experiments using palmitate at pathophysiological concentrations (i.e. 0.5 mM) (18, 30) is shown in **Fig. 1*D***. As shown in **Fig. 1*E***, palmitate triggered the ER stress-derived UPR in hepatocytes in a time-dependent manner, up-regulating ATF4 and EDEM1 (a member of the ER-associated protein degradation (ERAD)) at early stages (4 h), while increasing ATF3, CHOP, BiP and Erdj4 at later stages (8 h).

MaR1 ameliorates lipotoxic ER stress in primary mouse hepatocytes.

MaR1 is a biologically active lipid mediator that belongs to the pro-resolving SPM family (20). In humans, MaR1 is generated from DHA through the activity of the 12-LOX enzyme, which mouse homolog is 12/15-LOX (21-23). In mouse liver, constitutive expression of 12/15-LOX was detected (**Fig 2*A***), which was not altered under obese conditions (**Fig 2*B*)**. MaR1 exerts potent cytoprotective actions and promotes tissue homeostasis in several disease models (20-24). To investigate whether MaR1 also exerts protective actions against obesity-induced hepatic ER stress, we incubated hepatocytes with nanomolar concentrations of MaR1 before initiating the palmitate lipotoxic model. As shown in **Fig. S1,** in the absence of the lipotoxic stimulus (i.e. palmitate), MaR1 did not induce any change on markers of ER stress in resting hepatocytes. In contrast, increased peif2α/eif2α ratio and spliced XBP1 (XBP1s), a transcription factor generated through the action of IRE1α endoribonuclease domain (31), was seen in hepatocytes treated for 18 h with palmitate (**Fig. 2*C***). Moreover, lipotoxicity induced ATG7 and LC3-II protein levels, while repressing p62 (**Fig. 2*C***). Importantly, MaR1 pre-treatment resulted in a return of hepatocytes to homeostasis by reducing ER stress markers such as peif2α and XBP1s and the protein levels of the autophagic markers p62, ATG7 and LC3-II (**Fig. 2*C***). These changes in p62 and LC3-II are consistent with those previously described by Borgeson et al. (32) in adipose tissue from HFD-induced obese mice in response to the SPM LXA_4 and its stable analog benzol-LXA_4. In addition, MaR1 returned the palmitate-induced expression of ATF3, CHOP, BiP and Erdj4 to normal (**Fig. 2*D***). MaR1 did not modulate EDEM1 and ATF4 in response to palmitate (**Fig. S2*A***). Down-regulation of the ER stress markers ATF3, BiP and Erdj4 was also seen when MaR1 was applied therapeutically during the last 6 hours of palmitate lipotoxic damage (**Fig 2*E*)**, although the response was not as intense as when the cells were pre-treated with MaR1.

MaR1 protects hepatocytes from lipoapoptosis.

We next investigated the actions of MaR1 at different time points of lipotoxicity (early, 4 h; middle, 8 h and late, 18 h). As shown in **Fig. 3*A***, MaR1 treatment up-regulated the chaperone BiP and its cochaperone Erdj4 at early stages (4 hours), while reducing ATF3 and CHOP at later stages (8 and 18 hours) of lipotoxic damage. The observation that MaR1 activated pro-survival mechanisms such as the chaperone BiP and Erdj4 at early (but not later) stages of palmitate lipotoxicity can be interpreted as this SPM contributes to prevent the excessive activation of down-stream pro-apoptotic factors such as ATF3 and its putative target CHOP. Consistent with this view, we detected a substantial reduction in the number of TUNEL-positive apoptotic hepatocytes when MaR1 was added to the cell culture medium (**Fig. 3*B***). Of interest, the cytoprotective actions of MaR1 were accompanied by a reduction in the number of intracellular lipid vacuoles, as seen in hepatocytes stained with Oil Red-O (**Fig. S2*B***). Together, these findings add new value to previous observations with RvD1, another SPM generated from DHA, which displays protective actions against obesity-induced hepatic steatosis and inflammation (33, 34).

MaR1 ameliorates lipotoxic ER stress in PCLS and promotes phagocytosis in Kupffer cells.

PCLS are a very useful and reliable tool to study the global response of liver cells to injury because this model preserves cell-cell interactions in the original three-dimensional hepatic architecture (35). In these experiments, PCLS were exposed to palmitate for 18 h in the presence or absence of MaR1. A schematic diagram of the experimental design is shown in **Figure 4*A***. As expected, palmitate induced UPR in PCLS by up-regulating ATF4, BiP, EDEM1 and Erdj4 (**Figure 4*B***). Of note, MaR1 significantly reduced ATF3 and ATF4 expression, while up-regulating the pro-survival chaperone BiP. This is of importance because BiP is a master regulator of the UPR due to its role as a major ER chaperone with

anti-apoptotic properties as well as its ability to control the activation of transmembrane ER stress sensors (IRE1α, PERK and ATF6) (36). On the other hand, palmitate was not a strong inducer of inflammation in this organotypic model and only induced a modest increase in IL-β expression (**Fig. S2*C***). Nevertheless, under these circumstances, MaR1 significantly reduced TNFα, NLRP3 and IL-β expression in palmitate-challenged PCLS (**Fig. S2*C***). We also explored the actions of MaR1 on individual liver cells, namely hepatocytes, which are the predominant liver cell type in PCLS, and Kupffer cells, which are the primary liver resident macrophages engaged in inflammatory responses (37). As expected no major changes in inflammatory markers were observed in hepatocytes (data not shown), whereas Kupffer cells responded to MaR1 with an enhanced phagocytic activity, which is a cardinal sign of active resolution of inflammation (**Fig. 4*C***) (38, 39). Indeed, MaR1 induced a concentration-dependent increase in Kupffer cell phagocytosis with a maximal response at 10 nM, diminishing at higher concentrations (**Fig. 4*C***), in a bell-shaped like curve characteristic for SPMs as well as for other lipid mediators (40, 41). This observation indicates that MaR1 potentiates the phagocytic function of resident liver macrophages, which are responsible for engulfing (phagocyte) and removing cell debris and pathogens under homeostatic conditions Interestingly, MaR1 was able to enhance phagocytosis in a concentration-dependent manner in Kupffer cells even after these cells were challenged with palmitate (**Fig. 4*D***). In this case, the concentration-response curve was shifted to the right, suggesting that higher doses of MaR1 were required to achieve a similar pro-resolutive response to that obtained in the absence of palmitate. Representative photomicrographs of these experiments are shown on the left side of **Fig. 4*D***.

MaR1 induces a specific microRNA (miRNA) signature in hepatocytes exposed to lipotoxic damage.

Recently, miRNAs have emerged as key regulators of ER homeostasis and important players of ER-induced UPR (42). The endoribonuclease activity of IRE1α, in a process termed regulated IRE1-dependent decay (RIDD), is also connected to degradation of mRNA and miRNA substrates (3). Therefore and given that previous studies have assigned specific miRNA signatures to SPM actions (33, 43), we next profiled miRNA by next generation sequencing in primary hepatocytes challenged with palmitate in the absence or presence of MaR1. **Fig. 5*A*** shows a heat-map of the miRNAs differentially expressed in response to the incubation of hepatocytes with palmitate. A total of 57 miRNAs (34 up-regulated and 23 down-regulated) were identified after palmitate challenge (**Fig. 5*B***). These miRNAs are listed in **Table S3**. Pre-treatment of palmitate-challenged hepatocytes with MaR1 yielded a specific signature of 44 miRNAs, of which 24 were up-regulated and 20 down-regulated (**Fig. 5*B***). The fold changes of these 44 miRNAs are given in **Fig. 5*C*** and **Table S4**. Validation of a subset of the miRNA found to be differentially regulated by MaR1 in the RNAseq analysis was performed by real-time PCR (**Fig. 5*D*** and **Fig. S3**). Moreover, confirmation that MaR1 not only modulated the levels of these miRNAs but also the expression of their targets (i.e. BCL2, XBP1 and ATG12) is shown in **Fig. 5*D***. In particular, among the miRNA up-regulated by MaR1 (Pal+MaR1), we identified miRNA such as miR181a-1-3p and miR-129-2-3p that target ATG12 and BCL2 family members involved in ER stress and apoptosis (44). Another example is miR-125b which was up-regulated by MaR1 and has been described to repress translation of caspase-2 mRNA during sustained IRE1α RNase activation (3). The induction by MaR1 of a specific miRNA signature connected to the regulation of cellular processes such as protein folding and programmed cell death was confirmed by DAVID gene functional classification tool on the TOP target mRNAs of the 44 miRNAs differentially modulated by MaR1. This analysis revealed that the gene ontology category most impacted by MaR1 was cellular process (GO:0009987) (**Fig. 5*E***) with changes in 1493 genes, including

the GO terms protein folding (0006457; 35 genes) and programmed cell death (0012501; 348 genes).

Livers from obese mice show remarkable hypoxia. Association with ER stress.

In addition to lipotoxicity, hypoxia is recognized as an initiator of ER stress (44) and has been implicated as a causative factor in the progression of NAFLD. Indeed, hypoxic conditions have been reported in the liver of HFD-induced obese mice and intermittent hypoxia has been associated with a predisposition to liver injury (17, 45). In this regard, livers from obese mice showed increased hypoxia inducible factor-1α (HIF-1α) expression at both mRNA (**Fig. S4*A***) and protein (**Fig. S4*B***) levels. The observation that up-regulation of HIF-1α was more evident at protein level is consistent with the fact that HIF-1α is mainly regulated at the post-transcriptional level (46). Since HIF-1α is difficult to monitor *in vitro* due to its short half-life upon re-oxygenation (47), in this study we measured carbonic anhydrase 9 (CA9), which is a useful surrogate marker of the transcriptional activity of HIF-1α under hypoxic conditions (48, 49). As shown in **Fig. 6*A*** and **Fig 6*B***, livers from obese mice and hepatocytes cultured under hypoxic conditions (1% O_2) showed significantly increased CA9 expression. Moreover, hypoxic hepatocytes showed a time-response induction in GLUT-1 (**Fig. 6*C***), which is another surrogate marker of HIF-1α activity (50). No changes in HIF-1α mRNA expression were detected in cultures of hypoxic hepatocytes (**Fig. S4*C***). Of interest, hypoxia was associated with increased phosphorylation of the ER stress markers eif2α, IRE1α and JNK in primary hepatocytes (**Fig. 6*D***). The expression of the downstream effectors ATF3 and CHOP, which play a central role in mediating apoptotic cell death (18), were also increased in hypoxic hepatocytes (**Fig. 6*E***). In contrast, the ATF6 pro-survival target chaperone BiP was reduced by hypoxia (**Fig. 6*E***). Consistent with previous studies (51), hypoxia stimulated the

autophagic markers LC3-II and ATG7 in primary hepatocytes, while reducing p62 in a time-dependent manner (**Fig. 6*F***).

MaR1 ameliorates hypoxia-induced ER stress and promotes cell survival in primary hepatocytes.

As shown in **Fig. 7*A***, MaR1 markedly down-regulated hypoxia-induced peif2α and pIRE1α to a similar extent to that of the chemical chaperone 4-phenylbutyrate (4-PBA). Notably, MaR1 actions on peif2α and pIRE1α in hypoxic hepatocytes were concentration-dependent (**Fig. 7*B***). pJNK, which is activated by the cytosolic IRE1α kinase domain (31), was also attenuated by MaR1 in a concentration-dependent manner (**Fig. 7*B***). XBP1s was also down-regulated by MaR1 (**Fig. 7*B***). Importantly, attenuation of hypoxia-induced ER stress by MaR1 translated into reduced expression of ATF3 and its putative target and pro-apoptotic gene CHOP, without changes in the chaperone BiP or its cochaperone Erdj4 (**Fig. 7*C***). Furthermore, MaR1 reduced ATG7 in primary hepatocytes exposed to hypoxia (**Fig. 7*D***). Of interest, MaR1 protected hepatocytes from apoptotic cell death induced by TNFα + actinomycin, as revealed by a significant reduction in caspase 3/7 activity in cells incubated with this SPM (**Fig. 7*E***). As expected, the chemical chaperone 4-PBA completely blunted caspase 3/7 activity in hepatocytes undergoing the same treatment (**Fig. 7*E***).

Effects of MaR1 on PCLS upon hypoxic conditions.

Hypoxia is described as a potent trigger of hepatic inflammation (33), which together with lipotoxicity and cell injury are key features of progressive obesity-induced NASH (13, 14, 52). Consistent with previous studies (25, 33), obese mice showed increased serum markers of liver injury (**Table S2**) and up-regulated expression of inflammatory genes such as IL-1β, CCR7 and PAI-1 (**Fig. S5*A***) in the liver. Markers of inflammation were also triggered by

hypoxia in primary hepatocytes, although MaR1 was ineffective in modulating this response, except for a very modest increase in IL-10 expression **(Fig. S5*B*).** A more pronounced inflammatory response was observed in PCLS exposed to hypoxia, in which condition, MaR1 significantly down-regulated IL-1β and TNFα (**Fig. S5*C*).** MaR1 also significantly inhibited the expression of the NLRP3 inflammasome in PCLS challenged with hypoxia (**Fig. S5*D***). This is of particular interest because NLRP3 inflammasome, IL-1β and TNFα are key factors involved in immunity and inflammation linked to the ER-induced UPR (36).

MaR1 induces a specific miRNA signature in hepatocytes exposed to hypoxia.

Similar to hepatocytes exposed to palmitate lipotoxicity, we also profiled the miRNA signatures associated with MaR1 actions in primary hepatocytes challenged by hypoxia. Under hypoxic conditions, 102 miRNAs (36 up-regulated and 66 down-regulated) were differentially expressed (**Fig. S6*A*, Fig. S6*B and* Table S5**), whereas MaR1 differentially regulated 32 miRNAs (17 up-regulated and 15 down-regulated) in hypoxic hepatocytes **(Fig. S6*C*** and **Fig. 6*D*).** Under MaR1 treatment, the top ranked enriched pathways identified by the DAVID bioinformatics tool were the GO terms metabolic process, phosphorylation and protein modification process (**Fig. S6*E***).

MaR1 ameliorates lipotoxic ER stress in adipocytes.

Finally, we investigated whether the inhibitory actions of MaR1 on ER stress were restricted to the liver or were common to other insulin-sensitive tissues, such as the WAT. This is of relevance in the context of NASH because impaired WAT function in obesity exerts a direct impact on the liver (53). WAT from obese mice showed adipocyte hypertrophy accompanied by an induction of eif2α and JNK phosphorylation and an up-regulation of ATF3 **(Fig. S7*A*** and **Fig. S7*B*).** IRE1α phosphorylation was significantly decreased in obese adipose tissue, a

finding previously described in conditions of persistent ER stress (54). To investigate whether WAT is also sensitive to the MaR1 actions on ER stress, we performed experiments in differentiated 3T3 adipocytes incubated with palmitate as a model of lipotoxic damage (55). As shown in **Fig. S7*C***, MaR1 down-regulated Erdj4, ATF3, CHOP, EDEM1 and ATF4 in differentiated adipocytes exposed to lipotoxic ER stress. These findings confirm that the protective actions of MaR1 are not restricted to the liver.

In a previous study carried out in our laboratory, we had assessed the ability of another member of the SPM family, RvD1, to accelerate the resolution process during calorie restriction in a HFD-induced obesity model (33). In that study, we observed a remarkable improvement in the hepatic inflammatory component in mice receiving RvD1, in a large part related to the polarization of hepatic macrophages towards a pro-resolutive M2 phenotype (33). In the current study, we reinforced this concept by demonstrating that the SPM MaR1 promotes efferocytosis in Kupffer cells, the liver resident macrophages. Moreover, in the current study we added more value to the protective actions of SPMs on obesity-induced NAFLD by demonstrating that MaR1 was able to prevent ER stress and to properly manage the UPR response. ER stress is an early event in the inflammatory process, and therefore our current findings confirm that SPM not only promote resolution but also block early triggers of inflammation. Moreover, in our study with RvD1 we observed that this SPM was able to counteract hypoxia-induced inflammation in the PCLS model, meanwhile in the current study we observed that MaR1 counteracts both hypoxia- and lipotoxic-induced ER stress in PCLS. Interestingly, in both studies, SPM actions were related to a specific miRNA signature, suggesting that pro-resolving mediators exert their pleiotropic actions by regulating the transcription of a large set of genes.

In summary, the results of the current investigation identify the lipid mediator MaR1 as a potent modulator of the ER stress-triggered UPR. The highly-conserved UPR signaling pathway allows cells to adapt and respond to ER stressors, but if this response becomes chronic or it is too intense, as is the case of liver cells under NAFLD conditions, fail to adapt and the UPR activates inflammation and pro-apoptotic pathways leading to cell death (9). Therefore, our findings demonstrating that MaR1 is able to promote the resolution of lipotoxic and hypoxia-induced ER stress in hepatocytes are very relevant for the pathogenesis of NAFLD and the prevention of this metabolic syndrome-associated hepatic complication. There are some aspects of our study that deserve consideration. Firstly, we used physiological stimuli such as free fatty acids (lipotoxicity) to disturb protein folding and to activate UPR in liver cells, instead of pharmacological agents such as thapsigargin and tunicamycin, which triggers strong and typically unrecoverable ER stress (56-58). Secondly, we used very low concentrations of MaR1 (at the nanomolar range) to reduce ER stress in hepatocytes, levels that can be easily achieved endogenously during the intake of omega-3 fatty acids in the diet. This is therefore a feasible alternative to the use of chemical chaperones. Thirdly, we demonstrated that MaR1 attenuated lipotoxic ER stress not only in liver cells but also in adipocytes, indicating that this lipid mediator exerts protective actions in most insulin-sensitive tissues. Finally, our study also disclosed some unexpected findings such as the reduction in the intracellular accumulation of lipids in hepatocytes treated with MaR1 in the model of lipotoxicity. Although further investigations are needed, this anti-steatotic effect of MaR1 could be related to the involvement of ER stress in the impairment of lipid homeostasis is a similar way ER stress is associated with inflammation in NAFLD (29, 59, 60).

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: B.R. and J.C.; performed the experiments: B.R.; supervised procedures: E.T.; bioinformatics analysis: J.J.L.; GC analysis: C.L-V; contributed reagents and tools and provided assistance to experiments: A.L., J.A-Q, R.F-C, M.D-G and M.C.; wrote the paper: B.R. and J.C.

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FIGURE LEGENDS

Figure 1: Obesity-induced hepatic steatosis is associated with endoplasmic reticulum (ER) stress. Role of lipotoxicity. (A) Representative photomicrographs of liver tissue sections stained with H&E (*top*) and Oil Red-O (*bottom)* from lean (n=5) and obese (n=5) mice (magnification:200X). The quantification of the area stained with Oil Red-O is shown below. **(B)** Hepatic protein (*top*) and mRNA *(bottom)* expression of ER stress markers in lean and obese mice. **(C)** Hepatic index of C16:1/C16:0 measured by GC in lean and obese mice. **(D)** Schematic diagram of the *in vitro* experiments. **(E)** Time-course of mRNA expression in hepatocytes incubated with vehicle (Veh) or palmitate (Pal, 0.5 mM) as a model of lipotoxic damage. Results are mean±SEM from three independent experiments assayed in duplicate *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$ for obese versus lean or for Pal versus Veh.

Figure 2: Maresin 1 (MaR1) modulates lipotoxic-ER stress in primary hepatocytes. (A) Schematic diagram of the MaR1 biosynthetic route from DHA. The activity of 12/15-lipoxygenase (12/15-LOX) converts endogenous DHA into 14-hydroperoxyDHA (14-HpDHA), which is subsequently hydrolyzed into MaR1. A representative PCR analysis of 12/15-LOX and GAPDH mRNA expression in mouse liver is also shown. M, 100-bp DNA ladder; lane 1, positive control (WAT); lane 2, liver tissue; lane 3, negative control. **(B)** mRNA expression of 12/15-LOX in livers from lean and obese mice. **(C)** ER stress and autophagy in hepatocytes pre-treated with either Veh, MaR1 (1-50 nM) or 4-phenylbutyrate (4-PBA) and then exposed to palmitate (Pal)-induced lipotoxic damage for 18 hours. **(D)** mRNA expression for ATF3, CHOP, BiP i Erdj4 in hepatocytes treated as in *B*. **(E)** mRNA expression for ATF3, CHOP, BiP and Erdj4 in hepatocytes exposed to Pal-induced lipotoxic damage for 18 hours and then treated with MaR1 (1-50nM) for the last 6 hours.

Results are mean±SEM from three independent experiments assayed in duplicate. *, P<0.05 and **, P<0.01 for MaR1 versus Pal. a, P<0.05, b, P<0.01 and c, P<0.01 for Pal versus Veh.

Figure 3: MaR1 modulates lipotoxic-ER stress in a time-dependent manner and prevents hepatocyte apoptosis. (A) mRNA expression of ER stress markers in hepatocytes pre-treated with Veh or MaR1 (50 nM) and exposed to palmitate (Pal)-induced lipotoxic injury at different time points (4, 8 and 18 hours). **(B)** Representative photomicrographs of DAPI-stained nuclei (in blue), TUNEL-positive (apoptotic) nuclei (in red) and MERGE (in pink) in hepatocytes incubated with the same conditions as in *A* for 18 hours (magnification: 200X). Results are mean±SEM from three independent experiments assayed in duplicate. a, P<0.05; b, P<0.01 and c, P<0.001 versus Veh. *, P<0.05 and **, P<0.01 for MaR1 versus Pal.

Figure 4: MaR1 modulates lipotoxic ER stress in precision-cut liver slices (PCLS) and promotes phagocytosis in Kupffer cells. (A) Schematic diagram summarizing the PCLS experimental procedures. **(B)** mRNA expression in PCLS pre-treated with MaR1 (10 nM) and then incubated with Veh or palmitate (Pal) for 18 hours. **(C)** Percentage (%) of phagocytic capacity of Kupffer cells treated with MaR1 (1-50 nM) for 6 hours. **(D)** Percentage (%) of phagocytosis versus vehicle in Kupffer cells pre-treated with MaR1 (1-50 nM) and exposed to Pal for 6 hours. Representative photomicrographs of phase contrast microscopy of Kupffer cells treated with Veh, Pal or Pal+MaR1 (50 nM) are shown on the left. a, P<0.05 and b, P<0.01 versus Veh. *, P<0.05; **, P<0.01 and ***, P<0.001 versus Pal.

Figure 5: MaR1 induces a specific miRNA signature in hepatocytes exposed to lipotoxic damage. (A) Heat-map of the miRNA expression data. Red pixels correspond to increased abundance of miRNA in the samples indicated, whereas green pixels indicate decreased

miRNA levels. **(B)** Number of miRNAs differentially expressed in palmitate (Pal)-treated cells versus Veh *(left)* and when MaR1 was added *(right).* **(C)** List of miRNA modulated by MaR1 with the corresponding fold changes. **(D)** Selected miRNAs *(top)* and their targets *(bottom)* were validated by real-time PCR or Western blot in palmitate-treated hepatocytes in the presence of vehicle (Veh) or MaR1 (50 nM). **(E)** DAVID functional classification of gene ontology (GO) term enrichment in response to miRNA differentially modulated by MaR1.

Figure 6: Induction of hepatic ER stress in response to obesity and hypoxia. (A) Hepatic CA9 mRNA expression in lean (n=5) and obese (n=5) mice. **(B, C)** Time course of CA9 and GLUT-1 mRNA expression in hepatocytes incubated under normoxia (21% O_2) or hypoxia (1% O_2). **(D)** Changes in the expression of ER-stress markers in hepatocytes incubated in hypoxia for 2, 4 and 8 hours. **(E)** ATF3, CHOP and BiP mRNA expression in hepatocytes incubated under normoxia or hypoxia for 2, 4 and 8 hours. **(F)** Protein expression of autophagy markers in hepatocytes incubated as described above. Results are mean±SEM from three independent experiments assayed in duplicate. *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$ for obese versus lean or for hypoxia versus normoxia.

Figure 7: MaR1 modulates hypoxia-induced ER stress and cell death in primary hepatocytes. (A) Protein expression of ER stress markers in hepatocytes under normoxia or hypoxia for 8 hours treated with Vehicle (Veh), MaR1 or 4-PBA. **(B)** Protein expression of ER markers in hepatocytes treated with Veh or MaR1 (1-50 nM) and then incubated under normoxia or hypoxia for 8 hours. **(C)** mRNA expression of ER stress markers in hepatocytes treated with Veh or MaR1 and incubated under normoxia or hypoxia for 4 hours. **(D)** Protein expression of autophagy markers under the conditions described in C. **(E)** Luminescent signals corresponding to the measurement of caspase 3/7 activity in hepatocytes challenged

with TNFα (20 ng/mL)+actinomycin D (250 ng/mL) and treated with Veh, MaR1 or 4-PBA for 12 hours. RLU, relative light units. Results are mean±SEM from three independent experiments assayed in duplicate. a, $P<0.05$; b, $P<0.01$ and c, $P<0.01$ for hypoxia Veh versus normoxia Veh or for TNFα+ActD versus Veh. *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$ for MaR1 versus Veh under hypoxic conditions or versus TNFα+ActD alone.