Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Palmitoleate attenuates palmitate-induced Bim and PUMA up-regulation and hepatocyte lipoapoptosis☆

Yuko Akazawa[1,2], Sophie Cazanave[1], Justin L. Mott[1], Nafisa Elmi[1], Steven F. Bronk[1], Shigeru Kohno[2], Michael R. Charlton[1], Gregory J. Gores[1,*]

[1]*Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA;* [2]*Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852-8562, Japan*

Background & Aims: Saturated free fatty acids induce hepatocyte lipoapoptosis. This lipotoxicity involves an endoplasmic reticulum stress response, activation of JNK, and altered expression and function of Bcl-2 proteins. The mono-unsaturated free fatty acid palmitoleate is an adipose-derived lipokine which suppresses free fatty acid-mediated lipotoxicity by unclear mechanisms. Herein we examined the mechanisms responsible for cytoprotection.

Methods: We employed isolated human and mouse primary hepatocytes, and the Huh-7 and Hep 3B cell lines for these studies. Cells were incubated in presence and absence of palmitate (16:0), stearate (18:0), and or palmitoleate (16:1, n-7).

Results: Palmitoleate significantly reduced lipoapoptosis by palmitate or stearate in both primary cells and cell lines. Palmitoleate accentuated palmitate-induced steatosis in Huh-7 cells excluding inhibition of steatosis as a mechanism for reduced apoptosis. Palmitoleate inhibited palmitate induction of the endoplasmic reticulum stress response as demonstrated by reductions in CHOP expression, eIF2-α phosphorylation, XBP-1 splicing, and JNK activation. Palmitate increased expression of the BH3-only proteins PUMA and Bim, which was attenuated by palmitoleate. Consistent with its inhibition of PUMA and Bim induction, palmitoleate prevented activation of the downstream death mediator Bax.

Conclusions: These data suggest palmitoleate inhibits lipoapoptosis by blocking endoplasmic reticulum stress-associated increases of the BH3-only proteins Bim and PUMA.

Keywords: Apoptosis; Bcl-2 proteins; Endoplasmic reticulum stress; Human hepatocytes; Nile Red; Steatosis.

Received 19 August 2009; received in revised form 19 October 2009; accepted 5 November 2009; available online 13 February 2010

☆ This work was supported by NIH Grants DK 41876 to G.J.G., DK 79875 to J.L.M., DK 69757 to M.R.C., the Optical Microscopy Core of P30 DK 84567, and the Mayo Foundation. The authors have no potential conflict of reference in regards to this manuscript.

* Corresponding author. Tel.: +1 507 284 0686; fax: +1 507 284 0762.

E-mail address: gores.gregory@mayo.edu (G.J. Gores).

Abbreviations: FFA, free fatty acid; NAFLD, nonalcoholic fatty liver disease; ER, endoplasmic reticulum; PERK, protein kinase RNA-like ER kinase; CHOP, CCAAT/enhancer binding homologous protein; IRE-1, inositol-requiring protein-1; JNK, c-Jun-N-terminal kinase; BH3, Bcl-2 homology 3; PUMA, p53-upregulated mediator of apoptosis; Bim, Bcl-2-interacting mediator of cell death; PA, palmitate; SA, stearate; PO, palmitoleate; SCD-1, stearoyl-CoA desaturase-1; DMEM, Dulbecco's modified Eagle's medium with high glucose; PCR, polymerase chain reaction; GADD 34, growth arrest and DNA damage gene 34; Bax, BCL2-associated X protein; XBP-1, X Box binding protein-1; eIF2-α, eukaryotic initiation factor 2-alpha; DAPI, 4′,6-diamidino-2-phenylindole.

Introduction

The metabolic syndrome, characterized by obesity and insulin resistance, is associated with elevated levels of circulating free fatty acid (FFA) [1,2]. The excess serum FFA in the context of insulin resistance saturates the transport and storage capacity of adipocytes leading to their uptake by liver, skeletal muscle, heart, and pancreatic β-cells [2]. The surfeit of FFA results in their participation in non-oxidative pathways with potential deleterious consequences for the non-adipocyte including cellular demise, termed lipoapoptosis [3]. This phenomenon is particularly relevant to the liver where hepatocyte lipotoxicity contributes to the syndrome of nonalcoholic fatty liver disease (NAFLD) [4]. Indeed, hepatocyte lipoapoptosis is a pathologic feature of NAFLD and correlates with disease severity [5,6]. Thus, mechanistic insights regarding the initiation and prevention of hepatocyte lipoapoptosis are of biomedical interest.

Intracellular FFA are trafficked to and esterified within the endoplasmic reticulum (ER), and not surprisingly, inundation of the liver with FFA disturbs ER function resulting in an ER stress response [7], which is well documented in NAFLD [8]. A potent transducer of ER stress response in NAFLD is PERK [9]. PERK activation induces the transcription factor CHOP [10], which is increased in liver and hepatocytes from dietary models of hepatic steatosis [11]. Another transducer of ER stress is IRE-1, which leads to activation of JNK [10]. Consistent with an ER stress-induced pathway of FFA-mediated cytotoxicity, hepatocyte lipoapoptosis is also associated with JNK activation in both rodent and human steatohepatitis, and JNK inhibition attenuates lipoapoptosis in these models [12,13]. Thus, ER stress-associated JNK activation is a potential cellular mechanism contributing to hepatocyte lipoapoptosis.

Bcl-2 protein family member can act to induce or prevent apoptosis [14]. JNK activation can be linked to dysregulation of

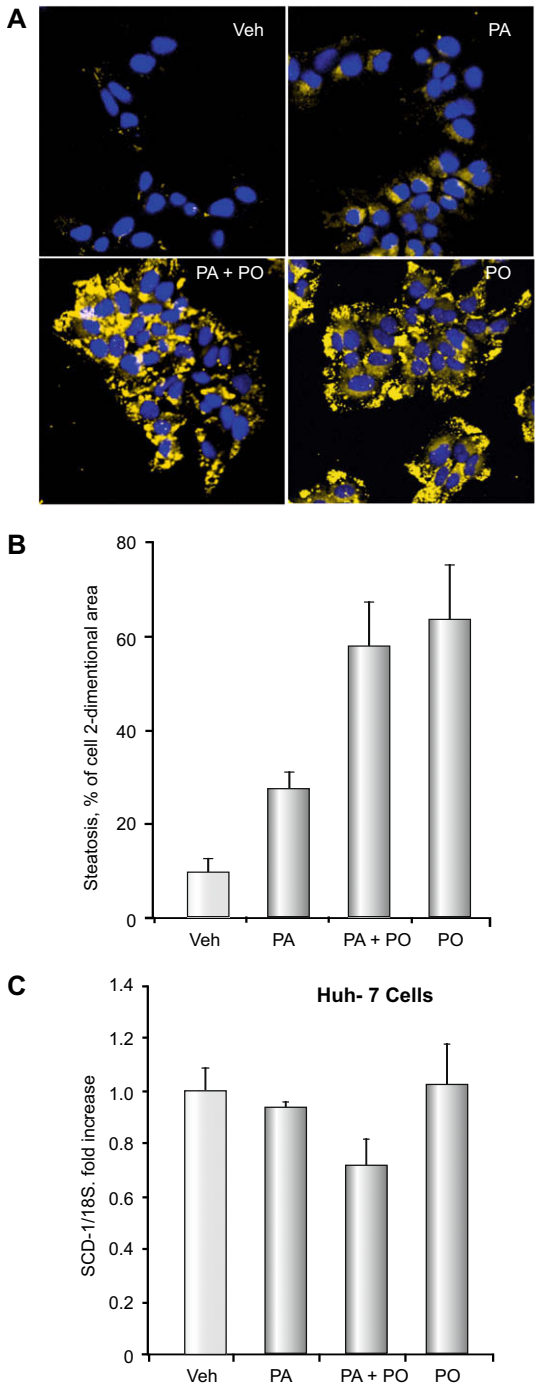

Fig. 1. PO does not attenuate PA-mediated steatosis. Nile Red staining was performed on Huh-7 cells treated with vehicle (Veh), 200 μM PA, 200 μM PO plus 200 μM PA, or 200 μM PO alone for 18 h. (A) Representative fluorescent photomicrographs (x 60) are depicted. Nile Red fluoresces as a yellow-gold at about 510 nm; cells were counter-stained for nucleic acids by DAPI (blue). (B) Cellular steatosis was quantified in 5 random low power fields for each condition with image analysis software. Total area of lipid per cell (pixels above threshold) is represented. The data represent the mean ± SEM for $n = 3$ studies. (C) Huh-7 cells were treated with vehicle (Veh), 800 μM PA, 400 μM PO plus 800 μM PA, or 400 μM PO alone for 8 h. SCD-1 mRNA was quantified by real time PCR. Fold induction was determined by normalization to 18S ribosomal RNA. Data represent the mean ± SEM of three independent experiments.

this core cell death machinery, promoting cell death [4]. In particular, JNK-1 but not JNK-2 activation by toxic FFA results in increased cellular levels of the pro-apoptotic BH3-only protein members of the Bcl-2 protein PUMA, and silencing this death mediator attenuates lipoapoptosis [15]. JNK is also known to activate Bim [16], which is another BH3-only protein contributing to lipoapoptosis [12]. Thus, ER stress can be directly coupled to dysregulation of Bcl-2 proteins which in turn trigger the mitochondrial pathway of apoptosis. Taken together, these observations suggest that FFA induce lipoapoptosis through both induction of PUMA and Bim, which in turn results in activation of the downstream death mediator Bax. Oligomerization of Bax within the outer mitochondrial membrane triggers mitochondrial dysfunction and cell death [17].

FFA and not their esterified product (triglyceride) appear to mediate lipotoxicity [18]. Experimental evidence indicates that saturated FFA such as PA (a 16 carbon FFA with no carbon–carbon double bonds, indicated 16:0) and stearate (18:0; SA) are more toxic than unsaturated FFA [12]. The mono-unsaturated FFA palmitoleate (PO; 16:1, n-7), is a unique product of adipocytes and functions as a lipid hormone, called a lipokine [19]. Although PO itself may effect minimal cytotoxicity, it is far less compared to saturated free fatty acids [12]. Furthermore, PO exerts a salutary effect in a murine model of the metabolic syndrome [19], in part by reducing expression of SCD-1, an enzyme which converts saturated FFA into mono-unsaturated FFA, a key step in channeling FFA into triglyceride synthesis [20]. PO has also been reported to reduce toxic FFA acid-induced apoptosis in rat hepatoma cell lines [21]. However, the mechanisms by which PO exerts its cytoprotective effects remain unclear and have not been directly examined in human hepatocytes. The elucidation of how this lipokine mitigates hepatocyte lipoapoptosis may help identify treatment strategies for human NAFLD.

The current work addresses the question of how PO reduces hepatocyte lipoapoptosis by toxic saturated FFA *in vitro*. We identify a pathway that involves PO inhibition of ER stress, and PUMA and Bim induction by PA. Inhibition of ER stress-mediated dysregulation of Bim and PUMA by PO and/or analogues may be salutary in human NAFLD.

Materials and methods

Cells. The human hepatocellular carcinoma cell lines, Huh-7, and Hep3B cells, and primary hepatocytes were employed for these studies. Detailed information regarding the culture conditions is in the Supplementary Materials And Methods Section.

Fatty acid treatment. Palmitic acid (catalog # P5585) and palmitoleic acid (catalog # P9417) were obtained from Sigma–Aldrich (St. Louis, MO). Free fatty acids were dissolved in isopropanol at a concentration of 20–80 mM. FFA were added to DMEM containing 1% bovine serum albumin to assure a physiologic ratio between bound and unbound FFA in the media [22]. The concentrations of individual FFA used in the experiments (200–800 μM) are similar to the fasting total FFA plasma concentrations observed in human nonalcoholic steatohepatitis [23–25].

Quantification of apoptosis. Apoptosis was quantified by assessing the characteristic nuclear changes of apoptosis using the nuclear binding dye DAPI (Molecular Probes, Eugene, OR) and fluorescence microscopy (Zeiss LSM 510, Carl Zeiss, Jena, Germany). Caspase 3/7 activation in cell cultures was measured using Apo-ONE homogeneous caspase 3/7 kit (Promega, Madison, WI) according to the manufacturer's instructions.

Nile Red staining. Huh-7 cells were grown on glass coverslips. Intracellular neutral lipid was stained with Nile Red (1 μg/ml) for 5 min at room temperature. Cells were then fixed with 3.7% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were mounted in Prolong Antifade (Invitrogen,