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MOLECULAR METABOLISM

ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload

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ABSTRACT

Palmitate overload induces hepatic cell dysfunction characterized by enhanced apoptosis and altered citric acid cycle (CAC) metabolism; however, the mechanism of how this occurs is incompletely understood. We hypothesize that elevated doses of palmitate disrupt intracellular calcium homeostasis resulting in a net flux of calcium from the ER to mitochondria, activating aberrant oxidative metabolism. We treated primary hepatocytes and H4IIEC3 cells with palmitate and calcium chelators to identify the roles of intracellular calcium flux in lipotoxicity. We then applied ^{13}C metabolic flux analysis (MFA) to determine the impact of calcium in promoting palmitate-stimulated mitochondrial alterations. Co-treatment with the calcium-specific chelator BAPTA resulted in a suppression of markers for apoptosis and oxygen consumption. Additionally, ^{13}C MFA revealed that BAPTA co-treated cells had reduced CAC fluxes compared to cells treated with palmitate alone. Our results demonstrate that palmitate-induced lipoapoptosis is dependent on calcium-stimulated mitochondrial activation, which induces oxidative stress.

Keywords Metabolic flux analysis; Lipotoxicity; Oxidative stress; ER stress; Fatty liver

1. INTRODUCTION

The obese and steatotic liver is marked by elevated fatty acids, ER stress, and metabolic alterations that give rise to hepatocyte dysfunction [1–5]. Non-alcoholic fatty liver disease (NAFLD) is a chronic condition resulting from excess lipid accumulation, which affects up to 30% of the U.S. population and is the leading cause of referrals to hepatology clinics [1,6]. Although simple steatosis does not always lead to complications, around 10% of NAFLD patients are at increased risk of developing more serious liver injuries such as nonalcoholic steatohepatitis (NASH) and hepatocellular carcinoma [7]. Free fatty acid (FFA) levels are present in higher concentrations in the plasma of these individuals, suggesting that *in vivo* alterations in FFA metabolism are linked to corresponding changes in disease severity [8,9].

Altered energy metabolism is a defining characteristic of both human and mouse fatty livers, which can be recapitulated in *in vitro* models of hepatocyte lipotoxicity [2,4,10,11]. However, the mechanism by which FFA overload induces metabolic dysfunction is undefined. *In vivo* flux analysis using $^{2}H/^{13}C$ NMR reported a ~2-fold increase in citric acid cycle (CAC) flux in NAFLD patients compared to patients with normal intrahepatic triglyceride content [2]. Complementary studies performed in high-fat diet (HFD) fed mice revealed similar increases in CAC activity that were associated with elevated markers of oxidative stress [4]. The authors hypothesized that CAC activation was required to meet energetic demands in the face of reduced respiratory efficiency resulting from mitochondrial oxidative damage. However, we have previously shown that saturated fatty acids (SFAs) can enhance mitochondrial metabolism independently of beta-oxidation in cultured hepatic cells through a mechanism that precedes the onset of oxidative damage [10,11]. Consistent with *in vivo* mouse studies, these changes in CAC fluxes coincided with enhanced reactive oxygen species (ROS) accumulation, suggesting that altered mitochondrial metabolism may be the cause, rather than a consequence, of enhanced oxidative stress observed in obesity and NAFLD. To confirm this, we performed experiments using antioxidants and mitochondrial inhibitors to demonstrate that CAC activation is critical for palmitate lipotoxicity but does not require prior ROS accumulation [10].

In vitro experiments have demonstrated that SFAs are also potent inducers of ER stress in hepatic cells, which precedes the onset of ROS accumulation and apoptosis [13]. It has been shown that markers of ER stress such as CHOP/GADD135 formation and depletion of ER calcium stores appear soon after cells are treated with long-chain SFAs, but not monounsaturated fatty acids (MUFAs) [14]. ER calcium is depleted shortly after SFA exposure, suggesting a mechanism of SFA toxicity that involves rapid disruption of ER homeostasis [12,15,16]. The exact

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Abbreviations: APE, atom percent enrichment; BSA, bovine serum albumin; CAC, citric acid cycle; FFA, free fatty acid; GC–MS, gas chromatography–mass spectrometry; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MFA, metabolic flux analysis; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OA, oleate; PA, palmitate; PI, propidium iodide; ROS, reactive oxygen species; SERCA, sarcoplasmic-endoplasmic reticulum calcium ATPase; SFA, saturated fatty acid

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Enzymes and metabolites			
AcCoA	acetyl-CoA	Suc	succinate
Akg	alpha-ketoglutarate	ADH	alpha-ketoglutarate dehydrogenase
Ala	alanine	CS	citrate syntase
Asp	aspartate	FUS	fumarase
Cit	citrate	GDH	glutamate dehydrogenase
Fum	fumarate	GLS	glutaminase
Gln	glutamine	IDH	isocitrate dehydrogenase
Glu	glutamate	LDH	lactate dehydrogenase
Lac	lactate	ME	malic enzyme
Mal	malate	PC	pyruvate carboxylase
Pyr	pyruvate	PDH	pyruvate dehydrogenase
		PK	pyruvate kinase
		SDH	succinate dehydrogenase

role of this calcium efflux in mediating lipotoxicity is unknown, although intracellular calcium levels impact many critical aspects of cell function. Calcium is integral in two important aspects of cell biology: oxidative metabolism and apoptosis. Calcium ions act as essential cofactors by activating several CAC enzymes, particularly dehydrogenases, and transporters involved in the malate–aspartate redox shuttle [17–20]. Calcium fluxes also initiate mitochondrial apoptotic pathways. Pro- and anti-apoptotic proteins of the Bax, Bcl, and Bim families have been shown to regulate the net movement of calcium into and out of the mitochondria [21–23].

Because of the rapid appearance of ER stress markers in response to palmitate treatment, we hypothesized that disruption of ER homeostasis may be the initial insult that is responsible for subsequent changes in mitochondrial function. We hypothesized that elevated levels of the SFA palmitate would compromise the ability of the ER to maintain calcium stores, resulting in net efflux of ER calcium that would enhance CAC flux, stimulate oxidative metabolism and ROS production, and ultimately lead to cellular dysfunction and apoptosis. To test this, we treated primary rat hepatocytes and immortalized H4IIEC3 hepatic cells with lipotoxic doses of palmitate, either with or without the intracellular calcium chelator BAPTA-AM. Palmitate-treated cells exhibited decreased ER calcium, elevated mitochondrial calcium, reduced mitochondrial potential, and enhanced oxygen consumption, all of which preceded the onset of apoptotic cell death. BAPTA co-treatment abrogated these lipotoxic phenotypes. ^{13}C metabolic flux analysis (MFA) revealed that palmitate-treated cells exhibited enhanced CAC flux and increased mitochondrial glutamine metabolism that were associated with ROS accumulation. BAPTA co-treatment also suppressed glutamine-dependent CAC activation and reduced oxidative stress. These results offer a mechanistic explanation for the close association between ER stress and ROS accumulation reported in prior lipotoxicity studies, in which calcium serves as a critical mediator linking changes in ER homeostasis to the onset of mitochondrial dysfunction.

2. MATERIALS AND METHODS

2.1. Materials

The fluorescent dyes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), propidium iodide (PI), Fura-2 AM, and JC-1 were purchased from Invitrogen (Carlsbad, CA, USA). The calcium-specific chelator BAPTA-AM was also obtained from Invitrogen. The fatty acids palmitate and oleate, bovine serum albumin (BSA), and low glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Aldrich (St. Louis, MO, USA). Primary hepatocytes were cultured on plates coated with Collagen I (Rat Tail) from BD Biosciences (San Jose, CA).

2.2. Primary rat hepatocyte isolation

Primary hepatocytes were isolated from male Sprague–Dawley rats as described previously [24]. The portal vein and inferior vena cava of anesthetized animals were cannulated and perfused with 37 °C oxygenated perfusion media, pH 7.4, containing 118 mM NaCl, 5.9 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.2 mM EGTA and 5 mM glucose. After 15 min, the liver was excised from the animal and perfused with liver digest medium (Invitrogen, Grand Island, NY). Then the cells were dispersed, washed four times, and suspended in attachment media, which consisted of 20 mM glucose DMEM supplemented with 30 mg/L proline, 100 mg/L ornithine, 0.544 mg/L $ZnCl_2$, 0.75 mg/L $ZnSO_4 \cdot 7H_2O$, 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 0.25 mg/L $MnSO_4$, 2 g/L bovine serum albumin (Sigma), 5 nM insulin, 100 nM dexamethasone, 100,000 U penicillin, 100,000 U streptomycin, and 2 mM glutamine. After 4 h of incubation in the attachment media, the primary hepatocytes were switched to a maintenance media identical to the attachment media except it had a concentration of 1 nM (instead of 5 nM) insulin.

2.3. H4IIEC3 cell culture

The H4IIEC3 rat hepatoma cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were cultured in 5 mM glucose DMEM supplemented with 2 mM glutamine, 10% FBS, and 1% penicillin/streptomycin antibiotic solution.

2.4. Fatty acid preparation

FFA stock solutions were prepared by coupling free fatty acids with BSA. First, palmitate or oleate was dissolved in pure ethanol at a concentration of 195 mM so that the final concentration of ethanol in our FFA stock solutions did not exceed 1.5% by volume. This solution was then added to a prewarmed 10% w/w BSA solution (37 °C) to achieve a final FFA concentration of 3 mM, and this solution was allowed to incubate in a water bath for an additional 10 min. The final ratio of FFA to BSA was 2:1. All vehicle treatments were prepared using stocks of 10% w/w BSA with an equivalent volume of ethanol added to match the concentration in FFA stocks. The final concentration of ethanol in all experimental treatments was less than 0.2% by volume.

2.5. ROS accumulation

Levels of intracellular ROS were assessed using the radical-sensitive dye H_2DCFDA, which is oxidized to the fluorescent 2, 7-dicholorofluorescein (DCF) upon exposure to ROS. Following

