Experimental Cell Research 354 (2017) 85–94

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Palmitate mediated diacylglycerol accumulation causes endoplasmic reticulum stress, Plin2 degradation, and cell death in H9C2 cardiomyoblasts

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ARTICLE INFO

Keywords:
Diabetic cardiomyopathy
Lipotoxicity
ER stress
Lipid droplet
Plin2

ABSTRACT

We have previously shown that palmitate causes ER stress in primary cardiomyocytes and this was associated with a diffuse lipid staining histology. In contrast, oleate, which was non-toxic, led to the formation of abundant, clearly delineated lipid droplets. The aberrant lipid histology in palmitate treated cells led us to hypothesize that perhaps there was an impairment in lipid droplet formation, which could lead to accumulation of lipids in the ER and consequent ER stress. To test this hypothesis we treated H9C2s (a cardiomyoblast cell line) with either 300 μM oleate or palmitate for 8 h. We found that palmitate resulted in significantly less lipid droplet abundance despite elevated intracellular lipid accumulation. Next we showed that palmitate was packaged primarily as diacylglycerol (DAG), in contrast oleate formed primarily triacylglycerol (TAG). Furthermore, the palmitate induced DAG accumulated mostly in the ER, while oleate treatment resulted in accumulation of TAG primarily in lipid droplets. The palmitate-induced accumulation of lipid in the ER was associated with a strong ER stress response. Interestingly, we found that ER stress induced by either palmitate, tunicamycin, or thapsigargin led to the degradation of Plin2, an important lipid droplet binding protein. In contrast palmitate had little effect on either Plin3 or Plin5. Furthermore, we found that acute MG132 administration significantly attenuated palmitate mediated ER stress and cell death. This protection was associated with a moderate attenuation of Plin2 degradation.

1. Introduction

Diabetic cardiomyopathy was first described by Rubler et al. in 1972 [1], and is now becoming increasingly recognized as a major contributor to morbidity and mortality in the diabetic population. One of the key pathological characteristics of this disease is cardiac steatosis. However, whether or not the accumulation of intracellular lipid is a contributing pathological factor or just a benign marker is still a matter of debate. There is a great deal of data showing that lipid accumulation is associated with insulin resistance, suggesting that lipid accumulation is pathological in nature. However, the athlete's paradox clouds this issue, as endurance athletes, which are insulin sensitive, have been shown to have abundant intramuscular lipid [2]. Therefore, lipid accumulation is likely too vague a parameter to predict the occurrence of pathophysiological mechanisms. As such, we need to more specifically characterize how lipids accumulate in cardiomyocytes as well as the downstream effects of said accumulation.

We previously demonstrated that palmitate-mediated lipotoxicity was associated with impaired lipid droplet formation, ER stress, and cell death in primary neonatal cardiomyocytes [3]. This was in stark contrast to treatment with oleate, which led to abundant lipid droplet formation without the occurrence of ER stress or cell death. Understanding the difference in how these two fatty acids accumulate may shed light on mechanisms of lipotoxicity.

Lipid droplets are storage depots for neutral lipids [4]. Lipid droplet dynamics are regulated in large part through the function of key lipid droplet binding proteins called Perilipins. There are 5 mammalian isoforms of Perilipins (Plin1-5). In the heart, Plin2, 3, and 5 are the most abundant, while Plin1 and 4 are primarily expressed in adipose tissue. Perilipins are important regulators of lipid metabolism and storage by either inhibiting or promoting lipolysis of stored triglycerides. This gatekeeping activity involves interaction with metabolic

Abbreviations: DAG, Diacylglycerol; TAG, Triacylglycerol; BSA, Bovine serum albumin; ER, endoplasmic reticulum

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http://dx.doi.org/10.1016/j.yexcr.2017.03.032
Received 11 July 2016; Received in revised form 13 March 2017; Accepted 14 March 2017
Available online 21 March 2017
0014-4827/

enzymes such as adipose triglyceride lipase (Atgl) [5].

Lipid droplets are produced by the ER before being released into the cytosol [6]. As such the ER is responsible not only for the translation of secreted proteins and calcium homeostasis, but lipid metabolism as well. As such perturbations in lipid metabolism can lead to ER stress. Not surprisingly ER stress has been demonstrated in both in vitro lipotoxicity studies [3,7,8], as well as in vivo studies of diabetic cardiomyopathy [9–12].

ER stress induces the unfolded protein response, a compensatory mechanism aimed at restoring ER homeostasis by up-regulation of several stress response genes including transcription factors (e.g. Atf6, Chop, Xbp1) and chaperones (e.g Grp78) [13]. Prolonged or severe ER stress leads to cellular de-compensation with consequent activation of apoptotic pathways. Therefore ER stress has emerged as a potentially important therapeutic target.

Here we aimed to further understand how differences in lipid accumulation, lipid droplet abundance, subcellular lipid distribution, and lipid profile might differentially affect lipotoxicity induced ER stress and cell death.

2. Methods

2.1. Reagents

Oleate (Sigma, O7501) was solubilized in anhydrous methanol, while palmitate (Sigma, P9767) was solubilized in 150 mM NaCl by heating to 70 °C. Both fatty acids were then complexed to bovine serum albumin (BSA) in a 6:1 ratio as previously described [3]. Radiolabelled oleate (American Radio-chemicals, ARC 0297) or palmitate (ARC 0172A) were also complexed to BSA. MG132 (M7449-200UL) was obtained from Sigma.

2.2. Histology

H9c2 cells (ATCC, CRL-1446) were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies). Cells were pre-treated with either 300 μM Oleate or 300 μM palmitate for 8 h and then washed and fixed in 4% paraformaldehyde for 20 min.

Cells were then stained with BODIPY 493/503 (Life Technologies), Rhodamine Phalloidin (Life Technologies) and DAPI (Life Technologies). Photomicrographs were taken using the IX83 inverted microscope from Olympus.

2.3. Lipid uptake assay

Cells were treated with either radiolabelled oleate or palmitate for 1 h. Cells were then washed with ice-cold PBS 4 times and then isolated. Cells were homogenized and added to scintillation fluid. Radioactivity was measured with the Scintillation counter (Beckman LS6500). Data are presented as picomol/mg protein.

2.3.1. Fatty acid oxidation assay

Following pre-treatment with either 300 μM oleate or 300 μM palmitate for 8 h, cells were treated with 0.4μCi of radiolabelled oleate (American Radio-chemicals, ARC 0297) or palmitate (American Radio-chemicals, ARC 0172A for 2 h. CO_2 was released from cellular media by addition of 6 N HCl to cell media and then captured by a filter paper soaked in 2 M NaOH. The filter paper was then added to a scintillation vial with scintillation fluid and radioactivity was read with the Beckman LS6500 scintillation counter.

2.3.2. Acyl-CoA synthetase activity assay

Acsl activity was measured as previously described [14]. Briefly, following pre-treatment with either 300 μM oleate or 300 μM palmitate for 8 h, cells were isolated and sonicated on ice. Reaction buffer containing ATP, DTT, Coenzyme-A, and radiolabelled oleate was added to 200 μg of cell sonicate. This was incubated for 20 min at 37 °C and then 1 ml of Doles reagent was added to each sample. 2 ml of heptane was then added to each sample and the samples were vortexed. Following this 500 μl of water was added and the samples were vortexed and then centrifuged (800*g* for 5 min). The aqueous fraction was isolated and then washed with another 2 ml of heptane. These samples were then vortexed and centrifuged again and radioactivity was measured from the aqueous phase with the Beckman LS6500 scintillation counter.

2.4. Thin Layer Chromatography (TLC)

Cells were treated with various conditions as outlined in the results. Lipid samples were isolated by Bligh and Dyer method then spotted on a silica gel plate. The silica plate was then placed in a TLC tank allowing the lipids to migrate with the mobile phase by capillary action. The TLC tank was equilibrated with 150 ml of 75:75:1.5 Chloroform:DiethylEther: Acetic acid. Lipid spots were then visualized on silica plate by rhodamine c (Sigma, R6626) labeling and quantified using Image J.

2.5. Sub-cellular distribution of lipid

Cells were treated as indicated in the results and then labelled with 20 μM radiolabeled oleate (C-14) for 20 min. Cells were isolated and then overlaid onto a sucrose density gradient (10–50% W/V) and centrifuged at 21,000*g* for 1 h. Radioactivity of the individual fractions was then measured using a Beckman scintillation counter (Beckman LS6500). Western blotting was performed on all fractions to determine content of ER (Calnexin) and lipid droplet (Plin2) markers.

2.6. Real-time RT-PCR

Following oleate or palmitate treatment RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad, 732–6820) as per manufacturer's instructions. cDNA was then synthesized using the 5X all-in-one RT MasterMix kit from (ABM, G485). Real-time PCR was carried out using the Go Taq® qPCR MasterMix 2X kit (Promega, A6002) on the Eco iIlumina real-time PCR machine. mRNA abundance was determined using the $2^{-\Delta\Delta}$Ct method. Gene expression was normalized to the housekeeping gene, Rpl34. Primers were designed using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in supplemental table S1. Xbp1 primers only amplify the spliced isoform, they do not recognize the un-spliced Xbp1 isoform.

2.7. Western blotting

Western blotting was carried out as previously described [3,15]. Antibodies used include Anti-Atf6 (Santa Cruz Biotechnology, SC-22799), anti-Caspase-3 (Abcam, Cat. #: ab2302), Anti-Chop (Santa Cruz Biotechnology, SC-7351), anti-Grp78(Santa Cruz Biotechnology, SC-13968), anti-Plin2 (Abcam, ab108323), anti-Plin3 (Santa Cruz biotechnology, sc-14726-R), anti-Plin5 (Abnova, PAB12542), anti-Tubulin (Santa Cruz Biotechnology, SC-23948), and anti-Xbp1 (Santa Cruz Biotechnology, SC-7160).

2.8. Viability assay

Viability assay was carried out by measuring propidium iodide fluorescence as previously described [3].

2.9. Immunofluorescence analysis

Mouse neonatal cardiomyocytes were isolated and cultured as previously described [3,16]. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton and 0.2% Tween in PBS, then cells were probed with anti-Plin2 antibody (also known as ADRP, SC32450