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Sequestration of fatty acids in triglycerides prevents endoplasmic reticulum stress in an in vitro model of cardiomyocyte lipotoxicity

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ABSTRACT

We used human cardiomyocyte-derived cells to create an in vitro model to study lipid metabolism and explored the effects of PPARγ, ACSL1 and ATGL on fatty acid-induced ER stress. Compared to oleate, palmitate treatment resulted in less intracellular accumulation of lipid droplets and more ER stress, as measured by upregulation of CHOP, ATF6 and GRP78 gene expression and phosphorylation of eukaryotic initiation factor 2a (EIF2a). Both ACSL1 and PPARγ adenovirus-mediated expression augmented neutral lipid accumulation and reduced palmitate-induced upregulation of ER stress markers to levels similar to those in the oleate and control treatment groups. This suggests that increased channeling of non-esterified free fatty acids (NEFA) towards storage in the form of neutral lipids in lipid droplets protects against palmitate-induced ER stress. Overexpression of ATGL in cells incubated with oleate-containing medium increased NEFA release and stimulated expression of ER stress markers. Thus, inefficient creation of lipid droplets as well greater release of stored lipids induces ER stress.

1. Introduction

Long chain fatty acids (FAs), either associated with albumin or contained in lipoproteins, are the main energy source for the heart, accounting for about 70% of its energy needs. Under normal conditions, the heart metabolizes FAs rather immediately; it has little capacity for storage [1]. However, obese and/or diabetic conditions lead to an excess influx of lipid to the heart, resulting in increased cardiac lipid accumulation, which is associated with impaired contractility [2] and cardiac hypertrophy (reviewed in [3]). Induction of endoplasmic reticulum (ER) stress by lipid oversupply has been proposed as one of the underlying mechanisms explaining lipid-driven cardiac dysfunction [4,5]. Induction of ER stress has been shown in vitro with conditions that mimic ischemia [6,7] and in vivo with infarction [7] and pressure overload [8]. Saturated FAs increase the saturated lipid content of the ER, leading to changes in ER structure and integrity, and contributing to the unfolded protein response (ER stress) [9]. Consequences of ER stress include mitochondrial dysfunction and reduced energy expenditure, activation of inflammatory pathways, impaired protein synthesis and cell growth, and apoptosis (reviewed in [10–12]).

Lipotoxicity is the result of an imbalance between lipid uptake and utilization. Saturated fatty acids (FAs) cause considerably more aggravating effects than unsaturated FAs. One possible reason for this is that the saturated FA palmitate leads to greater ceramide synthesis [13], triggers reactive oxygen species (ROS) generation [14], induces fusion/fission events of ER membranes [9], and impairs the synthesis of the mitochondrial membrane phospholipid cardiolipin, which causes mitochondrial dysfunction [15]. In combination these processes lead to apoptotic cell death [16,17]. Some of these effects are likely due to insufficient conversion of palmitate into triacylglycerol (TAG). Unsaturated FAs help prevent lipotoxic cell death via activation of cellular survival pathways and channeling of FAs towards storage as TAG in lipid droplets [5,18]. Storage of lipids in the form of inert TAG is considered harmless [2,18]. In contrast, accumulation of lipid intermediates like nonesterified FAs and signaling lipids such as

Abbreviations: ACSL1, acyl-CoA synthase 1; ATGL, adipose triglyceride lipase; DAG, diacylglycerol; ER, endoplasmic reticulum; FA, fatty acid; LD, lipid droplet; OA, oleic acid; PA, palmitic acid; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TAG, triacylglycerol

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ceramide and diacylglycerol (DAG) is associated with lipotoxicity [19–21].

In this report we describe studies of the effects of peroxisome proliferator-activated receptor γ (PPARγ) and acyl-CoA synthetase (ACSL1) on palmitate-induced ER stress in the human cardiomyocyte-like cell line AC16 [22], which was derived from adult ventricular heart tissue. PPARγ is a nuclear receptor involved in regulation of intracellular lipid storage, and ACSL1 catalyzes esterification of long chain FAs with co-enzyme A — the initial step in fatty acid metabolism. Although cardiomyocyte specific overexpression of either PPARγ or ACSL1 causes lipid accumulation and cardiac dysfunction, both PPARγ and ASCL1 inhibit inflammation in FA-treated macrophages [23]. Our study shows that PPARγ and ACSL1 can protect cardiomyocytes from ER stress. Moreover we found that oleate (OA), which is usually a non-toxic lipid, induces toxicity if its storage is disrupted by excess intracellular lipolysis.

2. Materials and methods

2.1. Cells

The human cardiomyocyte cell line AC16, derived from primary cultures of adult ventricular heart tissue [22], was used for the experiments. Cells were grown in DMEM/F-12 medium (GIBCO Invitrogen Corporation, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a 5% CO_2 atmosphere at 37 °C. Prior to the infection of the cells with recombinant adenovirus, the medium was changed to DMEM/F-12 medium supplemented with 2% heat inactivated horse serum and 1% penicillin–streptomycin. When cells were treated with FA, the medium was changed to DMEM/F-12 medium supplemented with 1% FBS and 1% penicillin–streptomycin.

2.2. Construction of recombinant adenoviruses

The plasmid that contained the cDNA of the human ACSL1 (pBS-hACS) was purchased from Open Biosystems. The hACSL1 cDNA was isolated with double digestion using BamHI and XbaI restriction enzymes. The 5′ and 3′ ends of hACSL1 were blunted with DNA polymerase I, Large (Klenow) Fragment. Accordingly, pAd-TrackCMV plasmid was digested with SalI restriction enzyme and ends were blunted with Klenow fragment. The cDNA of hACSL1 was then cloned in pAd-TrackCMV. The pAd-TrackCMV-hACSL1 plasmid was used to produce adenoviral particles as previously described [24] using the Ad-Easy-1 system [25]. The recombinant adenoviral vectors were linearized with PacI and used to infect human embryonic kidney 293 cells. The recombinant adenoviruses were purified by two consecutive cesium chloride ultracentrifugation steps, dialyzed, and titrated. Usually, titers of 5×10^{10} plaque-forming units (pfu)/ml were obtained. The adenovirus expressing the human PPARγ cDNA (Ad-PPARγ) was purchased from Vector Biolabs (Philadelphia, PA, USA). The adenovirus expressing ATGL was constructed as described previously [26]. Recombinant adenoviruses expressing green fluorescent protein (Ad-GFP) served as control.

2.3. Infection of cell cultures with recombinant adenoviruses and treatments with FA

At day 1, AC16 cells were plated in 6-well plates. The next day, cells were infected with either one of the recombinant adenoviruses, or solely GFP (control group) (multiplicity of infection — MOI: 10). Two days post-infection the cells were treated with FAs (palmitate or OA diluted in methanol, or solely methanol as a control) at a concentration of 0.1 mM or 0.4 mM. FA-free bovine serum albumin (BSA) fraction V (Sigma-Aldrich, St. Louis, Missouri, USA) was added (1%) to serve as a FA carrier. Cells were treated for 15 h. Subsequently, cells were processed for RNA isolation, protein isolation, or Oil-red-O staining.

2.4. Western blots

Cellular protein was isolated using lysis buffer containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, 0.1 mM sodium vanadate, 50 mM NaF, and complete protease inhibitor cocktail (Roche Pharma, Nutley, New Jersey, USA). Protein concentration was determined using the Pierce® BCA protein assay kit (Thermo Scientific, Waltham, Massachusetts, USA) and equal amounts of protein were loaded per lane. GAPDH protein expression was used as loading control. Membranes were incubated with antibodies against ACSL1, PPARγ, eIF2a and phospho-eIF2a (Ser51) (all from Cell Signaling Technology, Danvers, Massachusetts, USA). Secondary antibodies (goat-anti-rabbit and goat-anti-mouse; Santa Cruz Biotechnology, Santa Cruz, California, USA) were horseradish peroxidase (HRP) conjugated and detected using an ECL Western blotting detection kit (GE Healthcare, Pittsburgh, Pennsylvania, USA).

2.5. RNA isolation, cDNA synthesis and gene expression analyses

Total RNA was purified using Trizol reagent and treated with DNase, following the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). cDNA was synthesized using the SuperScript cDNA synthesis system from Invitrogen (Carlsbad, California, USA) according to the supplier's protocol. Quantitative real-time polymerase chain reactions (qPCR) were conducted with the Stratagene Mx3005 qPCR System (Stratagene, La Jolla, California, USA), using the Brilliant SYBR Green qPCR kit of Stratagene (La Jolla, California, USA). Primer sequences are listed in Supplemental Table 1. All samples were analyzed in duplicates and standardized to β-actin expression. Relative quantification of gene expression was performed with the comparative C_t method.

2.6. Oil-red-O staining

Neutral lipids were stained using Oil-red-O as previously described [2]. Microscopy was performed using a Nikon Eclipse E200 microscope (Nikon, Meltville, New York, USA) and digital images were obtained with a SPOT Insight Firewire digital camera (Diagnostic Instruments Inc., Sterling Heights, Michigan, USA) using SPOT Advanced software (Diagnostic Instruments Inc., Sterling Heights, Michigan, USA).

2.7. Cellular lipid analyses

Cellular lipids were extracted and TAG and free fatty acids (FFAs) measurements were performed as described previously [2].

2.8. Statistical analyses

The data were analyzed using the statistical program SPSS (SPSS 16.0 for Windows, Chicago, Illinois, USA). Differences between groups were evaluated with two-sided t-tests. Results were considered significant when $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Palmitate induces ER stress in cultured human cardiomyocytes

AC16 cells were treated with 0.4 mM palmitate (PA; C16:0) or 0.4 mM monounsaturated fatty acid, oleate (OA; C18:1). Both treatments resulted in intracellular lipid accumulation (Fig. 1A). OA but not PA treatment significantly increases TAG accumulation (CTRL: 1.0-fold ± 0.19 vs PA: 1.46-fold ± 0.20, $p = 0.08$ vs OA: 2.12-fold ± 0.20, $p = 0.008$; Supplemental Fig. 1A). Treatment with PA induced the