Biochemical and Biophysical Research Communications xxx (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Impaired fatty acid oxidation as a cause for lipotoxicity in cardiomyocytes

T. Haffar [a, b], F. Bérubé-Simard [b], N. Bousette [a, b, *]

[a] Université de Montreal, Canada
[b] Montreal Heart Institute, Canada

ARTICLE INFO

Article history:
Received 28 October 2015
Accepted 30 October 2015
Available online xxx

Keywords:
Diabetic cardiomyopathy
Lipotoxicity
Fatty acid oxidation

ABSTRACT

A major cause for diabetic cardiomyopathy is excess lipid accumulation. To elucidate mechanisms of lipotoxicity mediated diabetic heart disease we need to further our understanding of how lipid metabolism is altered in the diabetic heart. Here we investigated the role of lipid clearance by oxidation as a regulator of lipid-mediated toxicity (lipotoxicity).

We evaluated the effect of pre-treating rat neonatal cardiomyocytes (NCMs) with either oleate (mono-unsaturated fatty acid) or palmitate (saturated fatty acid) on fatty acid oxidation (FAO) by measuring $^{14}C-CO_2$ production. We evaluated carnitine palmitoyltransferase (Cpt1b) expression by western blotting and mitochondrial membrane potential by quantitative and qualitative fluorescence analyses using the JC-1 dye. We inhibited the Cpt1b pharmacologically using etomoxir and genetically by knocking down its expression using LentiVector mediated transduction of siRNAs targeting the Cpt1b gene.

We found that palmitate had a slower clearance rate from NCMs than oleate, and this was associated with a significant decrease in FAO. This impairment in FAO was not the result of either loss of Cpt1b protein or mitochondrial integrity. Enhancing FAO with either oleate or carnitine was associated with a significant attenuation of palmitate mediated lipotoxicity. In contrast impairing FAO in oleate treated NCMs caused lipotoxicity.

Here we demonstrate that a major difference between non-toxic unsaturated fatty acids and toxic saturated fatty acids is there ability to stimulate or inhibit fatty acid oxidation, respectively. This has important implications for diabetic cardiomyopathy since diabetic hearts consistently exhibit elevated lipid accumulation.

1. Introduction

Cardiac disease is the primary cause for morbidity and mortality in the diabetic population [1]. Diabetes is an independent risk factor for atherosclerosis and hypertension. However, Diabetes also causes cardiomyopathy independently of the latter vascular pathologies. This diabetic cardiomyopathy is due to excess lipid accumulation and the ensuing lipotoxicity [2–7]. To understand mechanisms of lipotoxicity mediated diabetic heart disease we need to further our understanding of how lipid metabolism is altered in the diabetic heart.

Long chain fatty acids are made up of three main categories including saturated fatty acids (e.g. palmitate), mono-unsaturated fatty acids (e.g. oleate), and poly-unsaturated fatty acids (e.g. linoleate). Oleate and palmitate are the two most common fatty acids in the human diet. Importantly, *in vitro* studies have shown that lipotoxicity is due mainly to saturated fatty acids, whereas unsaturated fatty acids like oleate are non-toxic and can even be protective.

The importance of lipid metabolism in the heart is underscored by the fact that the heart derives ~75% of its energy from fatty acids. However, this number increases to >90% in the diabetic setting. The diabetic heart is characterized as having an excess of intracellular lipid (also known as cardiac steatosis) [8–12]. Lipids accumulate in the heart because uptake exceeds lipid clearance. The two main pathways for lipid clearance are lipid export and fatty acid catabolism through oxidation. While lipid export does occur from cardiomyocytes [13], it is likely a small fraction of lipid clearance

* Corresponding author. Université de Montréal, Department of Surgery, Institut de Cardiologie de Montréal, 5000, rue Bélanger, Suite S-4100, Montréal, Québec H1T 1C8, Canada.
E-mail address: nicolas.bousette@umontreal.ca (N. Bousette).

http://dx.doi.org/10.1016/j.bbrc.2015.10.162
0006-291X/

compared to oxidation. Interestingly, studies have shown that β-oxidation is increased in models of diabetic cardiomyopathy despite the accumulation of lipid. The fact that diabetic hearts consistently exhibit elevated lipid accumulation suggests that either the increased β-oxidation is insufficient to handle the elevated uptake; or that the oxidation is incomplete. Indeed β-oxidation represents only the first half of fatty acid oxidation (FAO). The second half being the oxidation of acetyl-CoA to CO_2 in the citric acid cycle. Impaired citric acid cycle activity could lead to a build up of acetyl-CoA, which is converted to malonyl-CoA, a key substrate in fatty acid biosynthesis.

Here we investigated the effect of palmitate, a saturated fatty acid, on FAO in primary cardiomyocytes. We found that palmitate impaired FAO, and that enhancing FAO attenuated palmitate mediated cell death. Conversely, impairing FAO induced cell death in primary cardiomyocytes treated with the non-toxic fatty acid oleate.

2. Methods

2.1. Rat neonatal cardiomyocyte (NCM) harvest and culturing

NCMs were harvested from 1-day-old Sprague-Dawley rat pups as previously described [15]. NCMs were fed daily with DMEM/F12 + 2% iFBS + 100 μM bromodeoxyuridine (Sigma). Cells were cultured for a minimum of six days before treated to promote differentiation.

2.2. Fatty acid clearance assay

We evaluated the rate of fatty acid clearance by treating NCMs with 300 μM oleate or palmitate (275 μM cold fatty acid + 25 μM hot ^{14}C fatty acid). After 24 h we measured CPMs using a Scintillation counter (Beckman LS6500). CPMs were normalized to total CPMs produced by 25 μM radiolabeled fatty acid not exposed to cells.

2.3. Viability assays

We determined cell viability by the propidium iodide (PI) exclusion assay. Briefly, following treatment, NCMs were incubated with PI for 30 min at 37 °C. Fluorescence was measured (535 nm excitation/617 nm emission) using the Synergy2 fluorescence plate reader from Bio-Tek. Assays were done in triplicate.

2.4. Fatty acid oxidation assays

NCMs were treated with non-radiolabelled BSA, oleate or palmitate for 8 h then with 25 μM radio-labelled ^{14}C-oleate for 2 h. Media from treated NCMs were transferred to glass tubes containing center wells and stopper tops. NaOH soaked Whatman filter paper was placed in the center well after addition of the cell media. Concentrated HCL was added to the media to release the CO_2 which was captured by the basic filter. Filter papers were transferred to scintillation vials containing Aquasol II (Perkinelmer, NE9529). Radiolabelled CO_2 was counted by the Scintillation counter (Beckman LS6500).

2.5. Mitochondrial membrane potential assay

NCMs were incubated with warm Krebs-Ringer with HEPES buffer (KHR) containing 7.6 μM JC-1 (Sigma, cat# 1130-5) for 10 min. After incubation, cells were washed twice with warm KHR before reading the fluorescence (530 nm excitation/590 nm emission for red dye and 485 nm excitation/528 nm emission for green dye) using the Synergy2 fluorescence plate reader from Bio-Tek and a fluorescence microscope (Olympus IX83).

2.6. Western blotting

Total cellular protein was harvested from NCMs using RIPA protein isolation buffer containing Proteinase inhibitor cocktail (ROCHE). Protein concentration was determined using the Bradford assay. Proteins were transferred to activated PVDF membranes and probed with Cpt1b antibody (Geneway, cat# GWB-MQ462C). Equal protein loading conditions were utilized and verified by Ponceau staining of the membrane.

2.7. qRT-PCR

Total cellular RNA was isolated using RNEasy isolation kit (Qiagen) and cDNA synthesis was carried out using the Quantitect reverse transcription kit (Qiagen). Real-time RT-PCR was carried out using the Eco illumina PCR cycler. Primers were designed using the NCBI primer BLAST tool. Exon/exon junctions were selected to prevent amplification of genomic DNA. PCR amplifications were done using the Bryt green master mix (Promega). Cpt1b was normalized to the housekeeping gene Rpl34 and quantification was carried out using the ΔΔCt method. Primer sequences: rCpt1b-F, TCGAGTTCAGAAACGAACGC, rCpt1b-R, GTGTGTCTCCTGGTCTCAGC, rRpl34-F, TGCTGTGAGACCCAAAGTCCTCA, rRpl34-R, TAAGGAAAGCCCGCTTGATCCTG.

2.8. Knockdown studies

LentiVector mediated transduction of NCMs was carried out as previously described [14]. Briefly, HEK-T293 cells were transfected with plasmids expressing packaging proteins (psPAX2), envelope proteins (pMD2.G) and siRNA expressing plasmids (pLenti-siRNA, cat# i057517, ABMGood). 48 h after transfection cellular media containing virus were harvested and centrifuged to remove cells. NCMs were treated with 10 μg/ml polybrene for 90 min and then exposed to virus containing media for 21 h 6 days after transduction cells were treated as described below.

2.9. Statistical analysis

Data are presented as the mean ± standard error. Student's T-test was used for comparison of experiments with two groups. For three or more group comparisons we performed one-way ANOVA with the Tukey post-hoc test. P-values of <0.05 were considered statistically significant.

3. Results

3.1. Palmitate impairs fatty acid oxidation in primary cardiomyocytes

We previously demonstrated that palmitate led to a marked difference in the qualitative nature of lipid staining compared to oleate treated NCMs [15]. Indeed, palmitate caused a diffuse lipid staining pattern, while oleate produced canonical lipid droplet staining. To assess if this qualitative difference in lipid accumulation also exhibited quantitative differences we measured the amount of oleate or palmitate remaining after a 24-h period. Interestingly, there was a significant increase in palmitate remaining after 24 h compared to oleate (Fig. 1A) suggesting that palmitate was not oxidized with the same efficiency. In support of this, a previous report demonstrated that palmitate decreased fatty acid oxidation (FAO) following 20 h exposure [16]. However, from

Please cite this article in press as: T. Haffar, et al., Impaired fatty acid oxidation as a cause for lipotoxicity in cardiomyocytes, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.10.162