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Cardiomyocyte lipotoxicity is mediated by Il-6 and causes down-regulation of PPARs

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ABSTRACT

Here we sought to evaluate the effect of palmitate on cytokine and PPAR activity/expression. We investigated the effect of BSA conjugated palmitate and oleate on PPAR activity, PPAR-α and δ expression, as well as the expression of cytokines and key factors responsible for β-oxidation by qRT-PCR and western blotting in primary rat neonatal cardiomyocytes (NCMs). Furthermore we evaluated the effect of anti-inflammatory actions of AICAR and PPAR agonists on cytokine expression and cell death in palmitate treated NCMs. We found that palmitate caused down regulation of PPARs and increased cytokine expression and cell death, all of which was significantly attenuated by the co-administration of either AICAR or PPAR agonists. This work supports the pro-inflammatory actions of intracellular lipid and provides further insight into the pathological mechanism of cardiac lipotoxicity as occurs in diabetic hearts.

1. Introduction

Diabetic cardiomyopathy is associated with cardiac lipid accumulation. Indeed, studies have shown a build-up of intramyocellular lipids or, cardiac steatosis, in diabetic hearts and this is believed to lead to cardiac lipotoxicity [1–5]. A key to understanding lipotoxicity is a delineation of how lipid metabolic pathways are perturbed in cardiac disease.

PPARs regulate the expression of several metabolic genes and are therefore key determinants of lipid metabolism. PPAR-δ deletion in the heart impairs β-oxidation and causes cardiomyopathy suggesting critical importance of this protein [6]. Similarly, PPAR-α null mice also exhibit cardiac functional defects [7,8]. In contrast PPAR activation can attenuate cardiac pathologies due to a variety of stress stimuli [9,10]. Interestingly, diabetic rats have been shown to have attenuated PPAR activity in the heart [11] as well as decreased PPAR-α and δ protein levels [12,13].

One of the key PPAR target genes is Carnitine palmitoyl transferase-1b (Cpt1b, muscle isoform) since it catalyzes the rate-limiting step in β-oxidation, namely the transport of fatty acids across the outer mitochondrial membrane. Cpt1b is transcriptionally regulated by PPAR-α and δ. On the other hand, activity of Cpt1b is regulated by the metabolic intermediate malonyl-CoA. Malonyl-CoA is synthesized by Acetyl-CoA carboxylase (Acc2 is the predominant isoform in the heart) while it is degraded by Malonyl-CoA decarboxylase (Mcd). Acc2 on the other hand is regulated by the master metabolic regulator AMP-activated protein kinase (AmpK). The importance of Cpt1b in normal cardiac physiology is underscored by a recent study showing that Cpt1b deletion leads to adult mortality associated with severe hypertrophy [14]. Furthermore overexpression of Cpt1b in skeletal muscle has been shown to enhance β-oxidation and attenuate high fat diet induced insulin resistance [15].

Inflammation is an important pathological component of diabetes. Several studies have demonstrated the association between inflammation and diabetic cardiomyopathy [16–22]. Diabetics frequently exhibit systemic inflammation as evidenced by increased circulating levels of Tnf-α, and Il-6 [23–25]. In addition, there is evidence to suggest that intracellular lipids can induce cardiac inflammation *in situ* independently of circulating cytokines. Indeed, palmitate, a saturated fatty acid, induces increased expression of TNF-α and Il-6 levels in skeletal muscle cells [26,27], adipocytes [28], endothelial cells [29], keratinocytes [30], and hepatocytes [31].

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Here we sought to further our understanding of cardiac lipotoxicity by evaluating the effects of palmitate, a toxic saturated fatty acid, on PPAR activity and expression as well as cytokine expression in primary cardiomyocytes. We show that palmitate causes down-regulation of PPARs which in turn impairs the expression of key proteins involved in β-oxidation. In addition, we show that the impaired PPAR levels may be due to induction of Il-6, and that anti-inflammatory treatments which attenuate cytotoxicity are associated with attenuated Il-6 expression. This report describes the effects of palmitate on PPAR and cytokine expression in primary rat neonatal cardiomyocytes (NCMs) thus providing new information on the mechanism of cardiac lipotoxicity.

2. Methods

2.1. Preparation of fatty acids

Bovine serum albumin (BSA) in 150 mM NaCl was used as fatty acid vehicle. Sodium oleate was dissolved in methanol and then added to 0.17 mM BSA (6:1 M ratio). Oleate was conjugated to BSA by gentle agitation at 37 °C for 1 h and then stored at −80 °C until used. Palmitate was dissolved in 150 mM NaCl at 70 °C. The latter solution was then slowly added to 0.17 mM BSA at 37 °C (6:1 M ratio). The palmitate-BSA conjugate was agitated for 1 h at 37 °C and then stored at −80 °C until used.

2.2. Primary cardiomyocyte harvest and cell culturing

All animal care protocols were approved by and conformed with the guidelines of the institutional animal care and use committees at the Montreal Heart Institute. Hearts from 1 to 2 day old neonatal Sprague–Dawley rats were removed aseptically and washed with Hanks buffer solution containing 137 mM NaCl, 5.36 mM KCl, 0.81 mM $MgSO_4$, 5.55 mM dextrose, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 20 mM HEPES, and 50 μg/ml gentamicin (Gibco). Ventricular tissue digestion was carried out through repeated incubations of the tissue with Hanks buffer solution containing 50 U/ml collagenase-2 (Worthington, NJ) and 0.36uM $CaCl_2$ for 5–10 min or until solution became noticeably turbid. Once turbid, the digestion solution was added to ice-cold fetal bovine serum (FBS), taking care to leave the non-digested ventricular tissue in the initial tube. This was repeated until ventricular tissue was completely digested. The resultant cell suspension was plated on a Falcon Primaria cell culture dish (Becton Dickenson) for 1 h at 37 °C to let cardiac fibroblasts adhere to the plate. After 1 h non-adherent cardiomyocytes were re-plated on the Primaria plate for 120 h. Cells were subject to daily media replacement with DMEM/F12 (50:50; Gibco, ON) with 2% FBS and 100 μM bromodeoxyuridine to inhibit growth of any contaminating cardiac fibroblasts.

2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated from mouse or rat neonatal cardiomyocytes using RNEasy (Qiagen). cDNA was synthesized using a commercially available kit (Qiagen). qPCR was performed using SYBR Green Master mix from Qiagen and the Eco Ilumina real-time qPCR system. Primer sequences are listed in supplemental table S1. Data was analyzed using the $2^{-\Delta\Delta Ct}$ method [32]. All PCR results represent the expression of the gene of interest relative to endogenous control (Rpl34) normalized to the control group, and are presented as mean ± standard error (SE). Primers were designed to span exon–exon regions to avoid amplification of contaminating DNA and primer specificity was verified by blasting all sequences using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

2.4. Western blot analysis

Total cellular protein was harvested from rat neonatal cardiomyocytes using protein isolation buffer (250 mM Sucrose, 50 mM Tris, 1 μM PMSF (protease inhibitor), 1 μM DTT, and Proteinase inhibitor cocktail (ROCHE)). Protein concentrations were determined using Bradford assay and equal protein loading conditions were used and verified by Ponceau staining of the membrane. Proteins were transferred to PVDF membranes and probed with the following antibodies: anti-Acadl (Sc-82466, Santa Cruz biotechnologies), anti-Cpt1b (GWB-MQ462C, GenWay Biotech), anti-PPAR-α (SC-9000, Santa Cruz Biotechnologies), anti-PPAR-δ (PA1-823A, Pierce antibodies).

2.5. Viability assays

We evaluated cell viability by propidium iodide (PI) exclusion assays. Briefly, rat NCMs were treated with palmitate for indicated time points (n = 4 per timepoint). The treated cells were then 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 duplicate.

2.6. Statistics

All data are presented as mean ± standard error. Student's T-test was used for comparison of two groups, while one-way ANOVA with the Tukey post-hoc test was used for multiple group comparisons. P-values of <0.05 were considered statistically significant.

3. Results

3.1. Time dependent effects of palmitate on PPARs

Because of the importance of PPARs in lipid metabolism and hence cardiac lipotoxicity, we sought to evaluate the effect of palmitate on PPAR activity in primary rat neonatal cardiomyocytes (NCMs). PPAR activity was measured indirectly by assessing the mRNA expression of PPAR target genes including Acyl-CoA dehydrogenase, long chain (Acadl), Acyl-CoA synthetase (Acsl1), and carnitine palmitoyltransferase (Cpt1b) [33]. To validate these as legitimate PPAR target genes in NCMs we administered PPAR-α and δ agonists (Wy-14643 and GW501516, respectively) to NCMs for 24 h. We found that both PPAR agonists significantly induced the expression of all 3 genes (Supplemental Fig. S1). Having validated these as legitimate PPAR targets in NCMs, we assessed the time dependent expression of these genes in palmitate treated NCMs. Interestingly, we found that 300 μM palmitate initially increased PPAR activity as evidenced by significantly increased expression of the 3 genes after 4 and/or 8 h exposure (Fig. 1A). However, after 24 h of palmitate exposure, we found that mRNA expression of PPAR target genes had significantly decreased from peak values down to levels below baseline. On the other hand, Oleate, a non toxic unsaturated fatty acid, significantly induced PPAR activity for more than 24 h (Fig. 1B). To assess if the attenuated mRNA expression translated into decreased protein levels we assessed protein levels for Acadl and Cpt1b. Interestingly, Cpt1b (Fig. 2A), but not Acadl (Supplemental Fig. S2A), was significantly decreased after 24 h palmitate exposure. To determine if the decrease in PPAR activity was due to decreased PPAR abundance we performed Western blots for PPAR-α and PPAR-δ. Interestingly, NCMs treated with

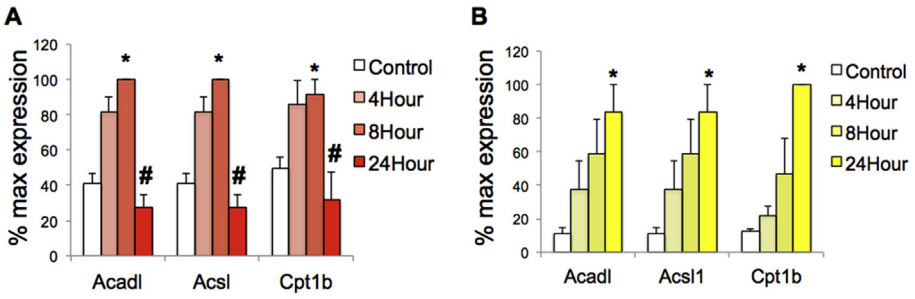

Fig. 1. PPAR target gene expression is induced early on, and then repressed later, in palmitate treated neonatal cardiomyocytes (NCMs). Graphs showing the % maximum expression of Acyl-CoA synthetase (Acsl), Acyl-CoA dehydrogenase (Acad), and Carnitine palmitoyl transferase (Cpt1b) in NCMs treated with (A) 300 μM palmitate or (B) 400 μM oleate for the indicated times. Control cells were treated with BSA (fatty acid vehicle) for 24 h. Values represent expression at indicated time relative to maximum expression of all time points (normalized to housekeeping gene Rpl34). * indicates $p < 0.05$ vs. control. # indicates $p < 0.05$ vs. 8 h time-point.

300 μM palmitate for 24 h showed significant reductions in both PPAR-α (Supplemental Fig. S2B) and -δ (Fig. 2B) isoforms.

3.2. Lipotoxicity induces marked cytokine expression in primary cardiomyocytes

Because of the well established link between PPARs and inflammatory cytokines we sought to determine if palmitate induced the expression of cytokines in primary cardiomyocytes. Palmitate has been shown to induce inflammatory markers in a variety of cell types but this has not been previously shown in NCMs. Therefore, we evaluated Tnf-α and Il-6 mRNA expression in NCMs treated with 300 μM palmitate for 24 h. We found that palmitate led to a robust induction of both Tnf-α (>20 fold) and Il-6 mRNA (>200 fold) levels (Fig. 3A–B) and this was dose dependent (Fig. 3C).

Because both AICAR (an AmpK activator) and PPAR agonists have previously been shown to attenuate cytokine expression in other cell types; we were interested if these treatments could attenuate palmitate induced cytokine expression in primary cardiomyocytes. In addition, we also tested the effect of oleate on palmitate induced cytokine expression because of the well established capacity for oleate to protect against palmitate induced lipotoxicity. Therefore, we tested the effect of oleate (50 μM), AICAR (2 mM), and PPAR agonists (Wy-14643 (240 μM) or GW501516 (10 μM)) on expression of Tnf-α and Il-6 in palmitate treated NCMs. Interestingly, we found that all four treatments had different effects on TNF-α mRNA expression. Specifically, oleate had no effect, AICAR caused a significant decrease, while PPAR agonists caused non-significant increases in TNF-α mRNA levels (Fig. 3D). In contrast, all four treatments concordantly led to significant decreases in Il-6 levels (Fig. 3E).

Oleate has been previously shown to attenuate palmitate mediated cell death but the effect of AICAR and PPAR agonists on palmitate induced cell death has not been previously described in primary cardiomyocytes. Therefore, we evaluated viability in cells treated with palmitate ± AICAR, Wy-14643, or GW501516. We found that all treatments significantly attenuated palmitate induced cell death (Fig. 4A and B).

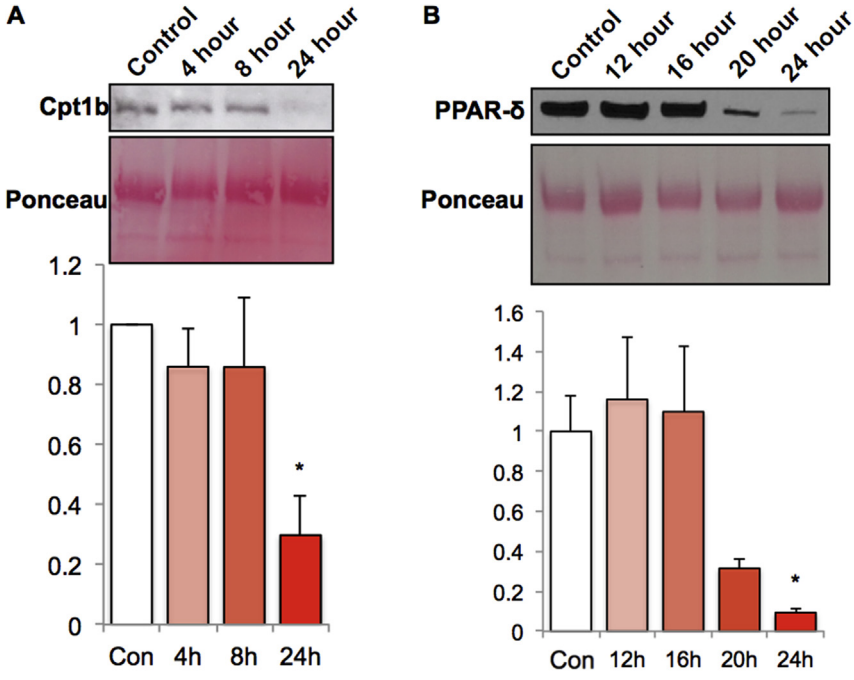

Fig. 2. Palmitate induces the down regulation of Cpt1b and PPAR-δ protein levels. (A) Western blot and associated ponceau staining of membrane demonstrate that Cpt1b protein significantly decreases after 24 h of palmitate exposure in NCMs. Graph at bottom represents quantification of Cpt1b protein abundance relative to total protein as determined by ponceau stain from 3 separate experiments (B) Western blot and associated ponceau staining of membrane demonstrate that PPAR-δ protein significantly decreases after 24 h of palmitate exposure in NCMs. Graph at bottom represents quantification of PPAR-δ protein abundance relative to total protein as determined by ponceau stain from 3 separate experiments. In all cases Control cells were treated with BSA (the fatty acid vehicle) in serum free media for 24 h * indicates $p < 0.05$ vs. control.

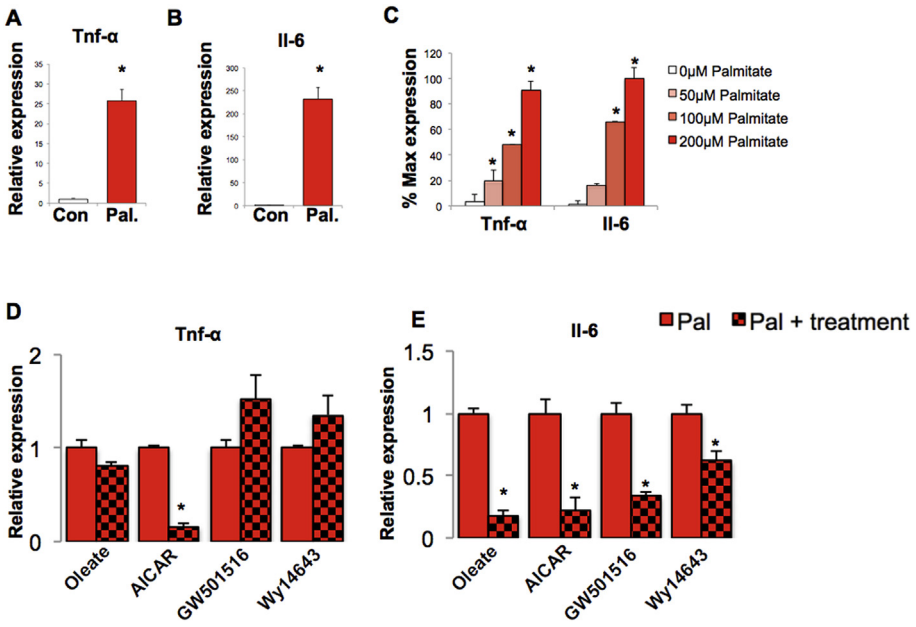

Fig. 3. Palmitate induces the expression of inflammatory mediators Tnf-α and Il-6 in neonatal cardiomyocytes (NCMs). (A–B) Graphs showing the expression of Tumor necrosis factor-alpha (Tnf-α) and Interleukin-6 (Il-6) relative to housekeeping gene Rpl34 in NCMs treated with 300 μM palmitate for 24 h (compared to BSA treated control NCMs). (C) Graph showing the % maximum expression of Tnf-α and Il-6 (relative to housekeeping gene Rpl34) in NCMs treated with palmitate at the indicated concentration. (D) Graphs showing the expression of Tnf-α (relative to housekeeping gene Rpl34) in NCMs treated with palmitate ± the indicated treatment for 24 h (E) Graphs showing the expression of Il-6 (relative to housekeeping gene Rpl34) in NCMs treated with palmitate ± the indicated treatment for 24 h * indicates $p < 0.05$ vs. control.

4. Discussion

Diabetes is a major contributor to cardiovascular disease. Indeed, the dyslipidemia in diabetics significantly increases risk for atherosclerosis. However, the accumulation of lipid within cardiomyocytes themselves, as opposed to in the vessels that supply them, may be equally pathological. Furthermore, the two pathological conditions can occur simultaneously, hence the substantial increase in heart failure and death by cardiovascular disease in diabetics.

Here we were interested in delineating the pathological mechanisms of lipotoxicity in an *in vitro* model of cardiac lipotoxicity. Indeed, we utilized primary rat neonatal cardiomyocytes which have the advantage of continuous spontaneous contraction which more closely mimics the high energy demand, and hence the energy metabolism of the beating heart, than would cardiomyocyte cell lines like H9c2 or AC16 cells. Although HL-1 cells can be differentiated to spontaneously contract, in our hands we found that this was highly variable.

Using primary cardiomyocytes we found several important aspects regarding mechanisms of cardiac lipotoxicity. Specifically, we show that (1) lipotoxicity causes impairment of PPAR activity due to loss of PPAR protein abundance and this results in Cpt1b down-regulation; (2) Cardiomyocyte lipotoxicity is associated with induction of cytokines, (3) Oleate, AICAR and PPAR agonists significantly decrease Il-6 but not Tnf-α levels in palmitate treated cardiomyocytes and this is associated with attenuated cell death. Together this data points to a key role for Il-6 in the pathological mechanism of cardiac lipotoxicity.

4.1. Palmitate causes PPAR down-regulation

A key finding of this current study was that palmitate induced PPAR activity at early time points but this activation was lost after

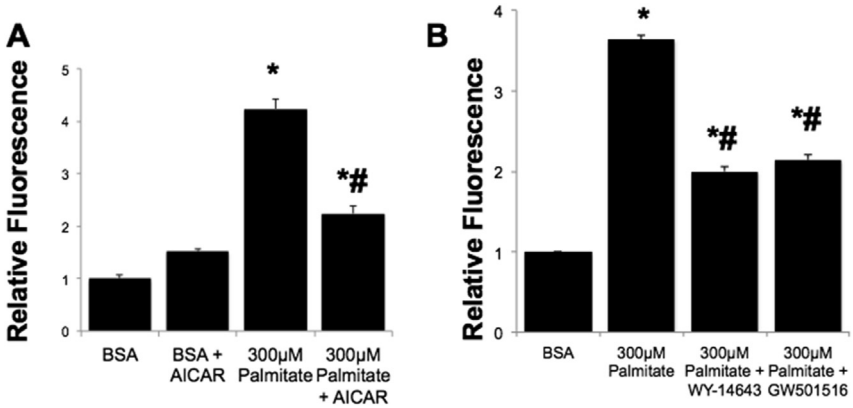

Fig. 4. Palmitate induced cell death in neonatal cardiomyocytes (NCMs) is attenuated with treatments that reduce Il-6 expression. Graphs (A–B) demonstrate relative fluorescence (y-axes) from NCMs treated as indicated in x-axes, followed by staining with propidium iodide, which is excluded from live cells but taken up by dead/dying cells. * indicates $p < 0.05$ vs. BSA treated NCMs (control). # indicates $p < 0.05$ vs. palmitate treatment alone.

24 h. In contrast oleate, the non-toxic fatty acid resulted in persistent PPAR activation. Indeed it is conceivable that persistent PPAR activity is necessary for the cell to manage the high lipid load and that loss of this metabolic capacity results in accumulation of toxic metabolites and cell death. The observed early PPAR activation by palmitate suggests that palmitate can act as a PPAR ligand, but that it simultaneously activates other pathways that ultimately lead to PPAR degradation. Indeed we show that both PPAR-α and δ are decreased in NCMs treated with palmitate for 24 h. The PPAR degradation results in decreased expression of genes responsible for β-oxidation of lipids thus allowing for the build-up of lipotoxic intermediates which ultimately result in cell death. Several lines of evidence suggest that the increased cytokine expression could be responsible for the PPAR degradation. For instance, inflammation was suggested to be the cause of attenuated PPAR activity in hearts of diabetic mice [12]. More specifically, Il-6 has been shown to decrease PPAR-α and/or γ activity and abundance in adipocytes and hepatocytes [34–38], but this has not been previously shown in cardiomyocytes. In addition, Tnf-α transgenic mice were shown to have decreased expression of PPAR-α and β-oxidation genes in the heart, resulting in decreased fatty acid oxidation [39]. Interestingly, they found that Tnf-α didn't have any direct effects on oxidation in cultured cardiomyocytes, but instead they attributed the impairment in oxidation in the Tnf-α transgenic hearts to the activation of the Tgf-β-Smad3 pathway. Unfortunately they did not evaluate Il-6 levels in that study, but since both Tgf-β [40,41] and Tnf-α [42] have been shown to induce Il-6, this supports the hypothesis that Il-6 is the key inducer of PPAR degradation. It is also important to note that the Il-6 expression precedes PPAR degradation, further supporting it as a cause rather than a consequence of metabolic dysfunction. Future studies will be focused on the mechanism of PPAR degradation in lipotoxic environments.

We also demonstrate that the loss of PPARs results in decreased Cpt1b protein levels, suggesting that PPAR down-regulation impairs oxidation through down-regulation of Cpt1b. The loss of Cpt1b protein after 24 h of palmitate exposure suggests that either Cpt1b protein half-life is very short and that protein levels mirror mRNA levels or that palmitate leads to the active degradation of Cpt1b protein. Importantly, although Acadl mRNA levels were down after 24 h; protein levels were unchanged, suggesting that this protein either has a longer half life than Cpt1b or that it is not specifically targeted for degradation.

Considering that our hypothesis revolves around the key beneficial role PPARs play in mitigating lipotoxicity, it is important to note the paradoxical finding that activation of PPAR-α by transgenic overexpression causes diabetic cardiomyopathy due to lipotoxicity. However it must be kept in mind that in these latter mice PPAR-α is expressed at supra-physiological levels (~50–100 fold) potentially leading to more lipid uptake than can be handled by the oxidative or storage capacities of hearts in these mice. Interestingly, PPAR-δ overexpressing mice did not exhibit lipid accumulation or develop cardiomyopathy. Both PPAR-α and PPAR-δ induce β-oxidation, however the key difference is the fact that PPAR-α induces lipid uptake to a much greater degree than PPAR-δ [43]. Thus perhaps the ratio of PPAR-α vs. δ activity can also have an impact on lipid accumulation and hence lipotoxicity in the heart.

4.2. Lipotoxicity induced cytokine expression, which was attenuated by oleate, AICAR and PPAR agonists

Palmitate has been shown to induce the expression of cytokines in a variety of cell types, but this has not been previously shown in NCMs. Here we showed that palmitate robustly induced the expression of both TNF-α and Il-6. This is highly relevant considering the important role inflammation has in the pathology of diabetes. Interestingly, oleate has been shown to attenuate palmitate induced Il-6 release from skeletal muscle cells [44]. Indeed, we showed that the protection from cell death afforded by oleate, AICAR, and PPAR agonists were consistently associated with a reduction in Il-6, but not Tnf-α. This suggests that the primary pathological culprit involves Il-6 signaling. Further studies will be required to demonstrate the mechanism of Il-6 induction in lipotoxic conditions.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.062.

Transparency document

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