Taha Haffar, Félix-Antoine Bérubé-Simard, Jean-Claude Tardif, Nicolas Bousette*

Saturated fatty acids induce endoplasmic reticulum stress in primary cardiomyocytes

Abstract:
Introduction: Diabetes is a major contributor to cardiovascular disease. There is a growing body of evidence pointing towards intra-myocellular lipid accumulation as an integral etiological factor. Here we aimed to determine the effect of two common fatty acids on lipid accumulation and cellular stress in primary cardiomyocytes.

Methods: We evaluated lipid accumulation biochemically (by triacylglyceride assay and radiolabeled fatty acid uptake assay) as well as histologically (by BODIPY 493/503 staining) in mouse and rat neonatal cardiomyocytes treated with saturated (palmitate) or mono-unsaturated (oleate) fatty acids. Endoplasmic reticulum (ER) stress was evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting. Cell viability was assessed by propidium iodide staining.

Results: We found that both oleate and palmitate led to significant increases in intracellular lipid in cardiomyocytes; however there were distinct differences in the qualitative nature of BODIPY staining between oleate and palmitate treated cardiomyocytes. We also show that palmitate caused significant apoptotic cell death and this was associated with ER stress. Interestingly, co-administration of oleate with palmitate abolished cell death, and ER stress. Finally, palmitate treatment caused a significant increase in ubiquitination of Grp78, a key compensatory ER chaperone.

Conclusion: Palmitate causes ER stress and apoptotic cell death in primary cardiomyocytes and this is associated with apparent differences in BODIPY staining compared to oleate treated cardiomyocytes. Importantly, the lipotoxic effects of palmitate are abolished with the co-administration of oleate.

Keywords: ER Stress, fatty acids, lipotoxicity, cardiomyocytes

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Abbreviations:
Atf: Activating transcription factor
BCA: Bicinchoninic acid
BSA: Bovine serum albumin
Chop: CCAAT/enhancer binding protein (c/ebp) homologous protein
CPM: Counts per minute
DAG: Diacylglyceride
ER: Endoplasmic reticulum
FBS: Fetal bovine serum
Grp78/Bip: Glucose regulated protein 78
Ire1: Inositol requiring enzyme-1
NCMs: Neonatal cardiomyocytes
PBS: Phosphate buffered saline
PI: Propidium iodide
PVDF: Polyvinylidene difluoride
TAG: Triacylglyceride
Xbp1: X-box binding protein-1

1 Introduction

Diabetes is a major contributor to cardiovascular disease. In fact, diabetes is an independent risk factor for coronary artery disease and hypertensive heart disease. However, diabetics can also develop isolated diabetic cardiomyopathy, characterized by ventricular dysfunction in diabetics without obvious macro-vascular disease or other etiological factors including alcoholism. Diabetic cardiomyopathy, with or without superimposed vascular disease, predisposes diabetic patients to heart failure. Indeed, the incidence of heart failure is four times higher in diabetic patients than the general population [1].
Furthermore, diabetes is an independent risk factor for death in patients with heart failure [2]. The specific cause of diabetic cardiomyopathy is likely multifactorial, however amounting evidence now suggests that intra-myocellular lipid accumulation is an important contributory factor. Several studies have demonstrated excess myocardial lipids or, cardiac steatosis, in diabetic humans and mice [3-7]. Indeed the cardiac lipotoxicity resulting from elevated intra-cellular lipids is believed to lead to cell death with ensuing cardiac dysfunction.

The two main fats in the human diet are oleate, a mono-unsaturated fatty acid, and palmitate, a saturated fatty acid. Importantly, lipotoxicity is predominantly due to saturated fatty acids like palmitate, whereas unsaturated fatty acids have been described as “lipo-protective”. A multitude of studies have demonstrated the toxic effects of palmitate in primary cardiomyocytes and cardiomyocyte cell lines. While the exact mechanism of palmitate has not yet been completely elucidated there is evidence to support the role of impaired β-oxidation [8-10], calcium dysregulation [11], loss of mitochondrial membrane potential [12], oxidative stress [13], alterations in membrane phospholipids [14], impaired cardiolipin synthesis [15], and finally loss of nuclear integrity [16].

Our aim here was to assess the qualitative nature of lipid accumulation in oleate and palmitate treated primary neonatal cardiomyocytes (NCMs) and their effects on endoplasmic reticulum (ER) stress and cell viability. ER stress results in the activation of several stress response genes including inositol requiring enzyme-1 (Ire1), and activating transcription factor-6 (Atf6), which are normally retained in the inactive state by glucose regulated protein 78 (Grp78/ Bip), an ER chaperone. Ire1 functions as an endoribonuclease which catalyzes the splicing of X-box binding protein-1 (Xbp1) mRNA. Spliced Xbp1 mRNA is translated into a nuclear transcription factor essential to the ER stress response. In addition, Atf6 release from Grp78 results in migration of this protein to the Golgi apparatus where proteases act to release the amino terminus. This amino-terminal portion of Atf6 is also a nuclear transcription factor.

Here we found distinct differences between oleate and palmitate induced lipid staining and ER stress activation in both mouse and rat NCMs thus providing mechanistic insight into the role of lipotoxicity in diabetic cardiomyopathy.

2 Methods

Preparation of fatty acid-albumin complex solutions. For proper fatty acid transport into cells, we used bovine serum albumin (BSA) dissolved in 150mM NaCl as vehicle. Sodium Oleate (dissolved in methanol) or palmitate (dissolved in 150mM NaCl solution heated to 70°C) were mixed with 0.17mM BSA/150mM NaCl solutions (6:1 molar ratio). Conjugation of oleate, or palmitate, to BSA was performed by gentle agitation at 37°C for 1 hour and conserved at -80°C. In all experiments, NCMs were treated with either oleate or palmitate for 24 hours unless otherwise indicated.

Isolation and culture of primary NCMs. Hearts were aseptically harvested from new born (1-2 day old) CD-1 mouse or Sprague-Dawley rats pups and washed with Hanks buffer solution (137mM NaCl, 5.36 mM KCl, 0.81mM MgSO₄, 5.55mM dextrose, 0.44mM KH₂PO₄, 0.34mM Na₂HPO₄, 20mM HEPES, and 50 µg/ml gentamicin). Next, ventricular tissue was isolated, cut in small pieces and washed 3 times in Hanks buffer solution. Ventricular tissue was digested by repeated incubations (for 10-20 mins at 37°C) in Hanks buffer solution containing 50U ml collagenase-2 and 0.36µM CaCl₂ until fully digested. Following each digestion round, cells were added to ice-cold fetal bovine serum (FBS). Post-digestion FBS suspensions were centrifuged at 800g for 5 min (4°C). The cell pellets were re-suspended in culture media (DMEM/ F12, 10% FBS) and plated on a 10 cm cell culture dish for 1 hour at 37°C to let cardiac fibroblasts adhere to the plate. Non-adhering cardiomyocytes were then re-plated in appropriate cell culture plates. Cells were incubated for a minimum of 120 hours with daily replacement of culture media (DMEM/F12, 2% FBS, 100µM bromodeoxyuridine). Bromodeoxyuridine was added to inhibit growth of cardiac fibroblasts. All animal experimentations were performed according to the guidelines of the Canadian Council on Animal Care and they were specifically approved by the institutional animal care committee at the Montreal Heart Institute. All experiments were carried out in rat NCMs unless otherwise indicated.

Histology. NCMs were seeded onto 18mm round coverslips coated with 2% gelatin. NCMs were treated as described and then fixed in 3% paraformaldehyde for 30 min. at room temperature. Cells were then stained with 1µM BODIPY 493/503 (Invitrogen), 1µM Rhodamine Phalloidin (invitrogen), and 1µM DAPI (Invitrogen).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments. Total RNA was isolated from NCMs. RNA extraction, cDNA synthesis and qPCR were performed using respective commercial kits (Qiagen) and Eco Ilumina real-time qPCR system. Data was analysed using the 2^(-ΔΔCt) method [17]. PCR results are presented as the expression of the indicated gene relative to an endogenous control gene (Rpl34) and normalized to the control group. Data are presented as mean ± standard
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Samples were analyzed in triplicate in three separate experiments. Primers were designed to span exons using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in supplemental table S1. Xbp1 primers do not amplify the non-spliced isoform, they only amplify the spliced Xbp1 isoform.

**Western blot analysis.** Total cellular protein was isolated from rat (or mouse where indicated) NCMs with isolation buffer (250mM Sucrose, 50mM Tris, 1µM PMSF (protease inhibitor), 1µM DTT, and Proteinase inhibitor cocktail (ROCHE)). Protein concentrations were measured by Bradford assay. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with the following antibodies: anti-Grp78 (SC-13968, Santa Cruz biotechnologies), anti-ATF6 (sc-22799, Santa Cruz biotechnologies), anti-Xbp1 (SC-7160, Santa Cruz Biotechnologies), anti-Chop (SC-7351, Santa Cruz biotechnologies), anti-Caspase3 (ab2302 (abcam)). Equal protein loading conditions were utilized for all blots, which was verified by Ponceau staining of the membrane.

**Co-immunoprecipitation.** Immunoprecipitations were carried out using protein A/G-agarose beads (Thermo Scientific). Briefly, freshly treated NCM lysate homogenates were incubated for 4 hours under continuous rotation at 4°C with anti-Grp78 antibody (SC-13968, Santa Cruz biotechnologies) in binding buffer (140 mM NaCl, 14 mM KCl, 0.1% Triton X-100 with 0.01% BSA). Simultaneously, protein A/G-agarose beads were blocked in 0.1% BSA in binding buffer for 2 h. Following this the antibody-protein complex formation was added to the agarose beads, and rotated overnight at 4°C. Samples were washed three times and eluted in 0.1 M glycine (pH 2.4). Immunoprecipitates were probed with anti-ubiquitin antibody (1/400, Santa-Cruz Biotechnology) and anti-Grp78 antibody (SC-13968, Santa Cruz biotechnologies).

**Fatty acid uptake assay.** NCMs were treated with either BSA (5µM), C14- radiolabelled oleate (100µM) or C14- radiolabelled palmitate (100µM) for 2 hours. Following this cells were washed twice with phosphate buffered saline (PBS), harvested by trypsinization and homogenized. Cell lysates were added to scintillation fluid and samples were counted with the Scintillation counter (Beckman LS6500). Data are presented as µmol/min based on the counts per minute (CPM) from isolated cells relative to CPMs from total amount of radiolabeled fatty acid that was added to the cells (divided by 120 since the cells were exposed for 2 hours).

**Triacylglyceride (TAG) assay.** Mouse NCMs were treated with BSA (n=3), 300µM (n=3) oleate or 300µM palmitate (n=3) for 24 hours and then assayed for TAG levels using the adipogenesis assay kit from Biovision (Catalog #K610-100) as per the manufacturer’s instructions. Values are presented as nmol glycerol (determined via a standard curve derived from increasing concentrations of pure glycerol) and normalized to protein concentration (determined using the bicinchoninic acid (BCA) assay) for each sample. Assays were done in duplicate.

**Cell viability assay.** Propidium iodide (PI) exclusion assays were carried out to evaluate cell viability. Briefly, NCMs were treated with palmitate (and oleate where indicated) for the indicated time points (n=4 per timepoint). After a 30 min incubation with PI at 37°C, we measured fluorescence (535nm excitation / 617nm emission) with the Synergy2 fluorescence plate reader (Bio-Tek). Assays were done in triplicate.

**Statistical analysis:** Numerical data were presented as the mean ± SE. Student’s T-test was used for two group experiments, while we performed one-way ANOVA with the Tukey post-hoc test for multiple group comparisons. P-values of <0.05 were considered statistically significant.

### Results

#### 3.1 Palmitate induces cell death of primary NCMs, which is prevented by the addition of oleate

Treatment of NCMs with 300µM palmitate caused a time dependent cell death as evidenced by increased PI staining, a marker of late cell death. The palmitate induced cell death was significantly increased at 18 hours and progressed thereafter until at least the 24-hour time point (Figure 1A). Importantly, 8 hours of palmitate did not cause cell death while there was only a trend for increased cell death following 16 hours exposure.

Because oleate has been shown to be protective against palmitate [18], we tested the effect of co-administration of various concentrations of oleate on the cytotoxic effect of palmitate. Interestingly, we found that concentrations as low as 50µM oleate were sufficient to completely abolish the cytotoxic effect of palmitate (Figure 1B).

#### 3.2 Distinct lipid staining in palmitate treated NCMs

The marked difference in cell death between oleate and palmitate prompted us to evaluate if there is a difference in lipid accumulation in NCMs caused by these two fatty acids. Excess non-metabolized fatty acids can be converted to TAG and stored in lipid droplets in
mammalian cells. To characterize the degree of oleate and palmitate induced lipid droplets in NCMs, we treated cells with either 400µM oleate or 300µM palmitate for 24 hours and then stained the cells with BODIPY 493/503, a stain for neutral lipids, as well as Rhodamine Phalloidin, an actin stain (Figure 2). Importantly we show that our NCM cultures are relatively pure as the majority of cells exhibited the striated sarcomeric staining pattern characteristic of cardiomyocytes. In agreement with the high degree of cell death following 24 hours of palmitate, we observed a loss of sarcomeric integrity in NCMs treated with palmitate (Figure 2G). We found that both oleate and palmitate led to increased BODIPY493/503 staining in NCMs. However there were some key differences in the staining pattern induced by the two different fatty acids. Firstly, oleate treated cells exhibited abundant BODIPY staining of spherical objects that were consistent with the morphology of typical lipid droplets (Figure 2D-F). On the other hand, BODIPY staining of palmitate treated NCMs was much fainter and more diffuse in appearance. (Figure 2G-I). Indeed the depicted micrographs of palmitate treated NCMs required brightness and contrast enhancement to visualize the BODIPY staining, despite being subjected to the identical staining protocol (similar results from >3 separate experiments). Secondly, while spherical stained objects could also be visualized in palmitate treated NCMs, there were also many stained objects that appeared non-spherical or irregular in shape, which is inconsistent with the morphology of lipid droplets and hence may represent non lipid droplet staining. Thus the difference in BODIPY staining between the two fatty acids suggests a difference in the chemical or physical nature of the resulting lipid accumulation.

In contrast to treatment with palmitate alone, the co-treatment of 50µM oleate and 300µM palmitate to NCMs for 24 hours resulted in a marked difference in cellular histology (Figure 2J-L). Firstly, phalloidin staining showed high integrity of sarcomeres similar to control and oleate treated NCMs. In addition, we found that oleate + palmitate co-treatment altered the BODIPY staining with an apparent decrease in the faintly diffuse staining pattern characteristic of palmitate treated NCMs and an increase in small brightly staining spherical objects characteristic of oleate treated NCMs.

Because there is a substantial degree of cell death in NCMs treated with palmitate for 24 hours, we aimed to determine if the apparent difference in BODIPY staining was due to cell death. Therefore we evaluated histology of NCMs treated with 300µM palmitate for 8 hours (Figure 3), a time point that is not associated with cell death. Indeed, at this time-point the palmitate treated NCMs exhibited sarcomeric integrity similar to control or oleate treated cells (Figure 3A,D,G). However, NCMs treated with
Figure 2. Marked differences in BODIPY staining in NCMs treated with oleate, palmitate, or oleate + palmitate for 24 hours. Images of NCMs treated with either BSA (control, A-C), 400µM oleate (D-F), 300µM palmitate (G-I), or 50µM oleate + 300µM palmitate (J-L) for 24 hours. All cells were stained with Rhodamine Phalloidin (actin stain, left column) and BODIPY 493/503 (neutral lipid stain, middle column) and DAPI (nuclear stain). Right column represents merged image of left and middle columns. All images exhibit cells at 1000x magnification.
Figure 3. Marked differences in BODIPY staining in NCMs treated with oleate, palmitate, or oleate + palmitate for 8 hours. Images of NCMs treated with either BSA (control, A-C), 400µM oleate (D-F), 300µM palmitate (G-I), or 50µM oleate + 300µM palmitate (J-L) for 8 hours. All cells were stained with Rhodamine Phalloidin (actin stain, left column) and BODIPY 493/503 (neutral lipid stain, middle column) and DAPI (nuclear stain). Right column represents merged image of left and middle columns. All images exhibit cells at 1000x magnification.
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Palmitate for 8 hours exhibited a similar staining pattern as observed for NCMs treated with palmitate for 24 hours (Figure 3H), suggesting that the altered BODIPY staining in palmitate treated NCMs was a characteristic of palmitate treatment and not an indirect result of cell death.

To verify that the altered BODIPY staining in palmitate treated NCMs was not due to alterations in lipid uptake, we measured fatty acid uptake and total cellular triglyceride levels. We found that there was no difference in uptake of radio-labeled oleate vs. palmitate (Figure 4A) and that both oleate and palmitate led to a ~3 fold increase in TAG levels (Figure 4B) confirming that both oleate and palmitate were being incorporated into the cells to similar degrees.

3.3 Palmitate induces ER stress

The altered BODIPY staining in palmitate treated cells suggested that perhaps the intracellular lipid may also be accumulating in the ER in addition to lipid droplets. Indeed, palmitate has been previously shown to cause pathological changes to the ER membrane by increasing the degree of saturation of ER phospholipids and consequent ER stress in a cardiomyoblast cell line [19]. In accordance with this, numerous studies have shown that palmitate induces ER stress, but this has not been shown in either mouse or rat primary NCMs. Therefore we aimed to characterize ER stress in NCMs. We found that palmitate caused a significant increase in mRNA levels of key ER stress mediators including Grp78, spliced Xbp1, DnaJ (Hsp40) homolog, subfamily B, member 9 (Dnajb9, a marker of Xbp1 activity), Atf4, and Atf6 in both rat and mouse NCMs (Figure 5A & Supplemental Figure S1A). Importantly, the induction of all of these ER stress markers exhibited both dose and time dependence in palmitate treated NCMs (Supplemental figure S2). In addition, we also found that palmitate induced time dependent activation of cleaved and full length Atf6 (Figure 5B).

Interestingly, Xbp1 protein (~30kDa) derived from the un-spliced Xbp1 mRNA decreased over time, indicating increased activity of Ire1. On the other hand, Xbp1 protein (~50kDa) derived from the spliced mRNA, increased over time indicating an increase in the active form of Xbp1 in palmitate treated NCMs (Figure 5C). It is important to mention that the protein derived from spliced Xbp1 mRNA is actually larger than the protein derived from the un-spliced mRNA because the splicing introduces a frame-shift which displaces the stop codon.

3.4 Palmitate induces apoptotic cell death

Severe or prolonged ER stress is believed to lead to activation of apoptotic pathways. CCAAT/enhancer binding protein (c/ebp) homologous protein (Chop) is a transcription factor implicated in ER stress induced apoptosis. Therefore we evaluated Chop expression and activation in palmitate treated NCMs. We found significantly increased mRNA levels of Chop in NCMs (Figure 5A). Furthermore, the

Figure 4. Oleate and palmitate exhibit similar uptake rates and intracellular lipid accumulation in NCMs. (A) Graph demonstrating the µmol fatty acid uptake/minute from cells treated with C14- radiolabelled oleate (100µM) or C14- radiolabelled palmitate (100µM) for 2 hours. (B) Graph demonstrating significant increase in intracellular triglyceride levels in mouse NCMs treated with 300µM oleate (n=3) or 300µM palmitate (n=3) compared to cells treated with BSA (control, n=3). * Indicates p<0.05 vs. control NCMs. # Indicates p<0.05 vs. palmitate treated NCMs.
Figure 5. Palmitate induces ER stress in NCMs. (A) Graph demonstrating the mRNA levels of 6 key markers and mediators of ER stress in NCMs treated with either BSA (control), 400µM oleate or 300µM palmitate for 24 hours (n= 9/group). (B) Western blot demonstrating the time dependent increase in full length and cleaved Atf6 protein following administration of 300µM palmitate for the indicated time points. NCMs were also treated with tunicamycin (0.1µg/ml) for 24 hours as a positive control. (C) Western blot demonstrating the time dependent increase in Xbp1 protein derived from the spliced mRNA (~54kDa) and the time dependent decrease in Xbp1 protein derived from the unspliced mRNA (~30kDa) in NCMs treated with 300µM palmitate for the indicated times. NCMs were also treated with tunicamycin (0.1µg/ml) for 24 hours as a positive control. (D) Graph demonstrating the mRNA levels of 6 key markers and mediators of ER stress in NCMs treated with 300µM palmitate, or 50µM oleate + 300µM palmitate for 24 hours (n= 3/group). * Indicates p<0.05 vs. control NCMs. # Indicates p<0.05 vs. palmitate treated NCMs.
palmitate mediated induction of Chop mRNA exhibited both time and dose dependency (Supplemental figure S2). Because Chop is active in the nucleus we evaluated nuclear levels of Chop protein. Interestingly, we show a dose dependent increase of Chop protein levels in the nucleus containing subcellular fraction of palmitate treated NCMs (Figure 6A). In addition we also demonstrate a time dependent increase of total Chop protein in palmitate treated NCMs (Figure 6B).

Chop activation should lead to induction of pro-apoptotic pathways. Therefore we assessed the degree of caspase-3 activation in palmitate treated NCMs. Indeed, we found that caspase-3 cleavage was significantly increased after 16 and 18 hours of palmitate exposure in NCMs compared to both oleate treated and control NCMs (Figure 6C). Importantly, the time point of Caspase-3 cleavage coincides with the commencement of cell death in these cells.

3.5 The protective effect of oleate is associated with a decrease in ER stress

Previous studies have demonstrated that unsaturated fatty acids, like oleate, prevent the toxicity associated with saturated fatty acids. In agreement with this, we showed that as little as 50µM oleate can prevent palmitate induced cell death. Expectedly, oleate alone, did not induce the mRNA levels of any of the ER stress markers in rat or mouse NCMs (Figure 5A and Supplemental Figure S1B). Furthermore, co-treatment of NCMs with 50µM oleate + 300µM palmitate was sufficient to significantly attenuate ER stress compared to NCMs treated with palmitate alone (Figure 5D). Altogether these data demonstrate that palmitate but not oleate, induces ER stress and apoptosis in primary NCMs.

3.6 Palmitate causes increased ubiquitination of Grp78

Grp78 is an important ER chaperone and a key marker of ER stress. In accordance with this we show that palmitate significantly induces Grp78 mRNA levels (Figure 7A), and this effect is both dose and time dependent (Supplemental Figure S2A-B). However, surprisingly Grp78 protein levels were only marginally elevated in palmitate treated rat NCMs (Figure 7B). As a positive control we evaluated the effect of tunicamycin, a known ER stressor, on Grp78 mRNA and protein expression. We found that tunicamycin (0.1µg/ml) substantially increased Grp78 mRNA (>10-fold, Figure 7A) and protein levels (Figure 7B). The fact that palmitate increased Grp78 mRNA but not protein suggested that perhaps the protein was being degraded. To test if this was via the ubiquitin-proteasome pathway we evaluated the degree of Grp78 ubiquitination in palmitate treated NCMs vs. untreated control and vs. tunicamycin treated cells. We immunoprecipitated Grp78 and then blotted for both Grp78 (to demonstrate equivalent loading of Grp78 immuno-precipitates) and for ubiquitin (Figure 7C). Semi-quantitative analysis of the immunoblots demonstrated that the Grp78 immuno-precipitate isolated from NCMs treated with 200µm palmitate exhibited a significantly increased ubiquitin signal compared to either control, tunicamycin, or low dose (50µM) palmitate treated NCMs (Figure 7C-D).

4 Discussion

Hearts of patients with diabetic cardiomyopathy have increased lipid levels compared to healthy non-diabetic patients [4,5,7,20,21]. The resulting lipotoxicity is likely to contribute to the increased cardiac events, heart failure and death in this patient population. Palmitate, a saturated fatty acid, is one of the most abundant lipids in human diets, and has been shown to cause lipotoxicity in a variety of cell types. In contrast, oleate, another major lipid in human diets, is non-toxic and has even been shown to reverse toxicity induced by palmitate.

While there is a large body of evidence demonstrating the toxic effects of palmitate in vitro, no studies have investigated the effect of oleate and palmitate specifically on lipid accumulation in NCMs. Here we show that oleate and palmitate led to distinctive BODIPY staining in spontaneously contracting mammalian cardiomyocytes. Specifically, BODIPY staining of oleate treated NCMs resulted in the appearance of distinct spherical objects, which is consistent with the presence of lipid droplets. On the other hand, BODIPY staining in palmitate treated NCMs was much fainter and less distinct. Although, spherical objects could be discerned in palmitate treated NCMs suggesting the presence of lipid droplets, they were much fainter than in oleate treated NCMs. In addition, there was also a large degree of staining of non-spherical or irregularly shaped objects, which is inconsistent with the morphology of lipid droplets. It is unlikely that this faint diffuse staining is background staining since it was not observed in control NCMs treated with BSA.

Interestingly we also show that 50µM oleate completely abolished palmitate induced cell death and ER stress; and this was associated with a change in lipid staining. Indeed co-administration of oleate and palmitate to NCMs attenuated the appearance of the faint
Figure 6. Palmitate induces apoptotic cell death in NCMs. (A) Western blot demonstrating the increase in Chop protein in the nuclear subcellular fraction of NCMs treated with palmitate for 24 hours at the indicated concentrations. Tunicamycin (0.5µg/ml for 24 hours) treated NCMs were used as positive control. (B) Western blot demonstrating time dependent increase in Chop protein in whole cell lysates of NCMs treated with 300µM palmitate for the indicated times. Tunicamycin (0.5µg/ml for 24 hours) treated NCMs were used as positive control. (C) Western blot and graph demonstrating increased levels of cleaved caspase-3 in NCMs treated with 300µM palmitate for the indicated time points compared to either BSA (Control), or 400µM oleate. * indicates p<0.05 vs. control and oleate treated NCMs.
Figure 7. Palmitate causes the ubiquitination of Grp78 protein in NCMs. (A) Both palmitate and tunicamycin significantly induce Grp78 mRNA levels. (B) Western blot demonstrating that tunicamycin strongly up-regulates Grp78 protein levels but palmitate does not. (C) Western blots demonstrating that Grp78 immuno-precipitated from NCMs treated with 200µM palmitate exhibit significantly increased ubiquitination. The IgG bands in (C) represents the antibody that is used to immunoprecipitate Grp78. (D) Graph demonstrating results from densitometry analysis of ubiquitin immuno-blot in (C). * indicates p<0.05 vs. control. # indicates p<0.05 vs. palmitate. † indicates p<0.05 vs. all other conditions.
diffuse staining pattern characteristic of palmitate only treated NCMs. Instead we observed the staining of small spherical objects similar to cells treated with oleate alone. The cause for this alteration in lipid staining is currently unknown but may be related to the increased induction of TAG formation by oleate compared to palmitate. Indeed, it has been previously suggested that oleate attenuates palmitate-induced lipotoxicity by inducing the formation of TAG, thus sequestering saturated fatty acids into inert moieties [40]. Specifically, the incorporation of saturated fatty acids into TAG, and subsequent storage of this TAG in lipid droplets, may reduce their bioavailability. This attenuated bioavailability is thus expected to decrease their subsequent metabolism into toxic metabolites and/or their incorporation into phospholipids and hence attenuate ER stress. In support of this, oleate has been shown to be a better substrate for TAG synthesis than palmitate in skeletal muscle cells [41]. Here we show that oleate produces brightly staining objects consistent with the morphology of lipid droplets, while palmitate produces a staining pattern that is fainter and more diffuse with the presence of irregularly shaped objects. This suggests that perhaps BODIPY has higher affinity for oleate derived TAG compared to DAG produced by palmitate. The nature of the irregularly shaped stained objects and the cause for the faint/diffuse staining pattern will be the focus of future studies. However, a potential caveat of these findings is that perhaps oleate and palmitate are differentially stained by BODIPY and hence the difference in staining pattern is due to differences in affinity of the dye for the respective fatty acids rather than differences in actual lipid accumulation.

The altered lipid staining in palmitate treated NCMs suggested to us that perhaps lipid may not be completely stored in lipid droplets resulting in potential accumulation of lipid in the ER. The accumulation of lipid in the ER has major implications for lipotoxic mechanisms. Indeed, lipid accumulation in the ER can perturb ER membrane physiology thereby leading to ER stress [19, 22]. ER stress has been shown to be induced by palmitate in different cell types [13, 19, 23-29], but this has not been previously demonstrated in primary NCMs. Altogether, we show that palmitate causes a robust induction of markers of ER stress with ensuing apoptosis in NCMs and this is associated with altered lipid staining patterns.

ER stress is an important pathological process in a variety of cardiovascular disorders including diabetic cardiomyopathy [30-32]. ER stress results in activation of a compensatory mechanism referred to as the unfolded protein response (UPR). As the name suggests, it can occur as a result of a build-up of unfolded proteins, but also results from other stress stimuli including calcium dysregulation [33], and membrane perturbations [19, 22]. Here we demonstrated activation of the UPR in palmitate treated mouse and rat NCMs. Indeed, we show significant increases in spliced Xbp1 mRNA (a marker of Ire1 activity) as well as full length and cleaved Atf6 protein levels in NCMs treated with 300µM palmitate. Of note, palmitate did not change the ratio of cleaved to un-cleaved Atf6, suggesting that palmitate induced ER stress did not cause an increase in the activity of the S1 and S2 proteases of the Golgi apparatus responsible for cleavage of Atf6. In addition, we showed that palmitate significantly induced active Xbp1 protein in NCMs. Finally we also demonstrated that palmitate induced significant increases in the expression of Dnajb9 mRNA (a marker of Xbp1 activity) as well as Grp78, Atf4 and Atf6 mRNA levels. Importantly, palmitate induction of all of the latter factors exhibited dose and time dependency.

The ER stress-dependent apoptotic pathway is believed to occur through the activation of Chop [34]. Atf6, a mediator of ER stress, has been shown to induce Chop expression [35]. This is in accordance with our data demonstrating increased expression and activation of both Atf6 and Chop in palmitate treated NCMs. While Chop up-regulation is consistently associated with apoptosis [36, 37], the underlying mechanism has not been fully elucidated. There is evidence to suggest that Chop down-regulates bcl-2 [38], and/or up-regulates death receptors [39]. We show here that palmitate leads to significant cell death after 18 hours exposure which is preceded by cleavage of caspase-3 at 16 hours suggesting that the mechanism of cell death is at least partially apoptotic.

It is important to bear in mind that the UPR is actually a compensatory response aimed at restoring ER function in part by up-regulation of ER chaperones. Indeed loss of key UPR chaperones results in cell death [42, 43], whereas their overexpression is protective [44, 45]. Interestingly, we found that palmitate treated NCMs exhibited only marginally elevated Grp78 protein levels despite a prominent induction of Grp78 mRNA levels. This suggests that there is an important post-transcriptional regulation of Grp78 in palmitate treated NCMs. In fact, we show significantly increased ubiquitination of Grp78 following high dose palmitate, suggesting this protein is actively degraded by the ubiquitin-proteasome pathway in palmitate treated NCMs. Loss of this important chaperone likely contributes to palmitate induced cell death. Palmitate has been shown to induce the ubiquitin-proteasome pathway via PKC activation in hepatocytes leading to degradation of anti-apoptotic proteins and subsequent lipoapoptosis [46]. Future studies are
required to determine whether this is the mechanism for ubiquitination of Grp78 in NCMs.

Here we aimed to characterize the effect of palmitate in NCMs. We found that palmitate led to significant increases in intracellular lipid accumulation, ER stress, and cell death. However the qualitative nature of the BODIPY staining in palmitate treated NCMs differed markedly from that of the oleate treated NCMs which may be due to differences in their inherent capacity to form DAG vs. TAG. Further studies are required to fully characterize the nature of palmitate induced lipid accumulation in cardiomyocytes and how this translates into ER stress.

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## Supplemental Table S1.

| Gene               | Primer sequence                                                                 |
|--------------------|---------------------------------------------------------------------------------|
| mAtf4-F            | ATG GCC GGC TAT GGA TGA TG                                                      |
| mAtf4-R            | TCT GGC ATG GTT TCC AGG TC                                                      |
| mAtf6a-F           | ACC ATA GCA AGC AGC CAC A                                                       |
| mAtf6a-R           | CTG GAA TGG GAC CAC CTG AC                                                      |
| mChop F            | GAG GAG GAA GAG CAA GGA AGA AC                                                  |
| mChop R            | TTC TCC TTC ATG GTG TGC TTC C                                                  |
| mDnajb9-F          | CGC CCT GTG GCC CTG ACT TG                                                      |
| mDnajb9-R          | AGC TTT CAG GGG CAA ACA GCC A                                                  |
| mGrp78-F           | CGA TAC TGG CCG AGA CAA CA                                                     |
| mGrp78-R           | GAC GAC GTG TCT GTG CTC AC                                                     |
| mHerp F            | GGC ATC CCT GAG GGC AGT CG                                                     |
| mHerp R            | TCA GTG GGG CCT TGA GGC GA                                                     |
| mRpl34 F           | AGC ACC TAA ATC TGC ATG TGG CG                                                 |
| mRpl34 R           | TAA GGA AAG CCC GCT TGA TCC TG                                                 |
| mXbp1 F (primer spans the splice site and therefore only amplifies spliced Xbp1) | TGA GTC CGC AGC AGG TGC A                                                    |
| mXbp1 R            | AGG CAA CAG TGT CAG AGT CC                                                     |
| rAtf4 F            | TTC CGG GAC AGA TTG GAT GTT GGA                                               |
| rAtf4 R            | CAT GTG TCA TCC AAC GTG GCC AAA                                               |
| rAtf6-F            | GTA CTG AGG AGA CAG CG                                                         |
| rAtf6-R            | GCC TCT GTG TCT CTG ACA CC                                                     |
| rChop F            | AGT CTC TGC CCT TGC CCT TTG AG                                                 |
| rChop R            | TGC AGG GTC AAG AGT GAA GAA                                                  |
| rGrp78-F           | TTC CGA GGA ACA CTG TGG TG                                                     |
| rGrp78-R           | GTC AGG GTG CGT TCA CCT TC                                                     |
| rDnajb9 F          | TTG GTC AGA AGA ACA ACA CTC GG                                                |
| rDnajb9 R          | GTA CTG TGC GTC GAT TGG TGC TA                                                 |
| rRpl34-F           | TGC TGT GAG AGC CAA AGT CCT CA                                                 |
| rRpl34-R           | TAA GGA AAG CCC GCT TGA TCC TG                                                 |
| rXbp1-F (primer spans the splice site and therefore only amplifies spliced Xbp1) | TGAGTCGGCAGCAGGTTGCA                                                        |
| rXbp1 R            | GCAATCTGGACAAAGTTGGACCC                                                        |