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Physiological and pathophysiological concentrations of fatty acids induce lipid droplet accumulation and impair functional performance of tissue engineered skeletal muscle

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Abstract

Fatty acids (FA) exert physiological and pathophysiological effects leading to changes in skeletal muscle metabolism and function, however, in vitro models to investigate these changes are limited. These experiments sought to establish the effects of physiological and pathophysiological concentrations of exogenous FA upon the function of tissue engineered skeletal muscle (TESkM). Cultured initially for 14 days, C2C12 TESkM was exposed to FA-free bovine serum albumin alone or conjugated to a FA mixture (oleic, palmitic, linoleic, and α -linoleic acids [OPLA] [ratio 45:30:24:1%]) at different concentrations (200 or 800 μ M) for an additional 4 days. Subsequently, TESkM morphology, functional capacity, gene expression and insulin signaling were analyzed. There was a dose response increase in the number and size of lipid droplets within the TESkM ($p < .05$). Exposure to exogenous FA increased the messenger RNA expression of genes involved in lipid storage (perilipin 2 [$p < .05$]) and metabolism (pyruvate dehydrogenase lipoamide kinase isozyme 4 [$p < .01$]) in a dose dependent manner. TESkM force production was reduced (tetanic and single twitch) ($p < .05$) and increases in transcription of type I slow twitch fiber isoform, myosin heavy chain 7, were observed when cultured with 200 μ M OPLA compared to control ($p < .01$). Four days of OPLA exposure results in lipid accumulation in TESkM which in turn results in changes in muscle function and metabolism; thus, providing insight into the functional and mechanistic changes of TESkM in response to exogenous FA.

KEYWORDS

contraction, insulin sensitivity, lipids

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1 | INTRODUCTION

Skeletal muscle is a major site of carbohydrate and lipid utilization, where efficient selection between these two substrates is paramount in maintaining metabolic homeostasis (Stinkens et al., 2015). The storage of fatty acids (FA) as triglycerides in skeletal muscle, which are referred to as intramyocellular lipids (IMCL), are elevated in skeletal muscle of trained endurance athletes (Amati et al., 2011). These IMCL provide an important energy source (Amati et al., 2011; Meex et al., 2009), yet paradoxically are associated with metabolic diseases, such as obesity and Type 2 diabetes (Jacob et al., 1999).

Dietary FA and their impact upon skeletal muscle IMCL accumulation and substrate metabolism is a highly debated area (Dirks et al., 2020; Goodpaster, 2020). Both rodent and human studies have shown that diets rich in FA can increase IMCL accumulation (Bachmann et al., 2001; Whytock et al., 2020) which can impact whole body metabolism, insulin sensitivity and glucose uptake (Krssak et al., 1999; Parry et al., 2019; Savage et al., 2019).

In addition, rodent models have shown a reduction in skeletal muscle quality and force production following high fat diet feeding (Hurst et al., 2019; Tallis et al., 2017). However, evidence that this translates to humans is lacking, although a relative reduction in muscle force production in patients who are obese compared to their lean peers has been observed (Abdelmoula et al., 2012; Hulens et al., 2001; Maffiuletti et al., 2007). Therefore, understanding the role of FA upon skeletal muscle metabolism and function using *in vivo* and *in vitro* experiments is important for improving skeletal muscle health and performance.

In vitro experiments, which are often in monolayer using cell lines and primary skeletal muscle cells, have established several short and long term adaptations to exogenous FA such as increased lipid oxidation (Meex et al., 2015), mitochondrial biogenesis (Turner et al., 2007) and the formation of lipid droplets (LD) as IMCL (Toledo et al., 2018). However, understanding the impact of exogenous FA upon skeletal muscle physiology and function in a monolayer culture system is challenging. Therefore, the use of advanced cell culture models can provide an alternative system which addresses some of the limitations of monolayer culture such as the ability to investigate the functional response of skeletal muscle to exogenous FA *in vitro*.

Advanced cell culture models, such as three dimensional tissue engineered skeletal muscle (TESkM), have provided physiologists with a tool to further enhance understanding of skeletal muscle development and maturation, its interaction with an extracellular matrix, and the impact of metabolic diseases, such as type 2 diabetes (Acosta et al., 2020; Cheng et al., 2014; Hinds et al., 2011; Kondash et al., 2020). Our research group, and others, have utilized TESkM to investigate how changes in the nutritional *milieu* can augment or attenuate skeletal muscle adaptation on both a molecular and functional level (Khodabukus & Baar, 2015; Martin et al., 2017).

Despite the importance of FA in skeletal muscle metabolism (Badin et al., 2013), quite how physiological and pathophysiological concentrations of FA influence TESkM function is yet to be defined. In order to offer some insight into these mechanisms, the TESkM model developed

by our research group (Capel et al., 2019), was exposed to physiological and pathophysiological concentrations of FA which have previously been shown to induce lipid accumulation in non-adipose cell lines (Green et al., 2015; Gunn et al., 2020; Hodson et al., 2008). This allowed investigation of the impact of FA overload upon LD formation, gene expression, insulin sensitivity, and functional capacity in TESkM.

2 | METHODS

2.1 | Skeletal muscle cell culture

C2C12 skeletal muscle myoblasts cells (ECACC 91031101, all below passage 10) were cultured in growth medium (GM); composed of high glucose Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific), supplemented with 20% fetal bovine serum (Pan Biotech Ltd), and 1% penicillin/streptomycin (P/S) (Thermo Fisher Scientific). Cells were incubated in a 5% CO₂ humidified atmosphere at 37°C until 80%–90% confluence was attained, before being detached from the culture vessel using trypsin–EDTA (Sigma-Aldrich) and counted in preparation for the formation of TESkM.

2.2 | Formation of tissue engineered skeletal muscle (TESkM)

TESkM were generated using C2C12 myoblasts cells as outlined previously by our group (Capel et al., 2019). TESkM were formed by the addition of 65% vol/vol Type I rat tail collagen (First Link; dissolved in 0.1 M acetic acid, protein at 2.035 mg/ml), 20% vol/vol Corning® Matrigel® Matrix (Thermo Fisher Scientific), with 10% vol/vol of 10X minimal essential medium (Thermo Fisher Scientific). This solution was subsequently neutralized by the addition of 5 and 1 M sodium hydroxide dropwise, until a color change to cirrus pink was observed. The cells were added in a 5% vol/vol GM solution, before being transferred to pre-sterilized 3D printed inserts to set for 10–15 min at 37°C. All relevant.stl files for the designs contained within this manuscript are freely available to download at the following domain: https://figshare.com/projects/3D_Printed_Tissue_Engineering_Scaffolds/36494. Constructs were cultured for 4 days in GM, before being changed to differentiation media (DM) (DMEM, 2% horse serum (Thermo Fisher Scientific), 1% P/S) for a further 10 days.

2.3 | Fatty acid conjugation and fatty acid exposure protocol

After a total of 14 days in culture, TESkM constructs were treated with 0.25% FA-free bovine serum albumin (FAF-BSA) alone or conjugated to oleic, palmitic, linoleic, and α -linoleic acid (OPLA) (physiological ratio 45:30:24:1%) at a concentration of 200 and 800 μ M, as used previously to induce lipid droplet accumulation in a

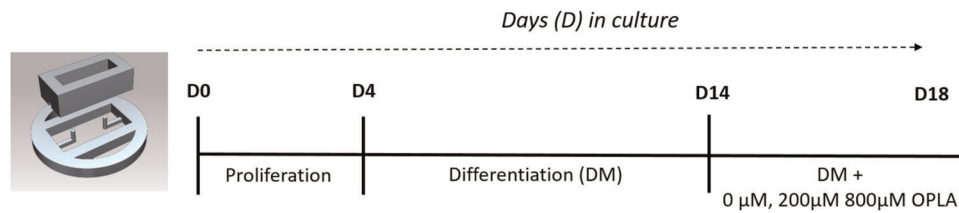


FIGURE 1 Experimental outline of development and exposure to exogenous fatty acid (FA) overload in 3D tissue engineered skeletal muscle. DM, differentiation media; OPLA, oleic, palmitic, linoleic, and α -linoleic acid

non-adipose cell line (Gunn et al., 2020). OPLA was supplemented into high glucose, phenol red free DMEM with 2% horse serum, 4 mM L-glutamine, 1 mM sodium pyruvate, and 1% P/S and were in addition to the concentrations of albumin and triglycerides contained in the horse serum. The control condition contained a concentration of FAF-BSA which was matched to the 800 μ M condition. Constructs were incubated for 4 days with the media changed 48 h before termination of the experiment (Figure 1).

2.4 | Acute insulin stimulation

The culture media was changed 1 h before acute insulin stimulation protocol. Constructs were washed twice in Krebs Ringer HEPES (KRH) buffer (10 mM HEPES pH 7.4, 138 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄, 5 mM glucose, and 0.05% BSA) and incubated in KRH buffer for 30 min twice before being stimulated for 30 min in KRH with or without insulin (100 nM).

2.5 | Protein quantification and immunoblotting

Protein was extracted using RIPA lysis buffer (Sigma-Aldrich) containing a protease and phosphatase inhibitor cocktail mix (Thermo Fisher Scientific). Lysis buffer was added to constructs before being mechanically lysed using a tissue lyser (Qiagen). Lysates were placed on a rotor for 1 h at 4°C to before being centrifuged at 12,000 g to further dissolve the cellular and pellet insoluble material. Protein concentrations were determined using the Pierce 660 nm protein assay (Thermo Fisher Scientific), and an equal amount of protein was mixed with 4X Laemmli buffer (Bio-Rad), boiled for 5 min at 95°C and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (Thermo Fisher Scientific) and blocked for 1 h at room temperature in 5% bovine serum albumin (BSA) or 5% blocking grade milk (Bio-Rad) in Tris-buffered saline with Tween-20 (TBST), before being incubated with the primary antibody overnight at 4°C in BSA or milk. Primary antibodies used for analysis were, Akt (1:2000, #9272), phospho Akt (Ser⁴⁷³) (1:2000, #4060), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000, #2118) GSK-3 β (1:2000, #9315) and phosphor GSK-3 β (Ser⁹) (1:2000, #9336) (Cell Signaling Technology). Following overnight incubation, membranes were washed in TBST and incubated with anti-rabbit (GE healthcare)

horseradish peroxidase-conjugated secondary antibody (1:5000) in blocking grade milk (Bio-Rad). Proteins were visualized using chemiluminescence substrate (Bio-Rad) and band densities were quantified using Quantity One image analysis software (Quality One 1-D analysis software version 4.6.8). Following visualization of phosphorylation proteins, membranes were washed in TBST and incubated in stripping buffer (Thermo Fisher Scientific) before being blocked and probed as outlined above for their corresponding total proteins. Phosphorylation was normalized to total protein with the exception of phospho AS160 (Ser⁵⁸⁸), which was normalized to GAPDH.

2.6 | RNA extraction and qPCR analysis

RNA was extracted using TRI Reagent® (Sigma-Aldrich) according to manufacturer's instructions and quantified using UV spectroscopy (NanoDrop; Thermo Fisher Scientific). Gene expression was analyzed by one-step reverse transcription-quantitative polymerase chain reaction (Quantifast SYBR Green Mix; Qiagen) using a Viia 7 thermocycler (Applied Biosystems). Each reaction consisted of 5 ng of RNA in a final 10 μ l reaction volume with master mixes made according to the manufacturer's instructions (Qiagen) and using primers sequences outlined in Table S1. Fluorescence was detected after every cycle (40 cycles) and data was analyzed using the 2^{- $\Delta\Delta$ Ct} method (Schmittgen & Livak, 2008) using RNA polymerase II beta (*polr2b*) as an endogenous control gene.

2.7 | Muscle function assessment

TESkM constructs were washed twice in phosphate-buffered saline (PBS) and one end of the construct was removed from the mold. The loose end of the construct was then attached to the force transducer (403A Aurora force transducer, Aurora Scientific) using the eyelet present in the construct. The construct was covered (4 ml) with KRH buffer solution and wire electrodes were positioned either side of the construct to allow for electric field stimulation. Impulses were generated using LabVIEW software (National Instruments) connected to a custom-built amplifier. Maximal twitch force was determined using a single 3.6 V/mm, 1.2 ms impulse and maximal tetanic force was measured using a 1 s pulse train at 100 Hz and 3.6 V/mm. Data was acquired using a Powerlab system (ver. 8/35) and associated software (Labchart 8, AD Instruments).

2.8 | Cryosectioning

Following completion of the muscle function assessment, constructs from each condition were washed in ice cold PBS before being fixed in 3.7% formaldehyde (Sigma-Aldrich). Fixed constructs were dehydrated in 20% sucrose (wt/vol) in Tris-buffered saline (TBS) for 24 h. Constructs were then embedded in Tissue-Tek® optimum cutting temperature mounting medium and frozen in isopentane at -80°C for a minimum of 2 h. Once frozen, $15\mu\text{m}$ sections were prepared, using standard cryostat protocols, perpendicular to the longitudinal axis of the construct and mounted onto SuperFrost Plus™ Adhesion Slides (Thermo Fisher Scientific) for immunocytochemistry.

2.9 | Immunocytochemistry

Sections were blocked and permeabilised (TBS, 5% goat serum, 0.1% triton X-100) for 1 h at room temperature before being incubated overnight at 4°C in blocking solution containing myosin heavy chain primary antibody at a concentration of 1:500 (MF 20 was deposited to the DSHB by Fischman, D.A. [DSHB Hybridoma Product MF 20]). Samples were washed with TBS before being incubated for 1 h at room temperature with secondary fluorescent conjugated antibody (1:500, Invitrogen Alexa Fluor™ 647 goat antimouse), counterstained with DAPI (1:2000) and Nile Red (NR) solution (1:1000, 1 mg/ml in DMSO) to visualize LD. Slides were then washed and mounted using Fluoromount™ mounting medium (Sigma-Aldrich). Images were collected on a Leica DM2500 microscope using Leica Application Suite X software. Fiji 1.52e (Schindelin et al. 2012) was used for image analysis, and an in-house macro performed automated myotube and lipid analysis macro which was developed and has previous been used by our group (Aguilar-Agon et al., 2019; Fleming et al., 2020) un blinded to the researcher.

To establish if the LD observed by NR were intracellular or extracellular, colocalisation analysis was conducted with MHC images. Images were converted to 16-bit before co-localization analysis which was conducted using the FIJI plugin, Just Another Co-Localization Plugin (Bolte & Cordelières, 2006). The threshold function was used to subjectively determine the myotubes and lipid droplets. Threshold corrected Manders' coefficient using five images per condition from three independent experiments.

2.10 | Statistical analysis

Data is presented as mean \pm SEM from 3 to 5 constructs per condition. Statistical analysis was performed using IBM® SPSS® Statistics version 23. One-way analysis of variance (ANOVA) with Bonferroni post hoc correction was used to identify significant differences between conditions. Statistical significance was set at $p < .05$.

3 | RESULTS

3.1 | Increases in lipid droplet size and number in TESkM following exposure to increasing concentrations of OPLA

To establish the presence of lipids in TESkM following exposure to OPLA, cross sections were probed with NR to identify LD (Figure 2). Image analysis showed that with increasing media concentration of OPLA, there was a dose response in the number of droplets present in the construct, with both 200 and $800\mu\text{M}$ concentrations significantly increasing the number of droplets ($p < .05$; Figure 3a). The average LD area was also increased in a dose dependent manner,

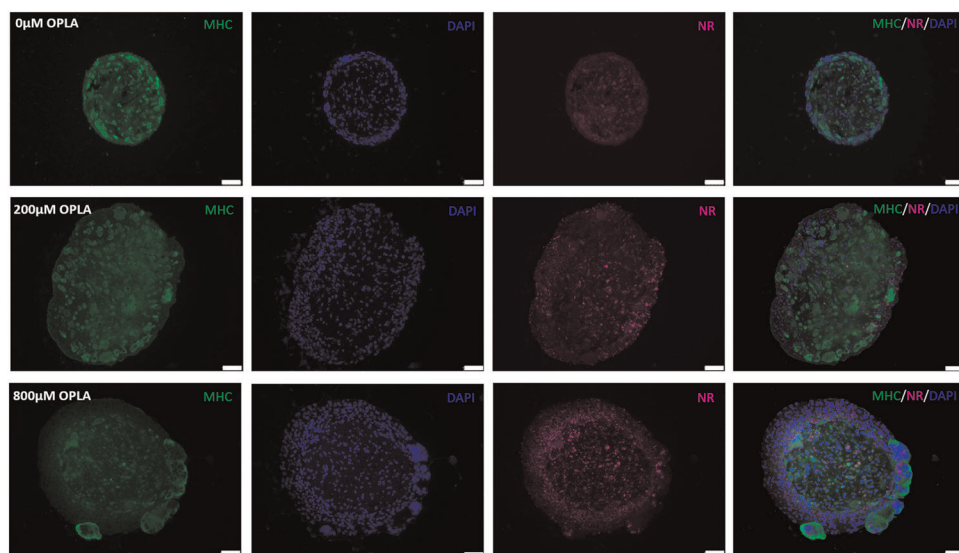


FIGURE 2 Exposure to exogenous FA overload induces intracellular lipid droplet formation in 3D tissue engineered skeletal muscle. Representative cross sections of tissue engineered skeletal muscle constructs (TESkM) cultured with ($200\mu\text{M}$ OPLA and $800\mu\text{M}$ OPLA) or without ($0\mu\text{M}$ OPLA) endogenous lipids for 4 days probed for pan myosin heavy chain (MHC) (Green), lipid droplets (Nile Red [NR], Magenta) and nuclei (DAPI, blue). Scale bar = $50\mu\text{m}$. DAPI, 4',6-diamidino-2-phenylindole; FA, fatty acid; OPLA, oleic, palmitic, linoleic, and α -linoleic acid

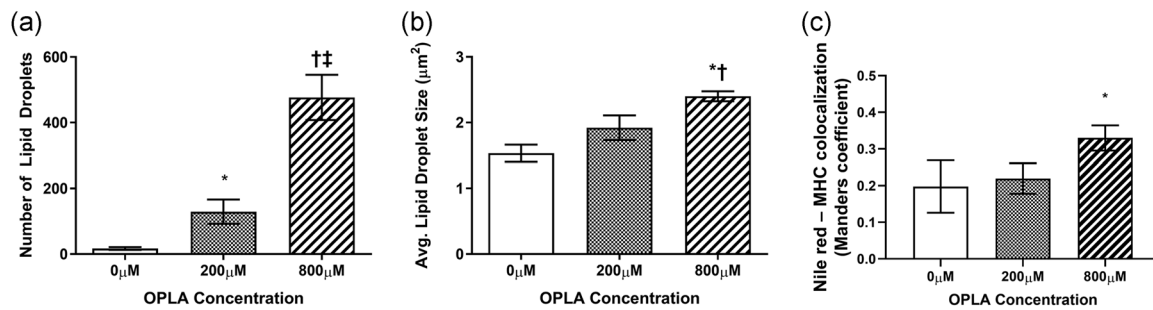


FIGURE 3 Exposure to exogenous FA overload increases the presence of intracellular lipid droplet in 3D tissue engineered skeletal muscle. Lipid droplet (LD) (Nile Red) image analysis (a, number of LD; b, average LD area) from skeletal muscle tissue engineered construct cross sections. (c) Colocalisation analysis using Manders' coefficient of Nile Red which overlapped with MHC. Data is presented as mean \pm SEM. Significantly different versus 0 μ M, (* p < .05, † p < .01), significantly different versus 200 μ M (‡ p < .05). FA, fatty acid; MHC, myosin heavy chain; OPLA, oleic, palmitic, linoleic, and α -linoleic acid

with only the 800 μ M concentration being significantly increased compared to 0 and 200 μ M, respectively (p < .05; Figure 3b).

To establish if the lipid droplets were intracellular or extracellular, colocalisation analysis was performed using a macro which has been previously published (Bolte & Cordelières, 2006). Using a threshold value to outline lipid droplets and myotubes, the Manders' coefficient of lipid droplets which overlapped with MHC increased with OPLA concentrations (M_1 0.197, 0.219, and 0.326 for 0, 200, and 800 μ M conditions, respectively) with a significant difference between 0 and 800 μ M conditions (p < .05; Figure 3c).

3.2 | Increased TESkM construct size but reducing in MHC coverage following exposure to increasing concentrations of OPLA

Exposure to exogenous FA did not alter the average number of myotubes per construct or average crosssectional area of the myotubes (p > .05; Figure 4a,b). However, on average the overall TESkM construct cross sectional area increased in response to 200 μ M OPLA (p < .05; Figure 4c) and to an extent in 800 μ M compared to control (p = .06). Consequently the MHC coverage, which was defined as the percentage area of the construct expressing MHC, and average myotubes per mm², were lower in response to both concentrations of OPLA (p < .001; Figure 4d,e).

3.3 | Attenuated TESkM force production with the addition of OPLA

Using a TESkM system enabled investigation of the functional capacity of skeletal muscle tissue to be investigated in response to OPLA supplementation. Increasing concentrations of OPLA resulted in a significant reduction in absolute maximal force generation (tetanic force) when exposed to 200 μ M compared to control (p < .05), with a trend toward a (p = .05) reduction in force of TESkM following 800 μ M compared to control (Figure 5a). Single contraction (twitch)

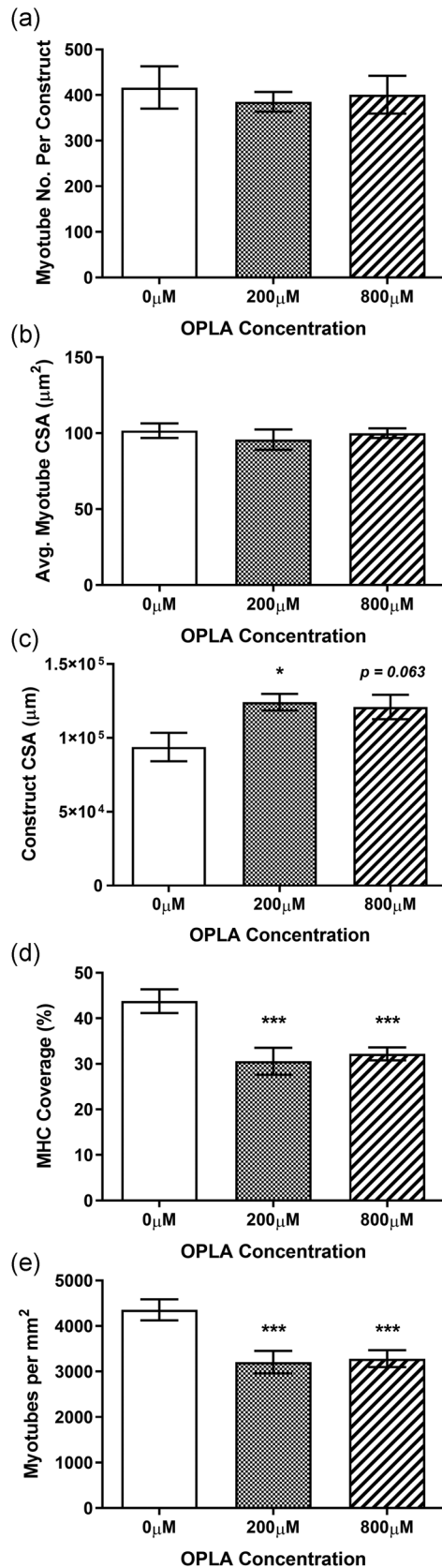
force production showed a similar pattern to tetanic force responses and was significantly lower when exposed to 200 μ M (p < .05) and had a tendency (p = .05) to be lower in the higher concentration of OPLA (Figure 5b).

There were no differences in the messenger RNA (mRNA) expression of *Myh1* (Type IIx) or *Myh2* (Type IIa) fast twitch isoforms between conditions (p > .05). The slow twitch isoform, *Myh7*, increased in both conditions, with a two-fold difference between 0 and 200 μ M OPLA (p < .001). The mRNA expression of the *Myh3* (embryonic) was lower between 200 μ M compared to 0 μ M OPLA (p < .05), with *Myh8* (Perinatal) expression elevated in 200 μ M compared to 0 μ M (p = .06) and significantly higher compared to constructs exposed to 800 μ M OPLA (p < .05; Figure 5c).

3.4 | OPLA modulates expression of fatty acid storage and oxidation regulatory mRNAs

Following 4 days of exposure to the different concentrations of OPLA, the mRNA expression of markers that regulate lipid metabolism were analyzed. An increase in the concentration of OPLA led to a significant (p < .01) increase in mRNA expression of *Pdk4*. The lipid droplet marker, *Pnpla2* was significantly (p < .05) increased following exposure to 200 μ M OPLA compared to control, however, no differences between these conditions were observed compared to exposure of 800 μ M OPLA (p > .05; Figure 6a). The increase in OPLA concentrations did result in a significant (p < .01) increase in the expression of LD marker Perilipin 2 (*Plin2*) over control, with no differences in the expression of Perilipin 5 (*Plin5*) mRNA between conditions (p > .05). Sterol regulatory element binding transcription factor 1 (*Srebp1f1*) expression was lower in constructs incubated in 800 μ M OPLA (p = .09), while *cd36* was not different between conditions (Figure 6a).

In addition, constructs were analyzed for the mRNA expression of the Peroxisome proliferator-activated receptor (*Ppar*) isoforms which are key transcriptional factors involved in the metabolism of FA. However, after short term exposure to exogenous FA did not



alter the mRNA expression of any of the *Ppar* isoforms (*Ppara*, *Ppar δ* , *Ppar γ*) or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1 α*) ($p > .05$; Figure 6b).

3.5 | Insulin signaling responses to OPLA exposure

Insulin stimulated Akt (Ser⁴⁷³) phosphorylation was not different between conditions ($p > .05$; Figure 7a), however insulin stimulated phosphorylation of AS160 (Ser⁵⁸⁸) and GSK-3 β (Ser⁹) relative to basal was attenuated with OPLA exposure between 0 and 800 μM OPLA ($p < .05$; Figure 7b,c).

4 | DISCUSSION

The use of TESkM to investigate the physiological and functional responses to nutritional substrates is well documented (Khodabukus & Baar, 2015; Khodabukus et al., 2007; Martin et al., 2017). Despite the role of FA in skeletal muscle physiology and pathophysiology (Meex et al., 2009), advanced in vitro models, such as TESkM, which enable investigation of both the molecular and functional responses to exogenous FA have yet to be utilized. Therefore, these experiments sought to determine if exposure to a mixture of FA would affect the accumulation of lipids within TESkM constructs, and ultimately alter the molecular and functional response of the construct.

Both skeletal muscle tissue and skeletal muscle cells have been shown to readily increase FA uptake in response to FA exposure (Bonen et al., 2004; Newsom et al., 2015). Using concentrations which have previously been shown to increase the accumulation of FA in C2C12 and primary skeletal muscle cells (Newsom et al., 2015), these experiments showed a dose dependent increase in the presence of lipid droplets in TESkM. Although we did not quantify TAG or diglyceride directly, it could be speculated that the increase in number and size of the LD in TESkM reflect an accumulation of neutral lipids, such as TAGs, previously shown in rodent skeletal muscle tissue and skeletal muscle cells (Lee et al., 2006; Newsom et al., 2015).

Based on the morphological findings that exogenous FA increased both the number and size of LD within our TESkM, we analyzed the mRNA expression of *plin2* and *plin5*, which are key

FIGURE 4 Exposure to exogenous FA overload alters myosin heavy chain coverage in 3D tissue engineered skeletal muscle skeletal muscle (MHC) image analysis (a, MHC coverage; b, myotubes per construct; c, Myotubes per mm^2 ; d, construct crosssectional area [CSA] [μm]; e, Avg. Myotubes CSA [μm^2]) skeletal muscle tissue engineered construct cross sections. Data is presented as mean \pm SEM. Significantly different versus 0 μM . FA, fatty acid; MHC, myosin heavy chain; OPLA, oleic, palmitic, linoleic, and α -linoleic acid. * $p < .05$, *** $p < .001$

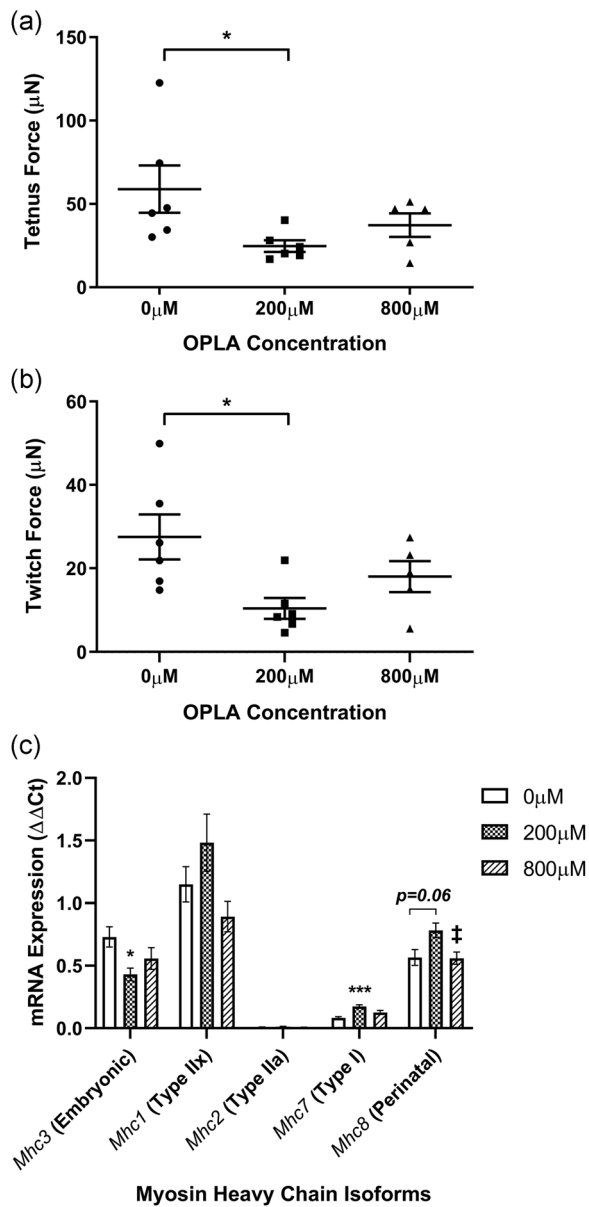


FIGURE 5 Exogenous FA overload induces functional changes in 3D tissue engineered skeletal muscle and alters myosin heavy chain messenger RNA (mRNA) expression. Force Analysis (a, tetanus and b, twitch) and (c), *Mhc* gene expression of skeletal muscle tissue engineered constructs cultured with different concentrations of OPLA. Data is presented as mean \pm SEM. Significantly different versus 0 μ M (* p < .05), ***(p < .001). Significantly different versus 200 μ M ($\ddagger p$ < .05). FA, fatty acid; OPLA, oleic, palmitic, linoleic, and α -linoleic acid

regulators of LD storage and mobilization in skeletal muscle (Bosma et al., 2012; Feng et al., 2017). Specifically *plin2* expression, which was increased in these experiments with increasing concentrations of OPLA, has been confirmed previously in vitro in response to saturated and unsaturated FA (Bosma et al., 2012). However, despite its role in lipid metabolism (Mason & Watt, 2015), there were no increases in *plin5* expression with exogenous FA. The disparity between our findings and previous in vitro studies, which have shown

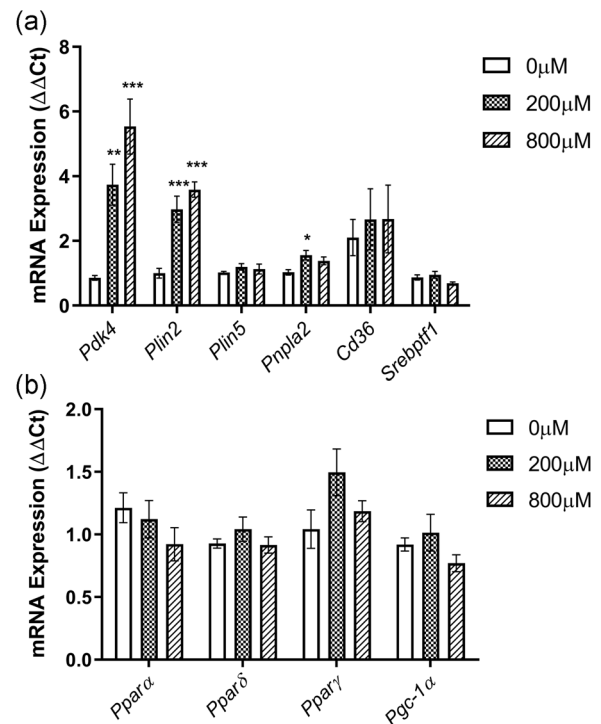


FIGURE 6 Exogenous FA overload alters mRNA expression of genes involved in lipid metabolism of 3D tissue engineered skeletal muscle. (a) Expression of lipid metabolism genes following exposure to OPLA for 4 days. (b) Peroxisome proliferator-activated receptor (*Ppar*) isoform mRNA expression following exposure to OPLA for 4 days. Data is presented as mean \pm SEM. Significantly different versus 0 μ M (* p < .05, ** p < .01, *** p < .001). FA, fatty acid; mRNA, messenger RNA; OPLA, oleic, palmitic, linoleic, and α -linoleic acid

FA to increase *plin5* in C2C12 cells overexpressing this gene (Bindesbøll et al., 2013), is potentially related to the low endogenous levels of *plin5* in this cell type (Daemen et al., 2018; Mason & Watt, 2015).

With the increased LD in our TESkM when cultured with exogenous FA, *pdk4* increased in a dose dependent manner. Increases in *pdk4* expression and activity are indicative of changes in nutritional status, such as increased exposure to lipids that induce metabolic switching toward FA metabolism. This is achieved through the pyruvate dehydrogenase complex and is correlated with PDK activity in skeletal muscle (Rodríguez et al., 2010). The increase in *pdk4* mRNA has been reported previously in human skeletal muscle cells obtained from healthy donors and patients who are obese and diabetic exposed to FA overload (McAinch et al., 2015), which although not directly measured in this system, indicates a potential metabolic shift in response to exposure of FA (Peters et al., 2001).

In skeletal muscle, exogenous FA are initially trafficked towards storage with the breakdown of LD mediated by lipases, such as adipose triglyceride lipase (ATGL) which is encoded by the *pnpla2* gene (Ji et al., 2006). *pnpla2* expression has been observed in human and rodent skeletal muscle (Sitnick et al., 2013), and to a lesser extent in C2C12 skeletal muscle cells (Meex et al., 2015). We observed only small increase

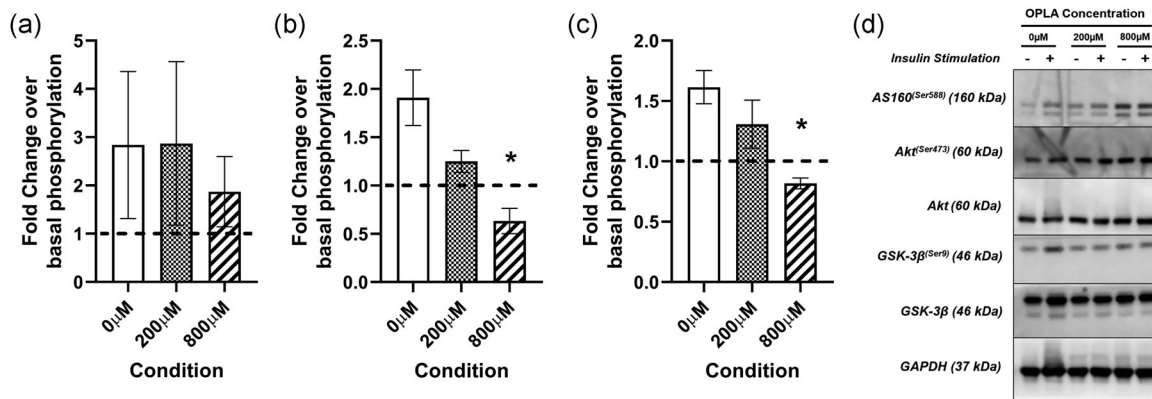


FIGURE 7 Insulin signaling response of 3D tissue engineered skeletal muscle in response to exogenous FA overload. Immunoblot analysis for (a) Akt, (b) AS160, (c) GSK-3 β , and (d) representative images of insulin signaling proteins in response to acute insulin stimulation (± 100 nM for 30 min) following exposure to different concentrations of OPLA. Data is presented as mean \pm SEM of the fold change to basal stimulation. Significantly different versus 0 μ M * ($p < .05$). FA, fatty acid; OPLA, oleic, palmitic, linoleic, and α -linoleic acid

in *pnpla2* expression with exogenous FA in this system which does not follow the trends in *pnpla2* (AGTL) protein expression with exogenous FAs (Newsom et al., 2015). Newsom and colleagues observed increases in AGTL protein expression following shorter incubation periods with FA that would suggest the translation to protein occurs within a relatively short period of time, with transcription preceding the increase in protein expression (Newsom et al., 2015). In addition, it is important to note that genes such as the lipid activated transcriptional factors, *Ppars*, are activated by dietary FA (Lee et al., 2003). Therefore, the transient expression of these transcription factors would account for the small changes observed in these experiments whereby the incubation time spanned the transcriptional peak of these genes in response to exogenous FA, which has been observed in C2C12 skeletal muscle cells (Rodríguez et al., 2010).

The impact of lipid accumulation IMCL on skeletal muscle insulin sensitivity is a highly debated area (Dirks et al., 2020; Goodpaster, 2020). Using a mixture of FA to mimic systemic concentrations has previously been shown to alter insulin signalling in hepatocytes and skeletal muscle cells using conventional culture methods (Green et al., 2015; Hodson et al., 2008; Newsom et al., 2015). We observed there to be a significant reduction in insulin stimulated phosphorylation of proximal insulin signaling proteins AS160 and GSK-3 β , although no significant reductions in Akt phosphorylation. The reductions observed in our experiments coincide with the modest reductions previously reported in skeletal muscle cells exposed to normal mixtures of FA (Newsom et al., 2015). A number of factors could contribute to this response, such as the cellular location of lipids and the type of lipids to which skeletal muscle is exposed. For example, the addition of polyunsaturated FA has been shown to have beneficial effects upon saturated FA induced insulin resistance (Lee et al., 2006) by driving an increase in TAG accumulation (Lee et al., 2006; Newsom et al., 2015). While some observed trends indicated changes in insulin signaling, these responses can occur following adaptations to, and increases in, IMCL following high fat diet feeding in skeletal muscle (Bonen et al., 2015).

Unlike monolayer culture methods, TESkM enables for investigation of the functional changes in skeletal muscle contraction in response to nutritional or electrical stimulation (Khodabukus et al., 2015; Martin et al., 2017). Furthermore, this in vitro model allows the maintenance of TESkM for prolonged culture periods (>14 days) and provides the flexibility to investigate the progressive metabolic changes to lipid exposure in skeletal muscle (Khodabukus & Baar, 2009; Madden et al., 2015). The absolute reduction in maximal tetanic and single twitch force in response to lipid exposure, despite an increase in construct size, could be attributed to changes in skeletal muscle fiber type which have been reported in rodent skeletal muscle following the feeding of dietary FA (Eshima et al., 2017; Shortreed et al., 2009). We analyzed the mRNA expression of myosin heavy chain isoforms, which identified a small but statistically significant increase in transcription of slow (*myh7*) type I fibers. This has previously been reported in rodents, and has been shown to have negative effects upon skeletal muscle force generation following short term dietary FA interventions (de Wilde et al., 2008; Tallis et al., 2018). However, other factors such as metabolic phenotype (Ciapaite et al., 2015; Tallis et al., 2017) and calcium handling (Bruton et al., 2002; Warmington et al., 2000), have been shown to contribute to changes in skeletal muscle force capacity with FA exposure and cannot be discounted as a contributing factor to the findings from these experiments. It should be noted that exposure to OPLA, independent of concentration, induced similar decrements in force generation compared to 0 μ M. Although we are unable to specifically identify the reason for these findings, this is an area requiring further investigation to understand how exogenous FA influences force transmission through changes in ECM expression and the implications it has for patients who are insulin resistant (Berria et al., 2006; Richardson et al., 2005).

Although our findings provide insight into some of the molecular responses to exogenous FA in TESkM, it is important to note that the changes in gene expression are not always representative of protein content or function. While our data provides evidence of the accumulation of lipids, further experiments are required to identify the neutral or bioactive lipid intermediates derived from exogenous

sources, as well as the cellular location of FA, as both are important factors in skeletal muscle insulin sensitivity (Amati et al., 2011; Bergman et al., 2012; Perreault et al., 2018). The relatively short term exposure to the physiological and pathophysiological concentrations are representative of an initial adaptation to increases in exogenous FA. However, the effects of long-term exposure are yet to be defined in our model, as prolonged exposure to FA could result in an exacerbated pathophysiological change in TESkM, similar to that observed in mouse skeletal muscle in vivo (Eshima et al., 2017).

Our findings show that TESkM is responsive to different concentrations of exogenous FA, through the increase in accumulation of lipids in the construct, as well as increased expression of genes involved in lipid breakdown and oxidation. These changes are accompanied by adaptations in the expression of myosin heavy chain isoforms that may contribute to the absolute reduction in functional capacity of TESkM in response to short term FA exposure. This study describes an advanced 3D cell culture model to investigate the impact of exogenous FA upon skeletal muscle physiology and function.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Mark C. Turner and Mark P. Lewis conceived the idea of the experiments. Mark C. Turner conducted the experiments. Andrew J. Capel, Jacob W. Fleming, Rowan P. Rimington supported the collection and analysis of the functional data. Mark C. Turner and Neil R. W. Martin analyzed the data. Leanne Hodson provided the fatty acid formulation. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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