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

Turner, M. C., Rimington, R. P., Martin, N. R. W., Fleming, J. W., Capel, A. J., Hodson, L. & Lewis, M. P.

Original citation:
Turner, MC, Rimington, RP, Martin, NRW, Fleming, JW, Capel, AJ, Hodson, L & Lewis, MP 2021, 'Physiological and pathophysiological concentrations of fatty acids induce lipid droplet accumulation and impair functional performance of tissue engineered skeletal muscle', Journal of Cellular Physiology.
https://dx.doi.org/10.1002/jcp.30365

DOI 10.1002/jcp.30365
ISSN 0021-9541
ESSN 1097-4652

Publisher: Wiley

Received: 12 August 2020 | Revised: 11 February 2021 | Accepted: 8 March 2021

DOI: 10.1002/jcp.30365

RESEARCH ARTICLE

Journal of Cellular Physiology WILEY

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

Mark C. Turner[1,2,3] | Rowan P. Rimington[1] | Neil R.W. Martin[1] | Jacob W. Fleming[1] | Andrew J. Capel[1] | Leanne Hodson[4] | Mark P. Lewis[1]

[1]School of Sport, Exercise and Health Sciences, National Centre for Sport and Exercise Medicine, Loughborough University, Loughborough, UK

[2]Leicester Biomedical Research Centre, University Hospitals of Leicester NHS Trust, Leicester, UK

[3]Centre for Sport, Exercise and Life Sciences, Research Institute for Health and Wellbeing, Coventry University, Coventry, UK

[4]Oxford Center for Diabetes, Endocrinology and Metabolism, Oxford Biomedical Research Centre, Radcliffe Department of Medicine, Churchill Hospital, University of Oxford, Oxford, UK

Correspondence
Mark C. Turner, Centre for Sport, Exercise and Life Sciences, Research Institute for Health and Wellbeing, Coventry University, Coventry, CV1 2DS, UK.
Email: mark.turner@coventry.ac.uk

Mark P. Lewis, School of Sport, Exercise and Health Sciences, National Centre for Sport and Exercise Medicine, Loughborough University, Loughborough, LE11 3TU, UK.
Email: m.p.lewis@lboro.ac.uk

Funding information
National Institute for Health Research, Grant/Award Number: IS-BRC-1215-20010; British Heart Foundation, Grant/Award Number: FS/15/56/31645

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 μM) 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 μM 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 ito the functional and mechanistic changes of TESkM in response to exogenous FA.

KEYWORDS
contraction, insulin sensitivity, lipids

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% CO_2 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 μM, as used previously to induce lipid droplet accumulation in a

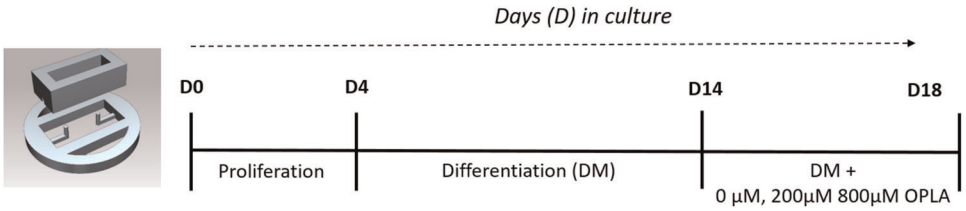


FIGURE 1 Experimental outline of development and exposure to exogenous fatty acid (FA) overload in 3D tissue engineered skeletal muscle. DM, differentiation media; OPLA, oleic, palmitic, linoleic, and α-linoleic acid

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 µM 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 $CaCl_2$, 1.25 mM $MgSO_4$, 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 (Ser^{473}) (1:2000, #4060), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000, #2118) GSK-3β (1:2000, #9315) and phosphor GSK-3β (Ser^{9}) (1:2000, #9336) (Cell Signaling Technology). Following overnight incubation, membranes were washed in TBST and incubated with ant-irabbit (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 (Ser^{588}), 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 µl 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, 15μm 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 lipids droplets. Threshold corrected Manders' coefficient using five images per condition from three independent experiments.

2.10 | Statistical analysis

Data is presented as mean ± *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 μM concentrations significantly increasing the number of droplets ($p < .05$; Figure 3a). The average LD area was also increased in a dose dependent manner,

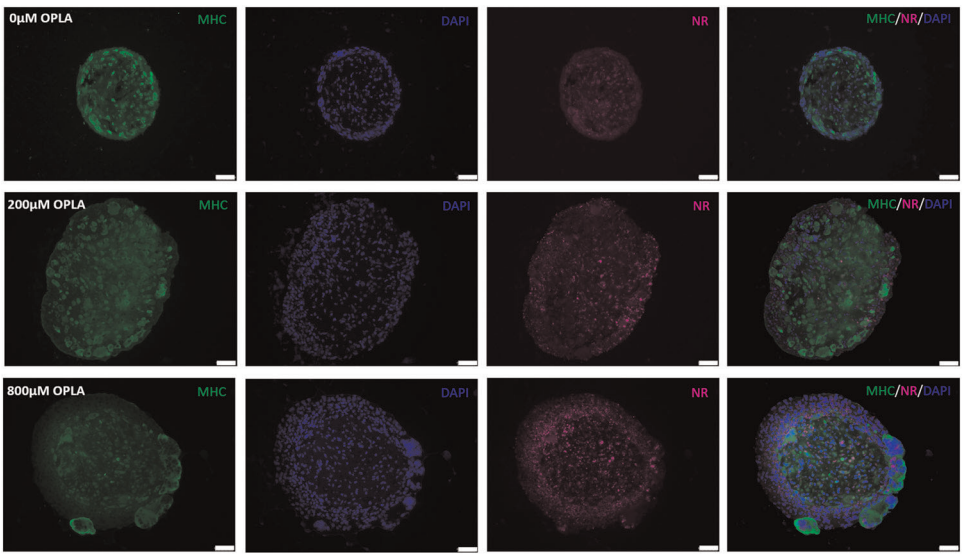


FIGURE 2 Exposure to exogenous FA overload induces intracellular lipid droplet formation in 3D tissue engineered skeletal muscle. Representative cross sections of tissue engineered skeletal muscle constructs (TESkM) cultured with (200 μM OPLA and 800 μM OPLA) or without (0 μM OPLA) endogenous lipids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

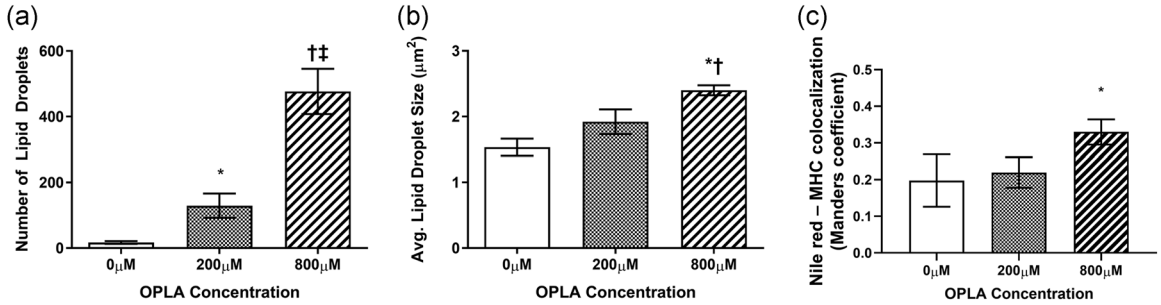


FIGURE 3 Exposure to exogenous FA overload increases the presence of intracellular lipid droplet in 3D tissue engineered skeletal muscle. Lipid droplet (LD) (Nile Red) image analysis (a, number of LD; b, average LD area) from skeletal muscle tissue engineered construct cross sections. (c) Colocalisation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

with only the 800 μM concentration being significantly increased compared to 0 and 200 μM, 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 μM conditions, respectively) with a significant difference between 0 and 800 μM 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 μM OPLA ($p < .05$; Figure 4c) and to an extent in 800 μM 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 mm^2, 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

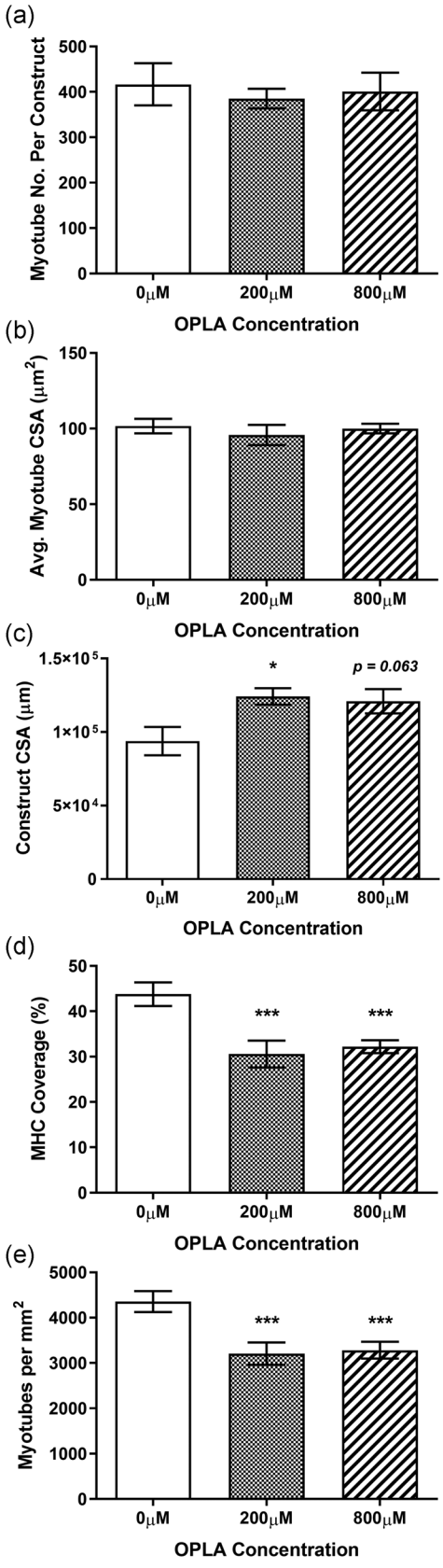
Using a TESkM system enabled investigation of the functional capacity of skeletal muscle tissue to be investigated in response to OPLA supplementation. Increasing concentrations of OPLA resulted in a significant reduction in absolute maximal force generation (tetanic force) when exposed to 200 μM compared to control ($p < .05$), with a trend toward a ($p = .05$) reduction in force of TESkM following 800 μM 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 μM ($p < .05$) 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 μM OPLA ($p < .001$). The mRNA expression of the *Myh3* (embryonic) was lower between 200 μM compared to 0 μM OPLA ($p < .05$), with *Myh8* (Perinatal) expression elevated in 200 μM compared to 0 μM ($p = .06$) and significantly higher compared to constructs exposed to 800 μM 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 μM OPLA compared to control, however, no differences between these conditions were observed compared to exposure of 800 μM 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 (*Srebptf1*) expression was lower in constructs incubated in 800 μM 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 (*Pparα*, *Pparδ*, *Ppary)* or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1α*) ($p > .05$; Figure 6b).

3.5 | Insulin signaling responses to OPLA exposure

Insulin stimulated Akt (Ser^{473}) phosphorylation was not different between conditions ($p > .05$; Figure 7a), however insulin stimulated phosphorylation of AS160 (Ser^{588}) and GSK-3β (Ser^{9}) 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 lipids 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 ± *SEM*. Significantly different versus 0 μM. FA, fatty acid; MHC, myosin heavy chain; OPLA, oleic, palmitic, linoleic, and α-linoleic acid. $^{*}p < .05$, $^{***}p < .001$

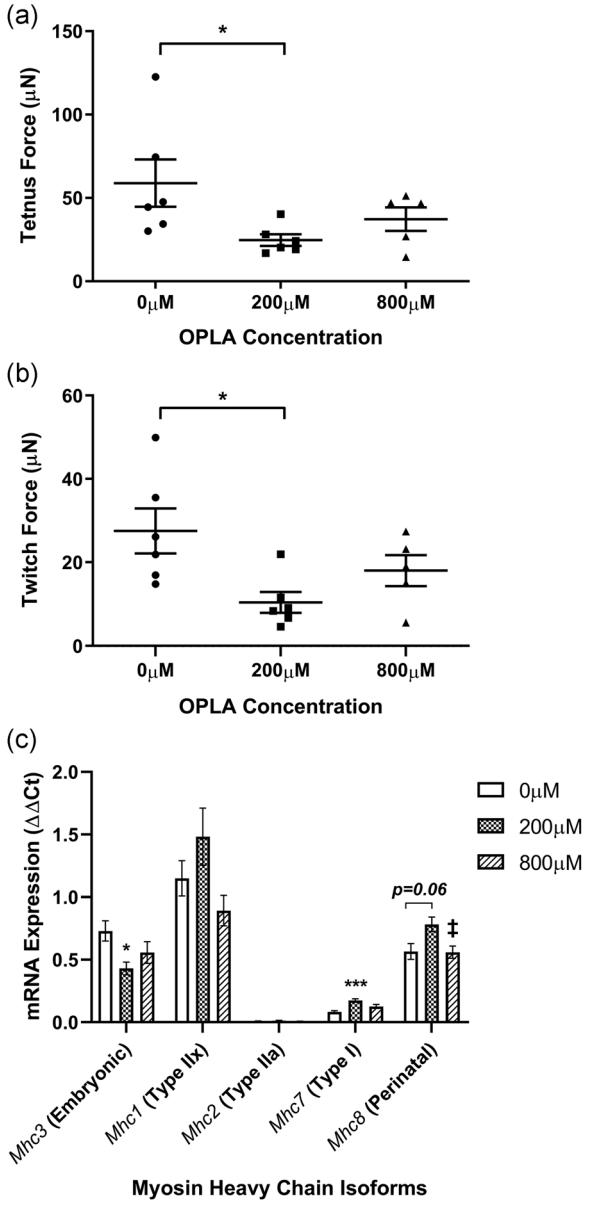


FIGURE 5 Exogenous FA overload induces functional changes in 3D tissue engineered skeletal muscle and alters myosin heavy chain messenger RNA (mRNA) expression. Force Analysis (a, tetanus and b, twitch) and (c), *Mhc* gene expression of skeletal muscle tissue engineered constructs cultured with different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

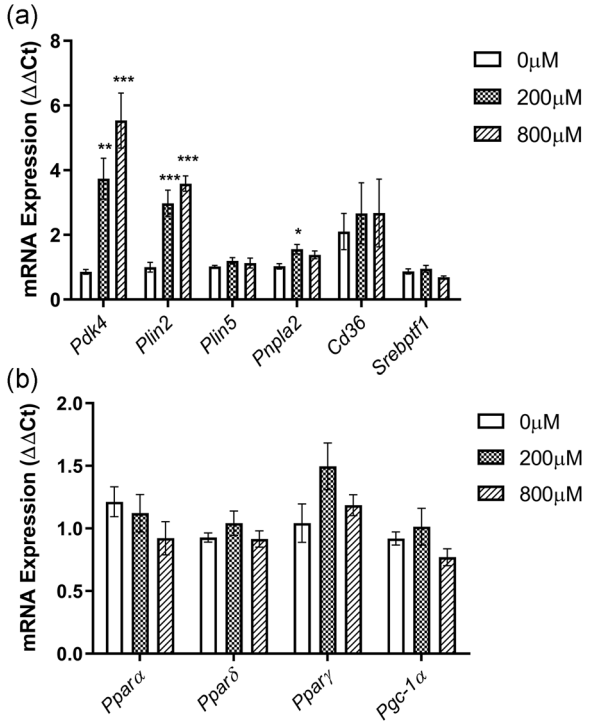


FIGURE 6 Exogenous FA overload alters mRNA expression of genes involved in lipid metabolism of 3D tissue engineered skeletal muscle. (a) Expression of lipid metabolism genes following exposure to OPLA for. (b) Peroxisome proliferator-activated receptor (*Ppar*) isoform mRNA expression following exposure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

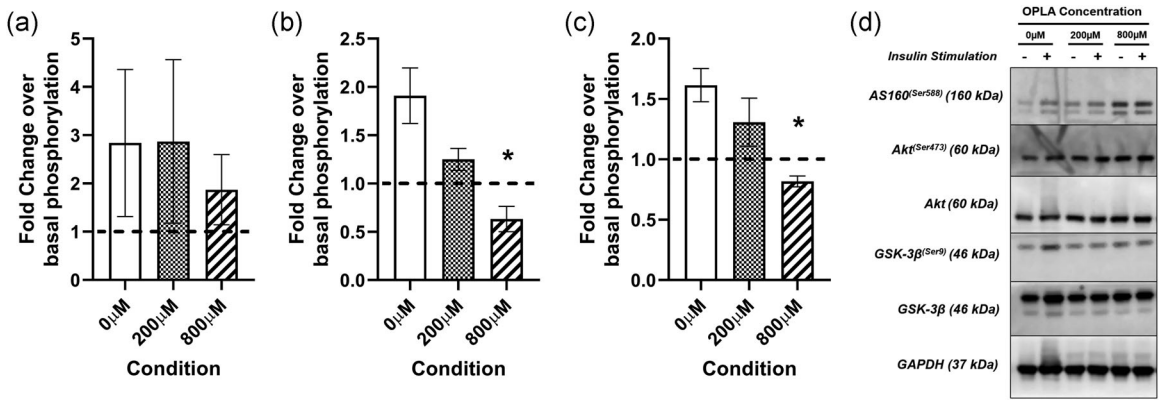


FIGURE 7 Insulin signaling response of 3D tissue engineered skeletal muscle in response to exogenous FA overload. Immunoblot analysis for (a) Akt, (b) AS160, (c) GSK-3β, and (d) representative images of insulin signaling proteins in response to acute insulin stimulation (±100 nM for 30 min) following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

ACKNOWLEDGMENTS

This study was funded by the NIHR Leicester Biomedical Research Centre. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care. Prof. L Hodson is a British Heart Foundation Senior Research Fellow in Basic Science (FS/15/56/31645).

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.

ORCID

Mark C. Turner https://orcid.org/0000-0002-5503-6188
Rowan P. Rimington https://orcid.org/0000-0002-8247-3922
Neil R.W. Martin https://orcid.org/0000-0002-1988-047X
Jacob W. Fleming https://orcid.org/0000-0003-1856-5176
Andrew J. Capel https://orcid.org/0000-0002-3043-0170
Leanne Hodson https://orcid.org/0000-0002-2648-6526

REFERENCES

Abdelmoula, A., Martin, V., Bouchant, A., Walrand, S., Lavet, C., Taillardat, M., Maffiuletti, N. A., Boisseau, N., Duché, P., & Ratel, S. (2012). Knee extension strength in obese and nonobese male adolescents. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquée, Nutrition et Métabolisme*, *37*(2), 269–275. https://doi.org/10.1139/h2012-010

Acosta, F. M., Jia, U.-T., Stojkova, K., Howland, K. K., Guda, T., Pacelli, S., Brey, E. M., & Rathbone, C. R. (2020). Diabetic conditions confer metabolic and structural modifications to tissue-engineered skeletal muscle. *Tissue Engineering. Part A*, ten.tea.2020.0138. https://doi.org/10.1089/ten.tea.2020.0138

Aguilar-Agon, K. W., Capel, A. J., Martin, N. R. W., Player, D. J., & Lewis, M. P. (2019). Mechanical loading stimulates hypertrophy in tissue-engineered skeletal muscle: Molecular and phenotypic responses. *Journal of Cellular Physiology*, *234*(12), 23547–23558. https://doi.org/10.1002/jcp.28923

Amati, F., Dubé, J. J., Alvarez-Carnero, E., Edreira, M. M., Chomentowski, P., Coen, P. M., Switzer, G. E., Bickel, P. E., Stefanovic-Racic, M., Toledo, F. G. S., & Goodpaster, B. H. (2011). Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: Another paradox in endurance-trained athletes? *Diabetes*, *60*(10), 2588–2597. https://doi.org/10.2337/db10-1221

Bachmann, O. P., Dahl, D. B., Brechtel, K., Machann, J., Haap, M., Maier, T., Loviscach, M., Stumvoll, M., Claussen, C. D., Schick, F., Häring, H. U., & Jacob, S. (2001). Effects of Intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. *Diabetes*, *50*(11), 2579–2584. https://doi.org/10.2337/diabetes.50.11.2579

Badin, P.-M., Langin, D., & Moro, C. (2013). Dynamics of skeletal muscle lipid pools. *Trends in Endocrinology and Metabolism*, *24*(12), 607–615. https://doi.org/10.1016/j.tem.2013.08.001

Bergman, B. C., Hunerdosse, D. M., Kerege, A., Playdon, M. C., & Perreault, L. (2012). Localisation and composition of skeletal muscle diacylglycerol predicts insulin resistance in humans. *Diabetologia*, *55*(4), 1140–1150. https://doi.org/10.1007/s00125-011-2419-7

Berria, R., Wang, L., Richardson, D. K., Finlayson, J., Belfort, R., Pratipanawatr, T., Filippis, E.aD.e, Kashyap, S., & Mandarino, L. J. (2006). Increased collagen content in insulin-resistant skeletal muscle. *American Journal of Physiology, Endocrinology and Metabolism*, *290*(3), E560–E565. https://doi.org/10.1152/ajpendo.00202.2005

Bindesbøll, C., Berg, O., Arntsen, B., Nebb, H. I., & Dalen, K. T. (2013). Fatty acids regulate perilipin5 in muscle by activating PPARδ. *Journal of Lipid Research*, *54*(7), 1949–1963. https://doi.org/10.1194/jlr.m038992

Bolte, S., & Cordelières, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy*, *224*(3), 213–232. https://doi.org/10.1111/j.1365-2818.2006.01706.x

Bonen, A., Jain, S. S., Snook, L. A., Han, X.-X., Yoshida, Y., Buddo, K. H., Lally, J. S., Pask, E. D., Paglialunga, S., Beaudoin, M.-S., Glatz, J. F. C., Luiken, J. J. F. P., Harasim, E., Wright, D. C., Chabowski, A., & Holloway, G. P. (2015). Extremely rapid increase in fatty acid transport and intramyocellular lipid accumulation but markedly delayed insulin resistance after high fat feeding in rats. *Diabetologia*, *58*(10), 2381–2391. https://doi.org/10.1007/s00125-015-3691-8

Bonen, A., Parolin, M. L., Steinberg, G. R., Calles-Escandon, J., Tandon, N. N., Glatz, J. F. C., Luiken, J. J. F. P., Heigenhauser, G. J. F., & Dyck, D. J. (2004). Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport increased sarcolemmal FAT/CD36. *FASEB Journal*, *18*(10), 1144–1146. https://doi.org/10.1096/fj.03-1065fje

Bosma, M., Hesselink, M. K. C., Sparks, L. M., Timmers, S., Ferraz, M. J., Mattijssen, F., Beurden, D., van, Schaart, G., Baets, M. H., de, Verheyen, F. K., Kersten, S., & Schrauwen, P. (2012). Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated

intramuscular lipid levels. *Diabetes*, *61*(11), 2679–2690. https://doi.org/10.2337/db11-1402

Bruton, J. D., Katz, A., Länneergren, J., Abbate, F., & Westerblad, H. (2002). Regulation of myoplasmic Ca2+ in genetically obese (ob/ob) mouse single skeletal muscle fibres. *Pflugers Archiv European Journal of Physiology*, *444*(6), 692–699. https://doi.org/10.1007/s00424-002-0882-1

Capel, A. J., Rimington, R. P., Fleming, J. W., Player, D. J., Baker, L. A., Turner, M. C., Jones, J. M., Martin, N. R., Ferguson, R. A., Mudera, V. C., & Lewis, M. P. (2019). Scalable 3D printed molds for human tissue engineered skeletal muscle. *Frontiers in Bioengineering and Biotechnology*, *7*(FEB), 20. https://doi.org/10.3389/fbioe.2019.00020

Cheng, C. S., Davis, B. N., Madden, L., Bursac, N., & Truskey, G.a (2014). Physiology and metabolism of tissue-engineered skeletal muscle. *Experimental Biology and Medicine*, *239*(9), 1203–1214. https://doi.org/10.1177/1535370214538589

Ciapaite, J., van den Berg, S. A., Houten, S. M., Nicolay, K., Willems van Dijk, K., & Jeneson, J. A. (2015). Fiber-type-specific sensitivities and phenotypic adaptations to dietary fat overload differentially impact fast- versus slow-twitch muscle contractile function in C57BL/6J mice. *Journal of Nutritional Biochemistry*, *26*(2), 155–164. https://doi.org/10.1016/j.jnutbio.2014.09.014

Daemen, S., Van Polanen, N., & Hesselink, M. K. C. (2018). The effect of diet and exercise on lipid droplet dynamics in human muscle tissue. *Journal of Experimental Biology*, *121*(Pt Suppl 1), jeb167015. https://doi.org/10.1242/jeb.167015

de Wilde, J., Mohren, R., Van Den Berg, S., Boekschoten, M., Van Dijk, K. W., De Groot, P., Müller, M., Mariman, E., & Smit, E. (2008). Short-term high fat-feeding results in morphological and metabolic adaptations in the skeletal muscle of C57BL/6J mice. *Physiological Genomics*, *32*(3), 360–369. https://doi.org/10.1152/physiolgenomics.00219.2007

Dirks, M. L., Wall, B. T., & Stephens, F. B. (2020). CrossTalk opposing view: Intramuscular lipid accumulation does not cause insulin resistance. *Journal of Physiology*, *598*(18), 3807–3810. https://doi.org/10.1113/jp278220

Eshima, H., Tamura, Y., Kakehi, S., Kurebayashi, N., Murayama, T., Nakamura, K., Kakigi, R., Okada, T., Sakurai, T., Kawamori, R., & Watada, H. (2017). Long-term, but not short-term high-fat diet induces fiber composition changes and impaired contractile force in mouse fast-twitch skeletal muscle. *Physiological Reports*, *5*(7), e13250. https://doi.org/10.14814/phy2.13250

Feng, Y. Z., Lund, J., Li, Y., Knabenes, I. K., Bakke, S. S., Kase, E. T., Lee, Y. K., Kimmel, A. R., Thoresen, G. H., Rustan, A. C., & Dalen, K. T. (2017). Loss of perilipin 2 in cultured myotubes enhances lipolysis and redirects the metabolic energy balance from glucose oxidation towards fatty acid oxidation. *Journal of Lipid Research*, *58*(11), 2147–2161. https://doi.org/10.1194/jlr.m079764

Fleming, J. W., Capel, A. J., Rimington, R. P., Wheeler, P., Davies, O. G., & Lewis, M. P. (2020). Bioengineered human skeletal muscle with a Pax7+ satellite cell niche capable of functional regeneration. *bioRxiv*. 05.04.076828. https://doi.org/10.1101/2020.05.04.076828

Goodpaster, B. H. (2020). CrossTalk proposal: Intramuscular lipid accumulation causes insulin resistance. *Journal of Physiology*, *598*(18), 3803–3806. https://doi.org/10.1113/jp278219

Green, C. J., Johnson, D., Amin, H. D., Sivathondan, P., Silva, M. A., Wang, L. M., Stevanato, L., McNeil, C. A., Miljan, E. A., Sinden, J. D., Morten, K. J., & Hodson, L. (2015). Characterization of lipid metabolism in a novel immortalized human hepatocyte cell line. *American Journal of Physiology, Endocrinology and Metabolism*, *309*(6), E511–E522. https://doi.org/10.1152/ajpendo.00594.2014

Gunn, P. J., Pramfalk, C., Millar, V., Cornfield, T., Hutchinson, M., Johnson, E. M., Nagarajan, S. R., Troncoso-Rey, P., Mithen, R. F., Pinnick, K. E., Traka, M. H., Green, C. J., & Hodson, L. (2020). Modifying nutritional substrates induces macrovesicular lipid droplet accumulation and metabolic alterations in a cellular model of hepatic steatosis. *Physiological Reports*, *8*(13). https://doi.org/10.14814/phy2.14482

Hinds, S., Bian, W., Dennis, R. G., & Bursac, N. (2011). The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. *Biomaterials*, *32*(14), 3575–3583. https://doi.org/10.1016/j.biomaterials.2011.01.062

Hodson, L., Skeaff, C. M., & Fielding, B. A. (2008). Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Progress in Lipid Research*, *47*(5), 348–380. https://doi.org/10.1016/j.plipres.2008.03.003

Hulens, M., Vansant, G., Lysens, R., Claessens, A. L., Muls, E., & Brumagne, S. (2001). Study of differences in peripheral muscle strength of lean versus obese women: An allometric approach. *International Journal of Obesity*, *25*(5), 676–681. https://doi.org/10.1038/sj.ijo.0801560

Hurst, J., James, R. S., Cox, V. M., Hill, C., & Tallis, J. (2019). Investigating a dose–response relationship between high-fat diet consumption and the contractile performance of isolated mouse soleus, EDL and diaphragm muscles. *European Journal of Applied Physiology and Occupational Physiology*, *119*(1), 213–226. https://doi.org/10.1007/s00421-018-4017-6

Jacob, S., Machann, J., Rett, K., Brechtel, K., Volk, A., Renn, W., Maerker, E., Matthaei, S., Schick, F., Claussen, C. D., & Häring, H. U. (1999). Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. *Diabetes*, *48*(5), 1113–1119. https://doi.org/10.2337/diabetes.48.5.1113

Ji, Y. K., Tillison, K., Lee, J. H., Rearick, D. A., & Smas, C. M. (2006). The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-α in 3T3-L1 adipocytes and is a target for transactivation by PPARγ. *American Journal of Physiology—Endocrinology and Metabolism*, *291*(1), E115–E127. https://doi.org/10.1152/ajpendo.00317.2005

Khodabukus, A., & Baar, K. (2009). Regulating fibrinolysis to engineer skeletal muscle from the C2C12 cell line. *Tissue Engineering Part C, Methods*, *15*(3), 501–511. https://doi.org/10.1089/ten.tec.2008.0286

Khodabukus, A., & Baar, K. (2015). Glucose concentration and streptomycin alter in vitro muscle function and metabolism. *Journal of Cellular Physiology*, *230*(6), 1226–1234. https://doi.org/10.1002/jcp.24857

Khodabukus, A., Baehr, L. M., Bodine, S. C., & Baar, K. (2015). Role of contraction duration in inducing fast-to-slow contractile and metabolic protein and functional changes in engineered muscle. *Journal of Cellular Physiology*, *230*(10), 2489–2497. https://doi.org/10.1002/jcp.24985

Khodabukus, A., Paxton, J. Z., Donnelly, K., & Baar, K. (2007). Engineered muscle: a tool for studying muscle physiology and function. *Exercise and Sport Sciences Reviews*, *35*(4), 186–191. https://doi.org/10.1097/jes.0b013e318156df01

Kondash, M. E., Ananthakumar, A., Khodabukus, A., Bursac, N., & Truskey, G. A. (2020). Glucose uptake and insulin response in tissue-engineered human skeletal muscle. *Tissue Engineering and Regenerative Medicine*, *17*(6), 801–813. https://doi.org/10.1007/s13770-020-00242-y

Krssak, M., Falk Petersen, K., Dresner, A., DiPietro, L., Vogel, S. M., Rothman, D. L., Shulman, G. I., & Roden, M. (1999). Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: A 1 H NMR spectroscopy study. *Diabetologia*, *42*(1), 113–116. https://doi.org/10.1007/s001250051123

Lee, C.-H., Olson, P., & Evans, R. M. (2003). Minireview: Lipid metabolism, metabolic diseases, and peroxisome proliferator-activated

Receptors. *Endocrinology*, *144*(6), 2201–2207. https://doi.org/10.1210/en.2003-0288

Lee, J. S., Pinnamaneni, S. K., Su, J. E., In, H. C., Jae, H. P., Chang, K. K., Sinclair, A. J., Febbraio, M. A., & Watt, M. J. (2006). Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: Role of intramuscular accumulation of lipid metabolites. *Journal of Applied Physiology*, *100*(5), 1467–1474. https://doi.org/10.1152/japplphysiol.01438.2005

Madden, L., Juhas, M., Kraus, W. E., Truskey, G. A., & Bursac, N. (2015). Bioengineered human myobundles mimic clinical responses of skeletal muscle to drugs. *eLife*, *2015*(4). https://doi.org/10.7554/elife.04885

Maffiuletti, N. A., Jubeau, M., Munzinger, U., Bizzini, M., Agosti, F., Col, A.D.e, Lafortuna, C. L., & Sartorio, A. (2007). Differences in quadriceps muscle strength and fatigue between lean and obese subjects. *European Journal of Applied Physiology and Occupational Physiology*, *101*(1), 51–59. https://doi.org/10.1007/s00421-007-0471-2

Martin, N. R. W., Turner, M. C., Farrington, R., Player, D. J., & Lewis, M. P. (2017). Leucine elicits myotube hypertrophy and enhances maximal contractile force in tissue engineered skeletal muscle in vitro. *Journal of Cellular Physiology*, *232*(10), 2788–2797. https://doi.org/10.1002/jcp.25960

Mason, R. R., & Watt, M. J. (2015). Unraveling the roles of PLIN5: Linking cell biology to physiology. *Trends in Endocrinology and Metabolism*, *26*(3), 144–152. https://doi.org/10.1016/j.tem.2015.01.005

McAinch, A. J., Cornall, L. M., Watts, R., Hryciw, D. H., O'Brien, P. E., & Cameron-Smith, D. (2015). Increased pyruvate dehydrogenase kinase expression in cultured myotubes from obese and diabetic individuals. *European Journal Nutrition*, *54*(7), 1033–1043. https://doi.org/10.1007/s00394-014-0780-2

Meex, R. C. R., Hoy, A. J., Mason, R. M., Martin, S. D., McGee, S. L., Bruce, C. R., & Watt, M. J. (2015). ATGL-mediated triglyceride turnover and the regulation of mitochondrial capacity in skeletal muscle. *American Journal of Physiology—Endocrinology and Metabolism*, *308*(11), E960–E970. https://doi.org/10.1152/ajpendo.00598.2014

Meex, R. C. R., Schrauwen, P., & Hesselink, M. K. C. (2009). Modulation of myocellular fat stores: lipid droplet dynamics in health and disease. *Am J Physiol Integr Comp Physiol*, *297*(4), R913–R924. https://doi.org/10.1152/ajpregu.91053.2008

Newsom, S. A., Everett, A. C., Park, S., Van Pelt, D. W., Hinko, A., & Horowitz, J. F. (2015). Lipid mixtures containing a very high proportion of saturated fatty acids only modestly impair insulin signaling in cultured muscle cells. *PLOS One*, *10*(3), e0120871. https://doi.org/10.1371/journal.pone.0120871

Parry, S. A., Turner, M. C., Woods, R. M., James, L. J., Ferguson, R. A., Cocks, M., Whytock, K. L., Strauss, J. A., Shepherd, S. O., Wagenmakers, A. J. M., Van Hall, G., & Hulston, C. J. (2019). High-fat overfeeding impairs peripheral glucose metabolism and muscle microvascular eNOS Ser1177 phosphorylation. *Journal of Clinical Endocrinology and Metabolism*, *105*(1), 65–77. https://doi.org/10.1210/clinem/dgz018

Perreault, L., Newsom, S. A., Strauss, A., Kerege, A., Kahn, D. E., Harrison, K. A., Snell-Bergeon, J. K., Nemkov, T., D'Alessandro, A., Jackman, M. R., MacLean, P. S., & Bergman, B. C. (2018). Intracellular localization of diacylglycerols and sphingolipids influences insulin sensitivity and mitochondrial function in human skeletal muscle. *JCI Insight*, *3*(3), e96805. https://doi.org/10.1172/jci.insight.96805

Peters, S. J., Harris, R. A., Heigenhauser, G. J. F., & Spriet, L. L. (2001). Muscle fiber type comparison of PDH kinase activity and isoform expression in fed and fasted rats. *American Journal of Physiology—Regulatory Integrative Comparative Physiology*, *280*(3 49-3), R661–R668. https://doi.org/10.1152/ajpregu.2001.280.3.r661

Richardson, D. K., Kashyap, S., Bajaj, M., Cusi, K., Mandarino, S. J., Finlayson, J., DeFronzo, R. A., Jenkinson, C. P., & Mandarino, L. J. (2005). Lipid infusion decreases the expression of nuclear encoded mitochondrial genes and increases the expression of extracellular matrix genes in human skeletal muscle. *Journal of Biological Chemistry*, *280*(11), 10290–10297. https://doi.org/10.1074/jbc.m408985200

Rodríguez, A. M., Sánchez, J., Tobaruela, A., Priego, T., Picó, C., & Palou, A. (2010). Time-course effects of increased fatty acid supply on the expression of genes involved in lipid/glucose metabolism in muscle cells. *Cellular Physiology and Biochemistry*, *25*(2–3), 337–346. https://doi.org/10.1159/000276566

Savage, D. B., Watson, L., Carr, K., Adams, C., Brage, S., Chatterjee, K. K., Hodson, L., Boesch, C., Kemp, G. J., & Sleigh, A. (2019). Accumulation of saturated intramyocellular lipid is associated with insulin resistance. *Journal of Lipid Research*, *60*(7), 1323–1332. https://doi.org/10.1194/jlr.m091942

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. http://dx.doi.org/10.1038/nmeth.2019

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*, *3*(6), 1101–1108. https://doi.org/10.1038/nprot.2008.73

Shortreed, K. E., Krause, M. P., Huang, J. H., Dhanani, D., Moradi, J., Ceddia, R. B., & Hawke, T. J. (2009). Muscle-specific adaptations, impaired oxidative capacity and maintenance of contractile function characterize diet-induced obese mouse skeletal muscle. *PLOS One*, *4*(10), e7293. https://doi.org/10.1371/journal.pone.0007293

Sitnick, M. T., Basantani, M. K., Cai, L., Schoiswohl, G., Yazbeck, C. F., Distefano, G., Ritov, V., DeLany, J. P., Schreiber, R., Stolz, D. B., Gardner, N. P., Kienesberger, P. C., Pulinilkunnil, T., Zechner, R., Goodpaster, B. H., Coen, P., & Kershaw, E. E. (2013). Skeletal muscle triacylglycerol hydrolysis does not influence metabolic complications of obesity. *Diabetes*, *62*(10), 3350–3361. https://doi.org/10.2337/db13-0500

Stinkens, R., Goossens, G. H., Jocken, J. W. E., & Blaak, E. E. (2015). Targeting fatty acid metabolism to improve glucose metabolism. *Obesity Reviews*, *16*(9), 715–757. https://doi.org/10.1111/obr.12298

Tallis, J., Hill, C., James, R. S., Cox, V. M., & Seebacher, F. (2017). The effect of obesity on the contractile performance of isolated mouse soleus, EDL, and diaphragm muscles. *Journal of Applied Physiology*, *122*(1), 170–181. https://doi.org/10.1152/japplphysiol.00836.2016

Tallis, J., James, R. S., & Seebacher, F. (2018). The effects of obesity on skeletal muscle contractile function. *Journal of Experimental Biology*, *221*(13), jeb163840. https://doi.org/10.1242/jeb.163840

Toledo, F. G. S., Johannsen, D. L., Covington, J. D., Bajpeyi, S., Goodpaster, B., Conley, K. E., & Ravussin, E. (2018). Impact of prolonged overfeeding on skeletal muscle mitochondria in healthy individuals. *Diabetologia*, *61*(2), 466–475. https://doi.org/10.1007/s00125-017-4496-8

Turner, N., Bruce, C. R., Beale, S. M., Hoehn, K. L., So, T., Rolph, M. S., & Cooney, G. J. (2007). Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: Evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. *Diabetes*, *56*(8), 2085–2092. https://doi.org/10.2337/db07-0093

Warmington, S. A., Tolan, R., & McBennett, S. (2000). Functional and histological characteristics of skeletal muscle and the effects of leptin in the genetically obese (ob/ob) mouse. *International Journal of Obesity*, *24*(8), 1040–1050. https://doi.org/10.1038/sj.ijo.0801357

Whytock, K. L., Parry, S. A., Turner, M. C., Woods, R. M., James, L. J., Ferguson, R. A., Ståhlman, M., Borén, J., Strauss, J. A., Cocks, M.,

Wagenmakers, A. J. M., Hulston, C. J., & Shepherd, S. O. (2020). A 7-day high-fat, high-calorie diet induces fibre-specific increases in intramuscular triglyceride and perilipin protein expression in human skeletal muscle. *Journal of Physiology*, *598*(6), 1151–1167. https://doi.org/10.1113/jp279129

SUPPORTING INFORMATION

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

How to cite this article: Turner, M. C., Rimington, R. P., Martin, N. R. W., Fleming, J. W., Capel, A. J., Hodson, L., & Lewis, M. P. (2021). Physiological and pathophysiological concentrations of fatty acids induce lipid droplet accumulation and impair functional performance of tissue engineered skeletal muscle. *Journal of Cellular Physiology*, 1–12. https://doi.org/10.1002/jcp.30365