Resolvin E1 (R_vE_1) attenuates LPS induced inflammation and subsequent atrophy in C2C12 myotubes

Luke A Baker[1], Neil R W Martin[1], Marc C Kimber[2], Gareth J Pritchard[2], Martin R Lindley[1*] and Mark P Lewis[1]

[1]*School of Sport Exercise & Health Sciences, Loughborough University, UK*

[2]*School of Science, Loughborough University, UK.*

**Correspondence to:* Dr Martin R Lindley

Email: M.R.Lindley@lboro.ac.uk

Telephone: +44(0) 1509 226349

Address: School of Sport, Exercise & Health Sciences, Loughborough University, Leicestershire LE11 3TU

Key Words:

- **Specialised pro-resolving mediators**
- **Skeletal muscle**
- **Muscle ageing**
- **Cytokines**
- **Resolution**
- **Omega-3 Fatty Acids**

Number of Tables: 1

Number of Figures: 4

Number of Supplementary Figures: 4

Abstract

Resolution of inflammation is now known to be an active process which in part is instigated and controlled by specialised pro-resolving lipid mediators (SPM's) derived from dietary omega-3 fatty acids. Resolvin E1 (R_vE_1) is one of these SPM's derived from the omega-3 fatty acid eicosapentaenoic acid. Using both molecular and phenotypic functional measures we report that in a model of Lipopolysaccharide (LPS) induced inflammation, R_vE_1 attenuated mRNA gene expression levels of both interlukin-6 and monocyte chemoattractant protein-1 whilst having no effect on tumour necrosis factor-α or Interlukin-1β in C2C12 skeletal muscle myotubes. Findings at the molecular level were transferred into similar changes in extracellular protein levels of the corresponding genes with the greatest attenuation being noted in IL-6 protein concentrations. R_vE_1 instigated beneficial morphological changes through the prevention of endotoxin induced skeletal muscle atrophy, thus resulting in a rescue of endotoxin force losses in tissue engineered skeletal muscle. These findings demonstrate, in our model of endotoxin induced inflammation in skeletal muscle, that R_vE_1 has pro-resolving properties in this cell type. Our data provides rationale for further investigation into the mechanistic action of R_vE_1 in skeletal muscle, with the vision of having potential benefits for the prevention/resolution of *in-vivo* skeletal muscle atrophy.

Introduction

A loss of skeletal muscle size (wasting) is common in a number of disease states as well as being prevalent in the 'healthy' ageing process (Romanick, Thompson & Brown-Borg, 2013). In turn skeletal muscle wasting impairs functional capacity, which is associated with impaired quality of life and increased mortality (Ruiz et al., 2008; Roshanravan et al., 2017). Systemic and local inflammation is often prevalent in elderly individuals (Argilés et al. 2005) and cachexic diseases (Saini et al., 2006; Candore et al., 2010) and is therefore thought to play a major role in mediating the loss of skeletal muscle size. Direct evidence for this has come from *in-vitro* studies, where incubation of myotubes with pro-inflammatory cytokines causes atrophy and up regulation of catabolic signalling pathways (Li et al., 1998; Girven et al., 2016; Alvarez et al., 2002). There is therefore a need to further develop intervention strategies aimed at dampening or resolving inflammation in order to prevent skeletal muscle wasting and offset subsequent decline in muscle function.

Omega-3 polyunsaturated fatty acids (ω-3) are bioactive lipids found in a variety of foods including fish oils. Nuts and seeds. The most common PUFA's are Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), which have shown to elicit a variety of potential health benefits in both clinical and laboratory based studies (Chagas et al., 2017; Calder et al., 2017; Poudyal et al., 2011). Though the beneficial effects of omega-3's is now widely accepted in specific disease states such as Cardiovascular disease (Tavazzi et al., 2008), the mechanisms of action are still poorly described. Research investigated the role of membrane make up both in terms of lipid raft formation and the ratio of omega-3:omega-6 in the lipid bilayer

structure (Calder, 2011). Though these factors may contribute to the anti-inflammatory role of omega-3 fatty acids, cellular based research proposed a novel set of naturally occurring ω-3 derived mediators termed Specialised Pro-Resolving Mediators (SPM's) which have been identified as having pro-resolving effects in several cell types, and may be critical in defining the health benefits of ω-3 (Serhan et al., 2000; Liu et al., 2017; Norling et al., 2016; Jeromson et al., 2015).

The 'E-series' resolvins are biosynthesised from the omega-3 fatty acid Eicosapentaenoic acid (EPA). In particular 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid (R_vE_1) is synthesised during the metabolism of EPA by either acetylated cyclooxygenase-2 (COX-2) or the mono-hydroxylase PY450 (Serhan et al., 2000; Serhan, 2004). Indeed, the detection of R_vE_1 at nanogram concentrations has been reported *in-vivo* (Ohira et al., 2010) and at these concentrations R_vE_1 has been shown to elicit pro-resolving actions *in-vitro* in an acute inflammatory model of leukocyte infiltration (Schwab et al., 2007). Further investigations have shown that R_vE_1 is able to attenuate Lipopolysaccharide (LPS) induced pro-inflammatory cytokine transcription in pancreatic islets (Lund et al., 2010) and can reduce circulating levels of Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) in animal (murine) models (Hasturk et al., 2007; Campbell et al., 2010; Seki et al., 2010). Importantly, Lund and colleagues found that inflammatory markers such as Tumour Necrosis Factor-alpha (TNF-α) and Monocyte chemotactic protein 1 (CcL-2) were reduced to the largest extent when R_vE_1 was co-incubated with LPS treated human pancreatic islets in vitro, which is of particular interest in skeletal muscle physiology as both play roles in the initiation and infiltration phases of the inflammatory process in skeletal muscle (Lund et al., 2010) highlighting the potential for advantageous properties of R_vE_1 in the resolution of skeletal muscle inflammation. Moreover, cyclic production of IL-6 through IL-1β signalling may further exacerbate atrophy in skeletal muscle (Haddad et al., 2005; Luo et al., 2003), thus highlighting the importance of attenuating levels of these cytokines. It is therefore plausible that R_vE_1 may have efficacy as a therapeutic intervention for the resolution of inflammatory signalling and prevent subsequent atrophy in skeletal muscle.

The aim of the current study was to investigate the preventative effects of R_vE_1 on LPS induced inflammation in C2C12 *in-vitro* muscle cultures to provide a first indication of its role skeletal muscle inflammation. Initially we explored the time course of TNFα, IL-6, IL-1β and CcL-2 induction following incubation of myotubes with LPS, and thereafter sought to determine if the elevations in pro-inflammatory cytokine production could be prevented by the addition of R_vE_1 to the cultures. Thereafter we investigated if R_vE_1 could prevent inflammation induced myotube atrophy and whether this would translate in to improvements in muscle function utilising '3D'-skeletal muscle tissue engineering techniques.

Methods

Cell Culture

The C2C12 murine myoblast cell line (C2C12) was used for all experiments. C2C12's were cultured at 37°C and 5% CO_2 (HeraCell, Thermo Scientific, UK) in growth medium (GM) composed of: Dulbecco's modified Eagle's medium (DMEM) (Fisher Scientific, UK) plus 20% FBS (PAN Biotech, Germany), and 1% penicillin–streptomycin (PS) solution (Invitrogen, Paisley, UK), until 80-95% confluency was attained. Cells were then enzymatically dissociated using Trypsin-EDTA (Sigma Aldrich, UK) and counted using the trypan blue exclusion method and subsequently seeded into 12 well plates (Thermo-Scientific) at a density of $12.5x10^{-3}$ cells/cm^2 in standard GM. Cells were grown to confluency (approximately 3 days), at which point medium was changed to differentiation media (DM) composed of DMEM (Fisher Scientific, UK) plus 2% Horse Serum (HS) (Fisher Scientific, UK), and 1% penicillin–streptomycin (PS) solution (Invitrogen, Paisley, UK) to initiate differentiation. Following three days of differentiation, myotubes were exposed to one of 3 conditions: i) Vehicle Control (CON): DM + 0.1 μl/ml EtOH; ii) LPS (LPS): DM + 100 ng/ml LPS (Sigma Aldrich, UK); iii) Resolvin E1 (R_vE_1): DM + 100 ng/ml LPS + 100 nM R_vE_1 (Bertin Pharma, France) and subsequently analysed for mRNA expression, myotube size and myokine release. Specifically, RNA was extracted at 0.5, 1.5, 3 and 6 h of experimental treatment. Based on the initial mRNA induction, following 3 hours of treatment, medias were removed and replaced with standard DM for a further 72 h, at which point cells were fixed for immunocytochemistry analysis and conditioned mediums harvested for further analysis. Furthermore, initial mRNA screening of the basal effect of R_vE_1 showed no effect on the genes of interest (Figure S4), thus authors deemed the condition unnecessary for subsequent experimentation.

To determine if the inflammatory stimulus could impact on muscle force production and the effects of R_vE_1, a tissue engineering approach was employed. Fibrin based hydrogels were fabricated as previously described (Martin, Aguilar-Agon, et al. 2017). Briefly, two 6mm sutures were pinned into PDMS (Sylgard 184 Elastomer, Dow Corning, UK) coated 35mm plates 12mm apart using 0.15mm minutien pins (Entomoravia, Czech Republic). Plates were sterilised using Ultraviolet light and washing with 70% ethanol and subsequently left to dry for 1 hour. Each plate then received 500µl of GM containing 10U/ml thrombin (Sigma-Aldrich) and 80µg/ml aprotinin (Sigma-Aldrich) which was spread evenly over the surface of the plate ensuring that the sutures were covered. 200µl of 20mg/ml stock fibrinogen (Sigma-Aldrich) solution was then added to the plate, and was agitated gently to ensure even distribution and then left to incubate for 10 minutes at room temperature before being transferred to the incubator (37°C) for one hour for polymerisation. Post incubation, 100,000 C2C12's seeded on the surface of each hydrogel in GM + 0.25 mg/ml of 6-Aminocaproic acid (AA). Once cells reached confluency (approximately 3

days) medium was changed to DM + 0.5 mg/ml AA for 2 days. Post differentiation, cells were maintained in maintenance medium consisting of DMEM, 7% FBS, 1%PS and 0.5 mg/ml AA (MM) for the remainder of the 14-day culture period. 72 h before the end of the 14-day culture period experimental hydrogels were changed into one of 3 conditions: i) CON; ii) LPS; iii) R_vE_1 as described above. After 3 h of incubation in the experimental medias, medias were removed and replaced with MM for the remainder of the culture period. All experiments were conducted with cells between passages 4 and 8 (n=9, across 3 biological repeats for each analysis method).

RNA Extraction

Cells isolated for mRNA analysis were lysed in 400 µl of TRI Reagent and frozen at -80˚C prior to further analysis. RNA extraction was performed as per the manufacturer's instructions. In brief, chloroform was added to ensure dissociation of nucleotide complexes (0.2 ml per 1 ml of TRI reagent), and samples were agitated and left to stand for 5 mins before being centrifuged at 12,000 g for 15 min. The aqueous phase was removed and 2-proponal (0.5 ml per 1 ml of TRI reagent) was added to the aqueous phase and mixed by inversion. Following 10 minutes' incubation at room temperature samples were centrifuged at 12,000 g for 10mins to pellet RNA. RNA pellets were washed in 75% ethanol, centrifuged for a further 5mins at 7,500 g, and air dried for 5-10 mins. Once isolated, RNA was suspended in 50 µl RNA storage solution (Ambion, Life Technologies) and stored in RNase free tubes for mRNA analysis.

RT-qPCR

One step quantitative RT-qPCR was used to determine expression of target mRNA's in C2C12 cultures. Primer sequences (Table 1) were checked for specificity and assay efficiency by performing standard curve analysis with a top standard of 200 ng of RNA. Output was analysed using ViiA 7 RUO Software where melt curve analysis was used to check for specificity of primers. Optimisation of standard curves was assumed with efficiencies of 100 ± 10%.

Reactions were made up in 384 well RNase free plates (Applied Biosystems, UK) and consisted of 20 ng (4 ng/µl) of RNA, 0.1 µl of both forward and reverse primers (Life Technologies) (Table 1), 0.1 µl of Quantifast Reverse Transcriptase kit (Qiagen) and 4.7 µl of SYBR green mix (Qiagen) to create 10 µl reactions. Once prepared, plates were transferred to the ViiA 7™ Real Time PCR thermal cycler (Applied Biosystems, Life Technologies) which was programmed to perform the following steps: 10 min hold at 50°C (reverse transcription), followed by a 5 min hold at 95 °C (activation of 'hot start' Taq polymerase), and cycling between 95 °C for 10s (denaturation) and 60 °C for 30 s (annealing and extension). Fluorescence was detected after every cycle and data was analysed using RPIIβ as the housekeeping gene. Data was made relative using the comparative Ct method (Livak and

Schmittgen 2001) with any changes in target genes being in comparison to that of the vehicle control condition for each experimental repeat.

Extracellular cytokine analysis

Cell supernatants were collected from culture wells for analysis of extracellular protein concentrations. Protein concentrations for IL-6, TNF-α, IL-1β and CcL-2 were measured using 'sandwich' based ABTS ELISA kits (PeproTech, US). In brief, 1.0 µg/mL of anti-murine capture antibody was added to an ELISA microplate (NUNC maxiSorp, Fisher, UK). Following overnight incubation at room temperature, the plate was washed 4 times with wash buffer (0.05% Tween-20 PBS) before being blocked (1% BSA in PBS) for 1 hour. The plate was washed 4 times and 100 µL of serially diluted standards or supernatant sample were added and incubated at room temperature for 3 hours with moderate agitation (500 rpm). The plate was again washed 4 times before being incubated with detection antibody for 2 hours at room temperature with agitation as above. Following 4 washes, 100 µL of ABTS liquid substrate (Sigma-Aldrich, Dorset, UK) was added to each well and the plate was loaded into a Varioskan™ Flash Multimode Reader (ThermoFisher). Colour development was monitored every 10 minutes for 1 hour at 405 nm with a wavelength correction of 650 nm.

Fluorescent staining

Cells grown on 13 mm coverslips in 12 well plates were fixed with 3.7% formaldehyde solution made up in PBS for 30 min. Fixed cells were then washed in TBS twice and cells were permeabilised using a 0.2% Triton X-100 (Sigma) solution made up in TBS for 1 hour. Following a further two washes cells were stained with Phalloidin (1:200 in TBS) to visualise the F-actin filaments in myotubes and DAPI (1:1000) to counter stain nuclei. After an hour of incubation, cells were washed 3 x with TBS and subsequently mounted onto microscope slides with Flouromount™ aqueous mounting medium (Sigma Aldrich). Images were captured using a Leica DM2500 fluorescent microscope (Leica, UK) at 20x magnification and 7 images were obtained per coverslip, equating to 21 images per condition, per biological repeat. Analysis of myotube width and number were conducted using Image J software (NIH, Bethesda, MD). Myotubes were identified as elongated structures containing 3 or more nuclei and expressing high levels of F-actin. Myotube number was counted per image and an average of 3 measures of each myotube was obtained to calculate myotube width.

Assessment of tissue engineered skeletal muscle function

Prior to functional tests, hydrogels were washed once in a Krebs Ringer HEPES buffer (KRH; 10mM HEPES, 138 mM NaCl, 4.7mM KCl, 1.25 mM $CaCl_2$, 1.25 mM MgSO, 5 mM Glucose, 0.05% Bovine Serum Albumin in dH_20) and attached to a

model 403A Aurora force transducer (Aurora Scientific, UK). Following the addition of 4 ml of KRH buffer, wire electrodes were positioned either side of the hydrogel in order to allow for electric field stimulation. 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 second pulse train at 100Hz and 3.6 v/mm, generated using labVIEW 2012 software (National Instruments, UK). Data was acquired using Powerlab (ver. 8/35) and associated software (Labchart 8, AD Instruments, UK).

Statistical Analysis

Data are presented as means ± SEM unless otherwise stated (n=9). Statistical analyses were performed using SPSS v.23 (SPSS Inc., Chicago, IL, US). Data were tested for normal distribution and homogeneity of variance. A one-way analysis of variance (ANOVA), or non-parametric equivalent, for a between-between design was used to analyse the differences between conditions at a single time point. For comparisons across time points, a one-way analysis of variance (ANOVA), or non-parametric equivalent, for a between-between design was used to analyse the differences between both time and condition and any interaction effect between the two. Where significance was reported, Bonferroni post-hoc tests or a series of non-parametric equivalents were used to identify where any significance lay between conditions and time points. Statistical significance was assumed if $p \leq 0.05$. Further analysis of effect sizes (r) were used to calculate the magnitude of effect: Trivial <0.02; Small 0.2-0.5; Moderate 0.5-0.8; Large >0.8 (Cohen, 1992).

Results

LPS induces elevations in mRNA of pro-inflammatory markers in skeletal muscle myotubes

To quantify the induction of pro-inflammatory markers, RT-qPCR was used to measure the mRNA expression of selected pro-inflammatory markers across a six-hour time course in response to LPS exposure. A pre-defined dose of 100 ng/ml of LPS was seen to induce significant increases across all four selected markers at differing time points in comparison to CON (See Figure S1). Significant increases in TNF-α were seen at 0.5 hrs (4.97 vs. 0.97) whereas the greatest induction of IL-6 (21.66 vs. 2.12) and CcL-2 (67.38 vs. 1.27) was observed at 3 hrs and IL-1β mRNA levels were significantly elevated at 6 hrs (4.00 vs. 1.98).

Resolvin E1 attenuates LPS induced mRNA induction of pro-inflammatory markers and related extracellular protein release

Utilising the time points optimised for maximal LPS induced mRNA expression in the initial experiments, the effects of an optimised dose of R_vE_1 (100 nM; See Figure S2) were investigated. Selected doses during preliminary experiments were based on

previous literature identifying R_vE_1 at nano-molar concentrations in-vivo (Ohira et al., 2010). Cultures were simultaneously exposed to R_vE_1 along with LPS had reduced levels of both IL-6 (23.44 vs. 14.92, $p<0.05$) and CcL-2 (45.10 vs. 32.68, $p<0.05$) in comparison to LPS alone (Figure 1). However, no changes were noted in TNF-α (3.56 vs. 3.89, $p>0.05$) or IL-1β mRNA expression (2.96 vs. 2.98, $p>0.05$). Subsequently, extracellular protein levels of all markers were measured in the conditioned media to investigate if myokine release reflected the transcriptional changes. Twenty-four hours post LPS exposure all proteins measured showed significant increases in the LPS condition compared to that of the CON (Figure 2). However, with the addition of R_vE_1, significant reductions were only noted in IL-6 (3900 ± 157 pg/m vs. 2500 ± 729 pg/ml, $p<0.05$, $r=1.92$). Reduced levels of both CcL-2 (20006 ± 4441 pg/m vs. 16014±4884 pg/ml, $p>0.05$, $r=0.82$) and IL-1β (11340 ± 3354 pg/m vs. 9223 ± 3580 pg/ml, $p>0.05$, $r=0.59$) were observed, although these changes did not reach statistical significance. No differences were noted in TNF-α extracellular protein following RvE1 addition in comparison with LPS alone (127.2 ± 35.8 pg/m vs. 124.8 ± 45.4 pg/ml, $p>0.05$, $r=0.05$).

RvE1 attenuates LPS induced atrophy

Myotubes incubated acutely with LPS for 3 hours displayed significant atrophy at later time points, with the most severe level of morphological atrophy noted at 72 hrs post LPS exposure displaying a 53.56 % reduction compared with 24 hrs and 48 hrs (24 % and 32 % reductions, respectively: See Figure S3). When R_vE_1 was added to myotubes acutely along with LPS, the resulting atrophy was resolved by 31.83 % compared to the LPS condition (9.71 ± 0.59 μm vs. 15.45 ± 1.21 μm, $p<0.05$). No difference was seen in myotube number across all three conditions confirming the phenotypic change was atrophic in nature as opposed to being hyperplasic (Figure 3).

Functional resolution of LPS induced force losses in tissue engineered skeletal muscle

A tissue engineering approach was adopted to investigate if LPS induced atrophy in skeletal muscle myotubes led to a decrement of its functional capacity in terms of force generation. LPS exposure was used as previously described as well as the 72 hrs post exposure time point where myotube atrophy was maximal. After LPS exposure, peak tetanic force (μN) of tissue engineered skeletal muscle was significantly reduced in comparison to that of the control (93.48 ± 3.27 % vs. 59.34 ± 7.07 %, $p<0.05$, $r= 1.80$). Furthermore, with the addition of R_vE_1 combined with LPS, force decrements were resolved by 13.35%, which although did not reach statistical significance, did represent a large effect (59.34 ± 7.07 % vs. 72.69 ± 5.15 %, $p>0.05$, $r=1.00$) (Figure 4).

Discussion

The resolution of inflammation is defined as an active process and one which has been shown to be in part mediated by lipid derived SPM's (Ohira et al., 2010). In the present investigation we sought to determine if the novel SPM, R_vE_1 can resolve inflammation in cultured C2C12 myotubes and thus act as a potential therapeutic for muscle wasting. This data is the first to show the pro-resolving action of R_vE_1 in skeletal muscle during endotoxin induced inflammation, resulting in the attenuation of muscle loss and attenuated loss of muscle function in a '3D' tissue engineered skeletal muscle model.

Diseases in which muscle wasting is noted are associated with elevated levels of circulating pro-inflammatory cytokines. In the present investigation, LPS (100ng) exposure resulted in elevations in mRNA levels of TNF-α, IL-6, CcL-2 and IL-1β across a 6-hour time course. These data are in agreement with Frost et al., (2002) who also found time dependent responses of these pro-inflammatory cytokines in response to a similar dose of LPS exposure in single cell myoblasts. Similar responses have also been identified in fused myotubes further supporting the findings of the current investigation (Boyd et al., 2006). Comparable findings have also been reported utilising cytokine exposure such as TNF-α to induce IL-6 mRNA gene expression (Alvarez et al., 2002). Moreover, in the present study conditioned medium samples from myotube cultures 24 hours after an acute 3 hour LPS exposure showed elevations in myokine levels of IL-6, TNF-α, IL-1β and CcL-2 which also mirrors responses previously reported in the *in-vitro* literature (McCoin et al., 2015; Podbregar et al., 2013; Peake et al., 2015) as well as displaying similar trends to *in-vivo* investigations (Lang et al., 2003). Therefore, exposure of C2C12 myotubes to 100ng LPS over a 3-hour time course provided an ideal model to explore the effect of R_vE_1.

Addition of R_vE_1 to the culture medium alongside LPS resulted in substantial attenuation of the mRNA induction of pro-inflammatory cytokines, and whilst these were not directly mirrored in the secreted extracellular levels of corresponding proteins, there was clear evidence of attenuated levels. Similar trend reductions have been seen in CcL-2 in regards to the effects of R_vE1 (Lund et al., 2010), though the current work showed contradictory effects on TNF-α. This suggests that R_vE_1 has tissue-specific effects however dampening of LPS induced myotube inflammation in our model highlights the efficacy of the use of R_vE_1 in this cell type/tissue indicating that it may be an important nutritional therapeutic for reducing inflammation in skeletal muscle.

Elevations in pro-inflammatory cytokines have previously been shown to induce an atrophic response in muscle cells in vitro (Magee, Pearson & Allen 2008; Romanick, Thompson & Brown-Borg, 2013) suggesting that the inflammation observed in

human diseases may be a major driver of the associated muscle wasting. In the present study, 3 hours of incubation with LPS resulted in 46% myotube atrophy 72 hours later, similar to that observed by both Yi-Ping *et al,* (2000) and Magee et al*.,* (2016) in response to TNF-α exposure. However, when LPS was co-incubated with R_vE_1 myotube size was preserved, with only a small (~14%) reduction compared to control cells. Interestingly, the current data set suggests that the attenuation of an atrophic phenotype utilising R_vE_1 is via an attenuation of IL-6 with no effect seen on TNF-α. This is contradictory to previous research identifying TNF-α as a key mediator of cachexia, thus future research should look to investigate these signalling differences across tissue type and based upon specific interventions. Furthermore, this ~14% reduction equated to a greater preservation of myotube size than that seen in response to EPA incubation in a pro-inflammatory state (Magee, Pearson & Allen 2008). Though the time course and inflammatory stimulus differed to the current investigation, our data provides initial evidence that R_vE_1 may have greater pro-resolving properties in skeletal muscle.

Tissue engineered muscle models have been shown to closely replicate the physiology and function of native tissue, with more recent studies utilising them to closely replicate muscle from diseases with associated changes in muscle phenotype (Martin et al., 2017; Martin et al., 2017). Further to this, work in tissue engineered constructs has defined the linear relationship between myotube phenotype in tissue engineered constructs and force output (Martin et al., 2013), as well as its ability to produce classic muscle mechanics closely replicative of native tissue (Huang et al., 2005). Thus, the use of this model as an assay of force production allowed us to contextualise our molecular and morphological findings, depicting that R_vE_1 prevents decrements in maximal force production associated with LPS exposure. This data suggests that R_vE_1 associated attenuation of inflammation is capable of having positive effects on muscle phenotype (size and function).

The proposed mechanism of action by which R_vE_1 impacts the inflammatory resolution process has been shown to differ between cell types. Two cell surface receptors have been identified for R_vE_1; Leukotriene B4 receptor 1 (BLTR1) and the G-protein coupled receptor Chemerin Receptor 23 (ChemR23) (Arita et al., 2007; Norling & Perretti, 2013; Cash, Norling & Perretti, 2014); the latter of which has previously been reported to be expressed on the sarcolemma of skeletal muscle (Sell et al., 2009). R_vE_1 has been shown to display specific binding to ChemR23 with a K_d of 11.3 nM, resulting in the attenuation of TNF-α mediated NFκB activation (Arita et al., 2005; Ohira et al., 2010). With the current findings providing the initial evidence for the pro-resolving capacity of R_vE_1 in skeletal muscle, future work needs to seek to understand whether beneficial effects of R_vE_1 are receptor dependent, and if so, provide an insight into the identity to direct future investigations for therapeutic strategies in cachexic disease states.

In conclusion, the current work has systematically developed an *in-vitro* method of LPS induced inflammatory atrophy in skeletal muscle myotubes for the screening of

anti-Inflammatory/pro-resolving compounds. The process used both mono-layer *in-vitro* approaches as well as recently defined ‘3D’ tissue engineered skeletal muscle culture, to enable the quantification of the effect of inflammatory atrophy on functional output in a controlled culture setting. Using these methodologies, we have shown potential application of R_vE_1 in this cell type. Future work should be directed towards investigating the specific binding potential of R_vE_1 in skeletal muscle, to identify its mechanism of action, furthering our understanding of its potential role as a naturally occurring pro-resolving mediator in diseases states with associated cachexia as well as in ageing populations.

References

Alvarez, Belén, LeBris S. Quinn, Silvia Busquets, Francisco J. López-Soriano, and Josep M. Argilés. 2002. “TNF-Alpha Modulates Cytokine and Cytokine Receptors in C2C12 Myotubes.” *Cancer Letters* 175 (2):181–85. https://doi.org/10.1016/S0304-3835(01)00717-0.

Argilés, Josep M, Sílvia Busquets, Antonio Felipe, and Francisco J López-Soriano. 2005. “Molecular Mechanisms Involved in Muscle Wasting in Cancer and Ageing: Cachexia versus Sarcopenia.” *The International Journal of Biochemistry & Cell Biology* 37 (5):1084–1104. https://doi.org/10.1016/j.biocel.2004.10.003.

Arita, Makoto, Francesca Bianchini, Julio Aliberti, Alan Sher, Nan Chiang, Song Hong, Rong Yang, Nicos a Petasis, and Charles N Serhan. 2005. “Stereochemical Assignment, Antiinflammatory Properties, and Receptor for the Omega-3 Lipid Mediator Resolvin E1.” *The Journal of Experimental Medicine* 201 (5):713–22. https://doi.org/10.1084/jem.20042031.

Arita, Makoto, Taisuke Ohira, Yee-ping Sun, Siva Elangovan, Nan Chiang, and Charles N Serhan. 2007. “Resolvin E1 Selectively Interacts with Leukotriene B4 Receptor BLT1 and ChemR23 to Regulate Inflammation.” *Journal of Immunology (Baltimore, Md. : 1950)* 178 (6):3912–17. https://doi.org/10.4049/jimmunol.178.6.3912.

Boyd, John H., Maziar Divangahi, Linda Yahiaoui, Dusanka Gvozdic, Salman Qureshi, and Basil J. Petrof. 2006. “Toll-like Receptors Differentially Regulate CC and CXC Chemokines in Skeletal Muscle via NF-kappaB and Calcineurin.” *Infection and Immunity* 74 (12):6829–38. https://doi.org/10.1128/IAI.00286-06.

Calder, Philip C. 2011. “Fatty Acids and Inflammation: The Cutting Edge between Food and Pharma.” *European Journal of Pharmacology* 668 (SUPPL. 1). Elsevier B.V.:S50–58. https://doi.org/10.1016/j.ejphar.2011.05.085.

Calder, Philip C, Alessandro Laviano, Fredrik Lonnqvist, Maurizio Muscaritoli, Maria Öhlander, and Annemie Schols. 2017. “Targeted Medical Nutrition for Cachexia in Chronic Obstructive Pulmonary Disease: A Randomized, Controlled Trial.” *Journal of Cachexia, Sarcopenia and Muscle*, September. https://doi.org/10.1002/jcsm.12228.

Campbell, Eric L, Christopher F MacManus, Douglas J Kominsky, Simon Keely, Louise E Glover, Brittelle E Bowers, Melanie Scully, Walter J Bruyninckx, and Sean P Colgan. 2010. “Resolvin E1-Induced Intestinal Alkaline Phosphatase Promotes Resolution of Inflammation through LPS Detoxification.” *Proceedings of the National Academy of Sciences* 107 (32):14298–303. https://doi.org/10.1073/pnas.0914730107.

Candore, G., C. Caruso, E. Jirillo, T. Magrone, and S. Vasto. 2010. “Low Grade Inflammation as a Common Pathogenetic Denominator in Age-Related Diseases: Novel Drug Targets for Anti-Ageing Strategies and Successful Ageing Achievement.” *Current Pharmaceutical Design* 16 (6):584–96. https://doi.org/10.2174/138161210790883868.

Cash, Jenna L, Lucy V Norling, and Mauro Perretti. 2014. “Resolution of

Inflammation: Targeting GPCRs That Interact with Lipids and Peptides." *Drug Discovery Today* 19 (8). Elsevier Ltd:1186–92. https://doi.org/10.1016/j.drudis.2014.06.023.

Chagas, T. R., D. S. Borges, P. F. de Oliveira, M. C. Mocellin, A. M. Barbosa, C. Q. Camargo, J. ?? G Del Moral, et al. 2017. "Oral Fish Oil Positively Influences Nutritional-Inflammatory Risk in Patients with Haematological Malignancies during Chemotherapy with an Impact on Long-Term Survival: A Randomised Clinical Trial." *Journal of Human Nutrition and Dietetics*, 1–12. https://doi.org/10.1111/jhn.12471.

Cohen, Jacob. 1992. "A Power Primer." *Psychological Bulletin* 112 (1):155–59. http://www.ncbi.nlm.nih.gov/pubmed/19565683.

Girven, Matthew, Hannah F. Dugdale, Daniel J. Owens, David C. Hughes, Claire E. Stewart, and Adam P. Sharples. 2016. "L-Glutamine Improves Skeletal Muscle Cell Differentiation and Prevents Myotube Atrophy After Cytokine (TNF-α) Stress Via Reduced p38 MAPK Signal Transduction." *Journal of Cellular Physiology* 231 (12):2720–32. https://doi.org/10.1002/jcp.25380.

Haddad, F, F Zaldivar, D M Cooper, and G R Adams. 2005. "IL-6-Induced Skeletal Muscle Atrophy." *Journal of Applied Physiology (Bethesda, Md. : 1985)* 98 (3):911–17. https://doi.org/10.1152/japplphysiol.01026.2004.

Hasturk, Hatice, Alpdogan Kantarci, Emilie Goguet-Surmenian, Amanda Blackwood, Chris Andry, Charles N Serhan, and Thomas E Van Dyke. 2007. "Resolvin E1 Regulates Inflammation at the Cellular and Tissue Level and Restores Tissue Homeostasis in Vivo." *Journal of Immunology (Baltimore, Md. : 1950)* 179 (10):7021–29. https://doi.org/10.4049/jimmunol.179.10.7021.

Huang, Yen-Chih, Robert G Dennis, Lisa Larkin, and Keith Baar. 2005. "Rapid Formation of Functional Muscle in Vitro Using Fibrin Gels." *Journal of Applied Physiology (Bethesda, Md. : 1985)* 98 (2):706–13. https://doi.org/10.1152/japplphysiol.00273.2004.

Jeromson, Stewart, Iain J Gallagher, Stuart D R Galloway, and D Lee Hamilton. 2015. "Omega-3 Fatty Acids and Skeletal Muscle Health." *Marine Drugs* 13 (11):6977–7004. https://doi.org/10.3390/md13116977.

Lang, Charles H, Christine Silvis, Nobuko Deshpande, Gerald Nystrom, and Robert a Frost. 2003. "Endotoxin Stimulates in Vivo Expression of Inflammatory Cytokines Tumor Necrosis Factor Alpha, Interleukin-1beta, -6, and High-Mobility-Group Protein-1 in Skeletal Muscle." *Shock (Augusta, Ga.)* 19 (6):538–46. https://doi.org/10.1097/01.shk.0000055237.25446.80.

Li, Y P, R J Schwartz, I D Waddell, B R Holloway, and M B Reid. 1998. "Skeletal Muscle Myocytes Undergo Protein Loss and Reactive Oxygen-Mediated NF-kappaB Activation in Response to Tumor Necrosis Factor Alpha." *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 12 (10):871–80. http://www.ncbi.nlm.nih.gov/pubmed/9657527.

Liu, Guizhu, Qian Liu, Yujun Shen, Deping Kong, Yanjun Gong, Bo Tao, Guilin

493 Chen, et al. 2017. “Early Treatment with Resolvin E1 Facilitates Myocardial
494 Recovery from Ischemia in Mice.” *British Journal of Pharmacology*, September.
495 https://doi.org/10.1111/bph.14041.

496 Livak, K J, and T D Schmittgen. 2001. “Analysis of Relative Gene Expression Data
497 Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method.”
498 *Methods (San Diego, Calif.)* 25 (4):402–8.
499 https://doi.org/10.1006/meth.2001.1262.

500 Lund, T., S. M. Mangsbo, H. Scholz, P. Gjorstrup, T. H. Tötterman, O. Korsgren, and
501 A. Foss. 2010. “Resolvin E1 Reduces Proinflammatory Markers in Human
502 Pancreatic Islets in Vitro.” *Experimental and Clinical Endocrinology & Diabetes :*
503 *Official Journal, German Society of Endocrinology [and] German Diabetes*
504 *Association* 118 (4):237–44. https://doi.org/10.1055/s-0029-1241825.

505 Luo, Guangjo, Dan D Hershko, Bruce W Robb, Curtis J Wray, and Per-Olof
506 Hasselgren. 2003. “IL-1beta Stimulates IL-6 Production in Cultured Skeletal
507 Muscle Cells through Activation of MAP Kinase Signaling Pathway and NF-
508 Kappa B.” *American Journal of Physiology. Regulatory, Integrative and*
509 *Comparative Physiology* 284 (5):R1249-54.
510 https://doi.org/10.1152/ajpregu.00490.2002.

511 Magee, Peter, Stephen Pearson, and Jeremy Allen. 2008. “The Omega-3 Fatty Acid,
512 Eicosapentaenoic Acid (EPA), Prevents the Damaging Effects of Tumour
513 Necrosis Factor (TNF)-Alpha during Murine Skeletal Muscle Cell Differentiation.”
514 *Lipids in Health and Disease* 7 (July):24. https://doi.org/10.1186/1476-511X-7-
515 24.

516 Martin, Neil R W, Kathyrn Aguilar-Agon, George P Robinson, Darren J Player, Mark
517 C Turner, Stephen D Myers, and Mark P Lewis. 2017. “Hypoxia Impairs Muscle
518 Function and Reduces Myotube Size in Tissue Engineered Skeletal Muscle.”
519 *Journal of Cellular Biochemistry* 118 (9):2599–2605.
520 https://doi.org/10.1002/jcb.25982.

521 Martin, Neil R W, Samantha L. Passey, Darren J. Player, Alastair Khodabukus,
522 Richard A. Ferguson, Adam P. Sharples, Vivek Mudera, Keith Baar, and Mark
523 P. Lewis. 2013. “Factors Affecting the Structure and Maturation of Human
524 Tissue Engineered Skeletal Muscle.” *Biomaterials* 34 (23). Elsevier Ltd:5759–
525 65. https://doi.org/10.1016/j.biomaterials.2013.04.002.

526 Martin, Neil R W, Mark C. Turner, Robert Farrington, Darren J. Player, and Mark P.
527 Lewis. 2017. “Leucine Elicits Myotube Hypertrophy and Enhances Maximal
528 Contractile Force in Tissue Engineered Skeletal Muscle in Vitro.” *Journal of*
529 *Cellular Physiology* 232 (10):2788–97. https://doi.org/10.1002/jcp.25960.

530 McCoin, Colin S., Trina A Knotts, Kikumi D. Ono-Moore, Pieter J Oort, and Sean H
531 Adams. 2015. “Long-Chain Acylcarnitines Activate Cell Stress and Myokine
532 Release in C2C12 Myotubes: Calcium-Dependent and -Independent Effects.”
533 *American Journal of Physiology. Endocrinology and Metabolism* 308 (11):E990–
534 1000. https://doi.org/10.1152/ajpendo.00602.2014.

535 Norling, Lucy V., Sarah E. Headland, Jesmond Dalli, Hildur H. Arnardottir, Oliver
536 Haworth, Hefin R. Jones, Daniel Irimia, Charles N. Serhan, and Mauro Perretti.

2016. “Proresolving and Cartilage-Protective Actions of Resolvin D1 in Inflammatory Arthritis.” *JCI Insight* 1 (5):e85922. https://doi.org/10.1172/jci.insight.85922.

Norling, Lucy V., and Mauro Perretti. 2013. “The Role of Omega-3 Derived Resolvins in Arthritis.” *Current Opinion in Pharmacology* 13 (3):476–81. https://doi.org/10.1016/j.coph.2013.02.003.

Ohira, Taisuke, Makoto Arita, Kazuhiro Omori, Antonio Recchiuti, Thomas E. Van Dyke, and Charles N. Serhan. 2010. “Resolvin E1 Receptor Activation Signals Phosphorylation and Phagocytosis.” *Journal of Biological Chemistry* 285 (5):3451–61. https://doi.org/10.1074/jbc.M109.044131.

Peake, Jonathan M., Paul Della Gatta, Katsuhiko Suzuki, and David C. Nieman. 2015. “Cytokine Expression and Secretion by Skeletal Muscle Cells: Regulatory Mechanisms and Exercise Effects.” *Exercise Immunology Review* 21 (32):8–25. http://www.ncbi.nlm.nih.gov/pubmed/25826432.

Podbregar, Matej, Mitja Lainscak, Oja Prelovsek, and Tomaz Mars. 2013. “Cytokine Response of Cultured Skeletal Muscle Cells Stimulated with Proinflammatory Factors Depends on Differentiation Stage.” *TheScientificWorldJournal* 2013:617170. https://doi.org/10.1155/2013/617170.

Poudyal, Hemant, Sunil K Panchal, Vishal Diwan, and Lindsay Brown. 2011. “Progress in Lipid Research Omega-3 Fatty Acids and Metabolic Syndrome : Effects and Emerging Mechanisms of Action.” *Progress in Lipid Research* 50 (4). Elsevier Ltd:372–87. https://doi.org/10.1016/j.plipres.2011.06.003.

Romanick, Mark, LaDora V. Thompson, and Holly M. Brown-Borg. 2013. “Murine Models of Atrophy, Cachexia, and Sarcopenia in Skeletal Muscle.” *Biochimica et Biophysica Acta* 1832 (9). Elsevier B.V.:1410–20. https://doi.org/10.1016/j.bbadis.2013.03.011.

Roshanravan, Baback, Kushang V Patel, Linda F Fried, Cassianne Robinson-Cohen, Ian H de Boer, Tamara Harris, Rachel A Murphy, et al. 2017. “Association of Muscle Endurance, Fatigability, and Strength With Functional Limitation and Mortality in the Health Aging and Body Composition Study.” *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences* 72 (2):284–91. https://doi.org/10.1093/gerona/glw210.

Ruiz, Jonatan R, Xuemei Sui, Felipe Lobelo, James R Morrow, Allen W Jackson, Michael Sjöström, and Steven N Blair. 2008. “Association between Muscular Strength and Mortality in Men: Prospective Cohort Study.” *BMJ (Clinical Research Ed.)* 337 (jul01 2):a439. https://doi.org/10.1136/bmj.a439.

Saini, Amarjit, Nasser Al-Shanti, Al-Shanti Nasser, and Claire E H Stewart. 2006. “Waste Management - Cytokines, Growth Factors and Cachexia.” *Cytokine & Growth Factor Reviews* 17 (6):475–86. https://doi.org/10.1016/j.cytogfr.2006.09.006.

Schwab, Jan M, Nan Chiang, Makoto Arita, and Charles N Serhan. 2007. “Resolvin E1 and Protectin D1 Activate Inflammation-Resolution Programmes.” *Nature* 447 (7146):869–74. https://doi.org/10.1038/nature05877.

Seki, H., K. Fukunaga, M. Arita, H. Arai, H. Nakanishi, R. Taguchi, T. Miyasho, et al. 2010. “The Anti-Inflammatory and Proresolving Mediator Resolvin E1 Protects Mice from Bacterial Pneumonia and Acute Lung Injury.” *The Journal of Immunology* 184 (2):836–43. https://doi.org/10.4049/jimmunol.0901809.

Sell, Henrike, Jurga Laurencikiene, Annika Taube, Kristin Eckardt, Andrea Cramer, Angelika Horrighs, Peter Arner, and Jürgen Eckel. 2009. “Chemerin Is a Novel Adipocyte-Derived Factor Inducing Insulin Resistance in Primary Human Skeletal Muscle Cells.” *Diabetes* 58 (12):2731–40. https://doi.org/10.2337/db09-0277.

Serhan, C N, C B Clish, J Brannon, S P Colgan, N Chiang, and K Gronert. 2000. “Novel Functional Sets of Lipid-Derived Mediators with Antiinflammatory Actions Generated from Omega-3 Fatty Acids via Cyclooxygenase 2-Nonsteroidal Antiinflammatory Drugs and Transcellular Processing.” *The Journal of Experimental Medicine* 192 (8):1197–1204. https://doi.org/10.1084/jem.192.8.1197.

Serhan, Charles N. 2004. “A Search for Endogenous Mechanisms of Anti-Inflammation Uncovers Novel Chemical Mediators: Missing Links to Resolution.” *Histochemistry and Cell Biology* 122 (4):305–21. https://doi.org/10.1007/s00418-004-0695-8.

Tavazzi, Luigi, Aldo P Maggioni, Roberto Marchioli, Simona Barlera, Maria Grazia Franzosi, Roberto Latini, Donata Lucci, et al. 2008. “Effect of N-3 Polyunsaturated Fatty Acids in Patients with Chronic Heart Failure (the GISSI-HF Trial): A Randomised, Double-Blind, Placebo-Controlled Trial.” *Lancet (London, England)* 372 (9645):1223–30. https://doi.org/10.1016/S0140-6736(08)61239-8.

Additional Information

Competing financial interests

The authors declare no competing financial interests.

Table Legends

Table 1. Primer sequences used for RT-qPCR measurements of pro-inflammatory cytokine mRNA expression in the present study.

Figure Legends

Figure 1: Pro-inflammatory cytokine mRNA expression in response to 100 ng/ml LPS or LPS + R_vE_1 100 nM in comparison with an unstimulated control (CON). R_vE_1 attenuated the elevations in IL-6 and CcL-2 mRNA levels whilst having no impact on TNF-α and IL-1β. Time points were selected from previous experiments (Figure S1) Data displayed as mean ± SEM. * denoting $p<0.05$ between the condition and CON; # denoting $p<0.05$ between LPS and LPS+ R_vE_1.

Figure 2: Pro-inflammatory cytokine extracellular protein concentrations in response to 100 ng/ml LPS or LPS + RvE1 100 nM in comparison with an unstimulated control (CON) 24h post stimulation. RvE1 attenuated the elevation in IL-6 in the cell culture

medium following LPS stimulation, whilst having little effect on TNF-α, CcL-2 and IL-1β. Data displayed as mean ± SEM, * denoting $p<0.05$ between the condition and CON. # denoting $p<0.05$ between LPS and LPS+ R_vE_1.

Figure 3: Myotube morphology 72 hrs following acute (3 hrs) exposure to 100 ng/ml LPS. The presence of LPS alone or in combination with RvE1 has no effect on myotube number (a). In comparison, myotube atrophy occurs (b) following LPS exposure which is prevented by co-incubation with RvE1. Data are expressed as mean ± SEM. * denoting $p<0.05$ between the condition and CON. # denoting $p<0.05$ between LPS and LPS+RvE1.

Figure 4: Maximal contractile force from engineered muscles cultured for 72 hrs following acute (3 hrs) incubation with 100 ng/ml LPS or LPS + R_vE_1 100 nM. Incubation of engineered muscle with LPS resulted in impaired muscle function which was partially prevented by co-incubation with R_vE_1. All conditions were compared to CON controls within individual experiments to calculate relative force. Data are expressed as mean ± SEM * denoting $p<0.05$ between the condition and CON.