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Resolvin E1 (RvE1) attenuates LPS induced inflammation and subsequent atrophy in C2C12 myotubes

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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 (RvE1) 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, RvE1 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. RvE1 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 RvE1 has pro-resolving properties in this cell type. Our data provides
rationale for further investigation into the mechanistic action of RvE1 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-
flammatory 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
cellular based research 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 (RvE1) 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
RvE1 at nanogram concentrations has been reported in-vivo (Ohira et al., 2010) and
at these concentrations RvE1 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 RvE1 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
(II-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
(CC-L-2) were reduced to the largest extent when RvE1 was co-incubated with LPS
in 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 RvE1 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 RvE1 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 RvE1 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 CC-L-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 RvE1 to the cultures.
Thereafter we investigated if RvE1 could prevent inflammation induced myotube
atrophy and whether this would translate into 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% CO₂ (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/cm² 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 (RvE1): DM + 100 ng/ml LPS + 100 nM RvE1 (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 RvE1 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 RvE1, 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
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) RvE1 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-propanol (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 10 mins to pellet RNA. RNA pellets were washed in 75% ethanol, centrifuged for a further 5 mins 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 Fluoromount™
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 CaCl₂, 1.25 mM
MgSO, 5 mM Glucose, 0.05% Bovine Serum Albumin in dH₂O) 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≤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 RvE1 (100 nM; See Figure S2) were investigated. Selected doses during preliminary experiments were based on
previous literature identifying RvE1 at nano-molar concentrations in-vivo (Ohira et al., 2010). Cultures were simultaneously exposed to RvE1 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 RvE1, 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 RvE1 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 RvE1 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).
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, RvE1 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 RvE1 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 RvE1.

Addition of RvE1 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 RvE1 (Lund et al., 2010), though the current work showed contradictory effects on TNF-α. This suggests that RvE1 has tissue-specific effects however dampening of LPS induced myotube inflammation in our model highlights the efficacy of the use of RvE1 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 RvE1 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 RvE1 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 RvE1 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 RvE1 prevents decrements in maximal force production associated with LPS exposure. This data suggests that RvE1 associated attenuation of inflammation is capable of having positive effects on muscle phenotype (size and function).

The proposed mechanism of action by which RvE1 impacts the inflammatory resolution process has been shown to differ between cell types. Two cell surface receptors have been identified for RvE1; 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). RvE1 has been shown to display specific binding to ChemR23 with a Kd 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 RvE1 in skeletal muscle, future work needs to seek to understand whether beneficial effects of RvE1 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 RvE1 in this cell type. Future work should be directed towards investigating the specific binding potential of RvE1 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.
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### 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 + RvE1 100 nM in comparison with an unstimulated control (CON). RvE1 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 + RvE1.

*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+ RvE1.

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 + RvE1 100 nM. Incubation of engineered muscle with LPS resulted in impaired muscle function which was partially prevented by co-incubation with RvE1. 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.