Redox Biology 6 (2015) 253–259

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

SS-31 attenuates TNF-α induced cytokine release from C2C12 myotubes

Adam P. Lightfoot, Giorgos K. Sakellariou, Gareth A. Nye, Francis McArdle, Malcolm J. Jackson, Richard D. Griffiths, Anne McArdle *

MRC-Arthritis UK Centre for Integrated Research into Musculoskeletal Ageing, University of Liverpool, United Kingdom

ARTICLE INFO

Article history:
Received 26 May 2015
Accepted 7 August 2015
Available online 10 August 2015

Keywords:
Skeletal muscle
TNF
Superoxide
C2C12
Myokine

ABSTRACT

TNF-α is a key inflammatory mediator and is proposed to induce transcriptional responses via the mitochondrial generation of Reactive Oxygen Species (ROS). The aim of this study was to determine the effect of TNF-α on the production of myokines by skeletal muscle. Significant increases were seen in the release of IL-6, MCP-1/CCL2, RANTES/CCL5 and KC/CXCL1 and this release was inhibited by treatment with Brefeldin A, suggesting a golgi-mediated release of cytokines by muscle cells. An increase was also seen in superoxide in response to treatment with TNF-α, which was localised to the mitochondria and this was also associated with activation of NF-κB. The changes in superoxide, activation of NF-kB and release of myokines were attenuated following pre-treatment with SS-31 peptide indicating that the ability of TNF-α to induce myokine release may be mediated through mitochondrial superoxide, which is, at least in part, associated with activation of the redox sensitive transcription factor NF-kB.

1. Introduction

Skeletal muscle can act as an endocrine organ and studies have identified that a large number of cytokines are expressed and secreted by muscle [1–4], a process which is thought to occur via the golgi network in other cell types [5]. Cytokines secreted by muscle are termed myokines [6]. The patterns of proteins released by muscle can change under different conditions and the precise function of myokines is currently unclear. Research has focused particularly on the role of IL-6, which is released by muscle following exercise and also appears to function as an energy sensor [7].

Systemic inflammation is thought to result from the release of cytokines from immune cells, but little is known regarding the effects of systemic inflammation on muscle cytokine release and the role that muscle-derived cytokines plays in compounding the overall systemic inflammation [8]. During systemic inflammation, tissues, including muscle, are exposed to a storm of inflammatory mediators. The catabolic effects of systemic inflammation on muscle mass and strength have been studied in a wide range of pathologies, such as sepsis [9], cancer [10], COPD [11] and ageing [12]. The impact of systemic inflammation on skeletal muscle is profound, inducing rapid muscle atrophy [13] and weakness [14].

Tumour necrosis factor alpha (TNF-α) is a key mediator of skeletal muscle catabolism and dysfunction in systemic inflammatory conditions [15]. Evidence suggests that TNF-α induces the activation of the Nuclear factor kappa B (NF-kB) canonical pathway in skeletal muscle and that this plays a key role in the well characterised TNF-α mediated skeletal muscle atrophy and dysfunction [16–19]. Evidence that loss of total muscle protein occurs in response to treatment of muscle with TNF-α occurs in an NF-kB-dependent manner further strengthens this association [20]. The mechanisms by which TNF-α results in activation of NF-kB are, as yet, unclear, but it has been proposed that the process is mediated, at least in part, by endogenous reactive oxygen species (ROS) production [21], and that mitochondria are a potential source of this endogenous ROS [22,23].

Exposure of skeletal muscle cells to TNF-α results in the increased synthesis of a number of inflammatory proteins, including: CCL2, CCL5, CXCL5, VCAM-1 and IL-6 [1], ROS are also proposed to play a key role in augmenting this myokine production and release [24]. Thus, N-acetyl-cysteine (NAC) treatment of L6 myotubes results in the attenuation of TNF-α-induced IL-6 release [25]. However, the precise site of generation and nature of the ROS species involved in such TNF-α mediated signalling remains poorly defined, with most studies using non-specific methods for detection of ROS [26].

We hypothesised that treatment of muscle cells with TNF-α would result in activation of the canonical NF-kB pathway, resulting in increased in myokine content of muscle cells and golgi-

* Corresponding author. Fax: +44 151 706 5802.
E-mail address: mdcr02@liv.ac.uk (A. McArdle).

http://dx.doi.org/10.1016/j.redox.2015.08.007

mediated release of these myokines and that this process is likely mediated, at least in part, by mitochondria-derived superoxide. We further hypothesised that modulation of mitochondria-generated superoxide would reduce activation of NF-kB and reduce the TNF-α mediated release of myokines.

2. Methods

2.1. Materials and methods

2.1.1. Chemicals and reagents

Unless stated otherwise, all chemicals used in this study were obtained from Sigma Chemical Company, Dorset, UK. The SS-31 peptide was obtained from W.M. Keck Fdn. Biotechnology Resource Laboratory at Yale (New Haven, CT).

2.1.2. Cell culture and treatments

C2C12 myotubes [27] were grown in culture in 5% CO_2 saturation at 37 °C, in Dulbecco's Modified Eagles Medium supplemented with 10% foetal bovine serum (v/v), with: 2 mM L-glutamine (Sigma Aldrich, Dorset, UK), 50 i.u. penicillin and 50 μg/ml streptomycin (Sigma Aldrich, Dorset, UK). Myotubes were grown to 60–70% confluence, then differentiated in growth media supplemented with 2% horse serum [28]. Myotubes were treated at 7 days post-differentiation in serum-free media for 3 h with 25 ng/ml carrier-free recombinant murine TNF-α (R&D Systems, Abingdon, UK), with or without 1 h of pre-treatment with 5 μM SS-31 peptide or in the presence of 1 μM sodium salicylate as an inhibitor of NF-kB [17]. Myotube viability was assessed following treatment with TNF-α (25 ng/ml) for 3 h, with or without pre-treatment with SS-31 (5 μM) peptide, using LIVE/DEAD staining (Invitrogen, Paisley, UK) in accordance with the manufacturer's protocol. To determine the mechanism of cytokine release from C2C12 myotubes, cells were pre-treated for 1 h with 1 μg/ml Brefeldin A (BFA), to inhibit the golgi-mediated release of protein.

2.1.3. Use of MitoSOX Red to monitor mitochondrial superoxide

C2C12 myotubes were incubated in 2 ml Dulbecco's phosphate-buffered saline (D-PBS) containing 250 nM MitoSOX Red (Invitrogen, Paisley, UK) for 30 min at 37 °C [29]. Myotubes were washed twice with D-PBS and were maintained in MEM without Phenol Red during the experimental period. MitoSOX Red is a derivative of dihydroethidium (DHE) designed for the selective detection of superoxide in mitochondria and exhibits fluorescence (MitoSOX Red fluorescence) upon oxidation and subsequent binding to mitochondrial DNA (Fig. 1) [30].

The imaging system consisted of a C1 confocal laser-scanning microscope (Nikon Instruments Europe BV, Surrey, UK) equipped with a 405 nm excitation diode laser, a 488 nm excitation argon laser and a 543 nm excitation helium–neon laser. Emission fluorescence was detected through a set of 450/35, 515/30 and 605/15-

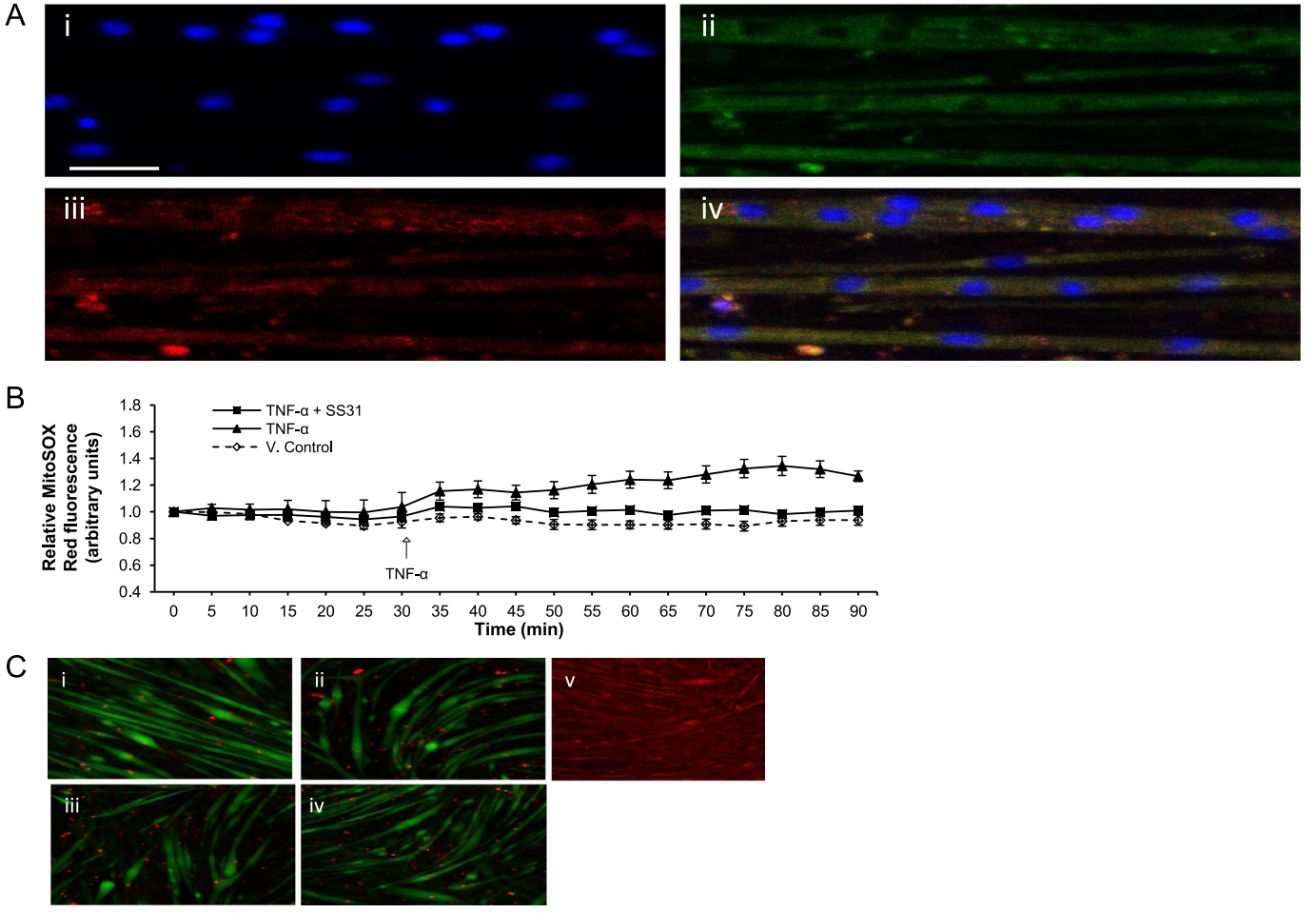

Fig. 1. (A) Confocal images of C2C12 myotubes. Fluorescent image following cells loaded with DAPI (i), fluorescence from MitoTracker Green FM (15 nM) (ii), fluorescent image from MitoSOX Red (iii) and a merged image of i, ii and iii (iv). 20× original magnification. (bar=40 μm). (B) Relative change in MitoSOX Red fluorescence from C2C12 myotubes either treated with TNF-α (25 ng/ml) alone, vehicle only (V control) or treated with TNF-α and the mitochondrial targeted SS-31 peptide (5 μM). (n=4–5 in each group). (C) Representative images of (i) control C2C12 myotubes or myotubes treated with (ii) TNF-α, (iii) SS-31 alone, (iv) pre-treated with SS-31 followed by treatment with TNF-α or (v) treated with 70% ethanol as a positive control, stained with LIVE/DEAD.

emission filters. Using a × 40 objective, fluorescence images were captured and analysed with the EZC1 V.3.9 (12 bit) acquisition software. To quantify the degree of co-localisation of fluorescent probes in C2C12 myotubes, NIH Image J software was used. Co-localisation coefficients: Pearson's correlation (Rr) coefficient, Mander's overlap (*R*) coefficient and Manders's co-localisation coefficient for each image; channel 1 (Mred) and channel 2 (Mgreen) were calculated over the entire confocal image [29].

2.1.4. RNA isolation and quantitative real-time PCR (qPCR)

RNA was isolated using standard Trizol® (Sigma Aldrich, Dorset, UK) extraction method [31] and purified using RNeasy clean-up kit (Qiagen); cDNA was synthesised using iScript first strand kit from 1 μg of isolated RNA (Bio-Rad, Hercules, USA). Primers were designed for the following genes: CCL2 (5′-TGAATGTGAAGTTGACCCGT-3′; 5′-TTAAGGCATCACAGTCCGAG-3′), CCL5 (5′-GTGCCCACGTCAAGGAGTAT-3′; 5′-CCCACTTCTTCTCTGGGTTG-3′), CXCL1 (5′-CTTGAAGGTGTTGCCCTCAG-3′; 5′-TCTCCGTTACTTGGGGACAC-3′), IL-6 (5′-AGGTGCTAAAGGGTCTCTTG-3′; 5′-TCCACGATTTCCCAGAGAAC-3′), S29 (5′-ATGGGTCACCAGCAGCTCTA-3′; 5′-GTATTTGCGGATCAGACCGA-3′). Targets were amplified from 1 μg of cDNA using SYBR Green master mix reagent and amplified using a Bio-Rad thermocycler (Bio-Rad icycler, Heracles, USA). The threshold cycle for target genes of interest was normalised to s29 and expressed as fold-change using the delta-delta ct ($2^{-\Delta\Delta ct}$) method.

2.1.5. Cytokine analyses

At 3 h following treatment of myotubes with TNF-α, serum-free cell culture media was analysed for the presence of cytokines using multiplex cytokine analysis (Bio-Rad, Hercules, USA). In brief, media was incubated with fluorescently dyed beads conjugated with monoclonal antibodies specific to IL-6, CCL2, CCL5 and CXCL1 (Bio-Rad, Hercules, USA). The bead-sample conjugate was then incubated with a biotinylated secondary detection antibody and a streptavidin-PE fluorophore. Samples were then analysed using a Luminex-200 platform using Bioplex software version 5 (Bio-Rad, Hercules, USA).

2.1.6. SDS-PAGE and Western blotting

Myotubes were washed, harvested, pelleted and re-suspended in ice-cold PBS, the cell lysate was sonicated and total protein content quantified using the BCA assay (Pierce, UK). Samples were prepared in 1% SDS, 1 mM Iodoacetamide, 1 mM benzithonium chloride, 5.7 mN phenylmethylsylphonyl fluoride. Fifty micrograms of total protein from C2C12 myotubes was applied to a 12% polyacrylamide gel with a 4% stacking gel (National Diagnostics, Atlanta, Georgia, USA). Separated proteins were transferred to a nitrocellulose membrane by Western blotting (Pharmacia, Uppsala, Sweden). The membranes were analysed using antibodies specific to IkappaB-alpha (IκBα) and beta-actin (Abcam Plc, Cambridge, UK) as previously described and incubated with species specific peroxidase-conjugated secondary antibodies (Sigma Aldrich, Dorset, UK). Enhanced chemiluminesence (Amersham, Cardiff, UK) was used to detect peroxidase activity. Bands were detected using a Bio-Rad Chemi-doc XRS system with QuantityOne software (Bio-Rad, Hercules, USA). The intensity of protein bands was quantified using densitometry [32].

2.1.7. Statistical analysis

Data was statistically analysed using Student's *t*-test and one-way ANOVA where appropriate using an alpha of $p \leq 0.05$ with Microsoft Excel and SPSS 20 software.

3. Results

3.1. Effect of treatment of myotubes with TNF-α and SS-31 on myotube viability

Myotube viability was determined using LIVE/DEAD staining (Invitrogen, Paisley, UK). Treatment of C2C12 myotubes with 25 ng/ml TNF-α resulted in no significant change in cell viability over the 3 h duration of the experiment compared with carrier (PBS) treated control myotubes (Fig. 1C).Treatment with SS-31 peptide alone, or in combination with TNF-α, did not result in any significant change in cellular viability compared with carrier treated control myotubes (Fig. 1C).

3.2. TNF-α-induced mitochondrial superoxide production: effect of SS-31 peptide

As expected MitoSOX Red fluorescence was co-localised with Mitotracker Green, indicating mitochondrial localisation (Fig. 1A). Myotubes incubated in the presence of TNF-α showed a small but significant increase in 2-hydroxyethidium (2-OH-E^+) fluorescence, following excitation at 405 nm, indicating an increase in superoxide production within the mitochondrial matrix. The TNF-α-induced increase in 2-OH-E^+ was reduced following prior incubation with SS-31 peptide (Fig. 1B).

3.3. TNF-α-induced changes in inflammatory gene expression: effect of SS-31 peptide

Elevated and substantial changes in the expression (fold-change) of CCL2 (59.1 ± 8.8), CXCL1 (2.1 ± 0.35), CCL5 (137.1 ± 24.6) were seen in myotubes following treatment with TNF-α compared with untreated cells; no change was detected in IL-6 expression (Fig. 2). Such dramatic fold-changes in mRNA were likely to be due to the very low levels of expression in untreated cells. Pre-treatment of C2C12 myotube with SS-31 peptide prior to TNF-α challenge resulted in attenuated expression of CCL2 but no effect of SS-31 was observed on the TNF-α mediated increase in mRNA for CCL5 or CXCL1 (Fig. 2).

3.4. TNF-α-induced myokine release: effect of SS-31 peptide, sodium salicyate and BFA

Treatment of C2C12 myotubes with TNF-α resulted in a significant release of IL-6, CXCL1, CCL2 and CCL5 into the media, compared with that of untreated control myotubes (Fig. 3). No detectable levels of the following: FGF-Basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, MIG & MIP1-α were seen in the media. Pre-treatment of myotubes with SS-31 peptide resulted in an attenuation in the release of all four cytokines measured compared with the levels released from myotubes treated with TNF-α alone (Fig. 3). Pre-treatment of myotubes with sodium salicyate (as an inhibitor NF-kB activation) abolished the TNF-α mediated increase in cytokine release (Fig. 3). Pre-treatment with BFA (to block golgi-mediated release) for 1 h prior to treatment with TNF-α also abolished cytokine release from C2C12 myotubes (Fig. 4).

3.5. TNF-α-induced activation of NF-kB in C2C12 myotubes: effect of SS-31 peptide

A decrease in IκBα protein was seen in myotubes following treatment with TNF-α, suggesting activation of NF-kB; this decrease in IκBα was not evident when cells were treated with SS-31 peptide prior to treatment with TNF-α (Fig. 5).

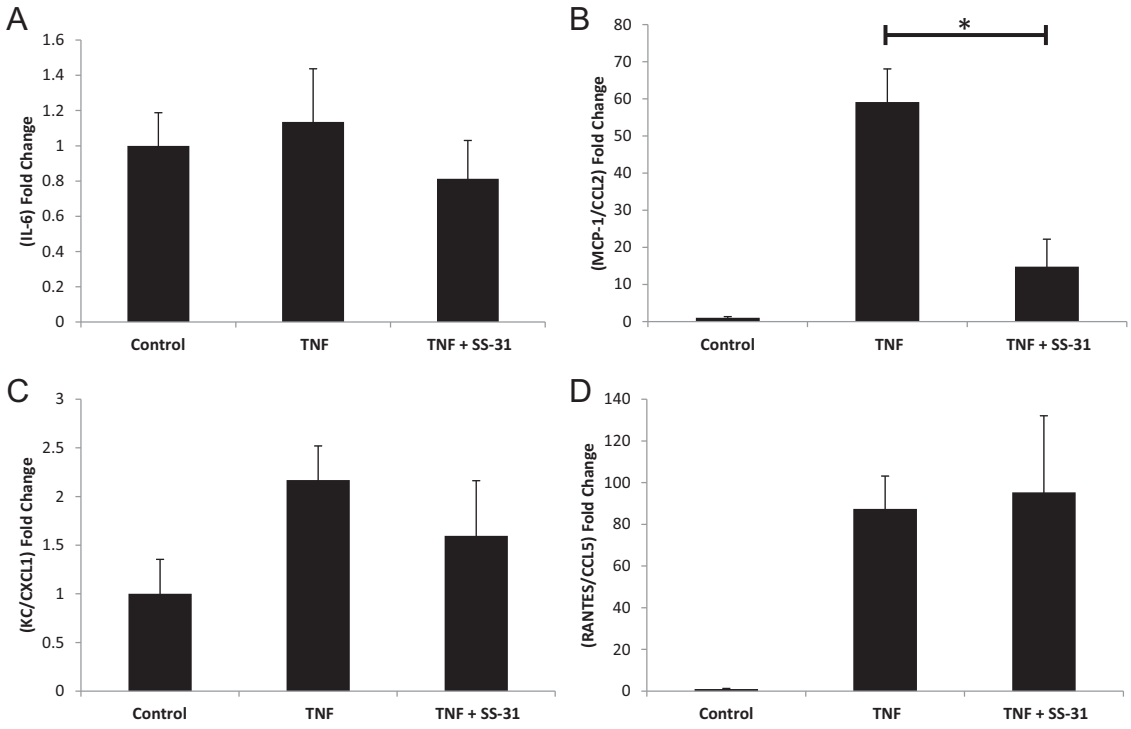

Fig. 2. Fold changes in gene expression for IL-6 (A), MCP-1/CCL2 (B), CXCL1 (C) and RANTES/CCL5 (D) in myotubes following treatment with TNF-α (25 ng/ml) with or without 1 h pre-treatment with SS31 (5 μM). Data are represented as mean fold change normalised to s29 housekeeper gene ± SEM, $^{*}p < 0.05$ ($n = 4$–6 in each group).

4. Discussion

The ability of skeletal muscle to act as a source of cytokines (myokines) is an area of increasing research interest. Such research has focused on the beneficial systemic effects of the production of anti-inflammatory cytokines by muscle, particularly during exercise [33]. However, less is known about the mechanisms responsible for muscle cell cytokine production and release. The

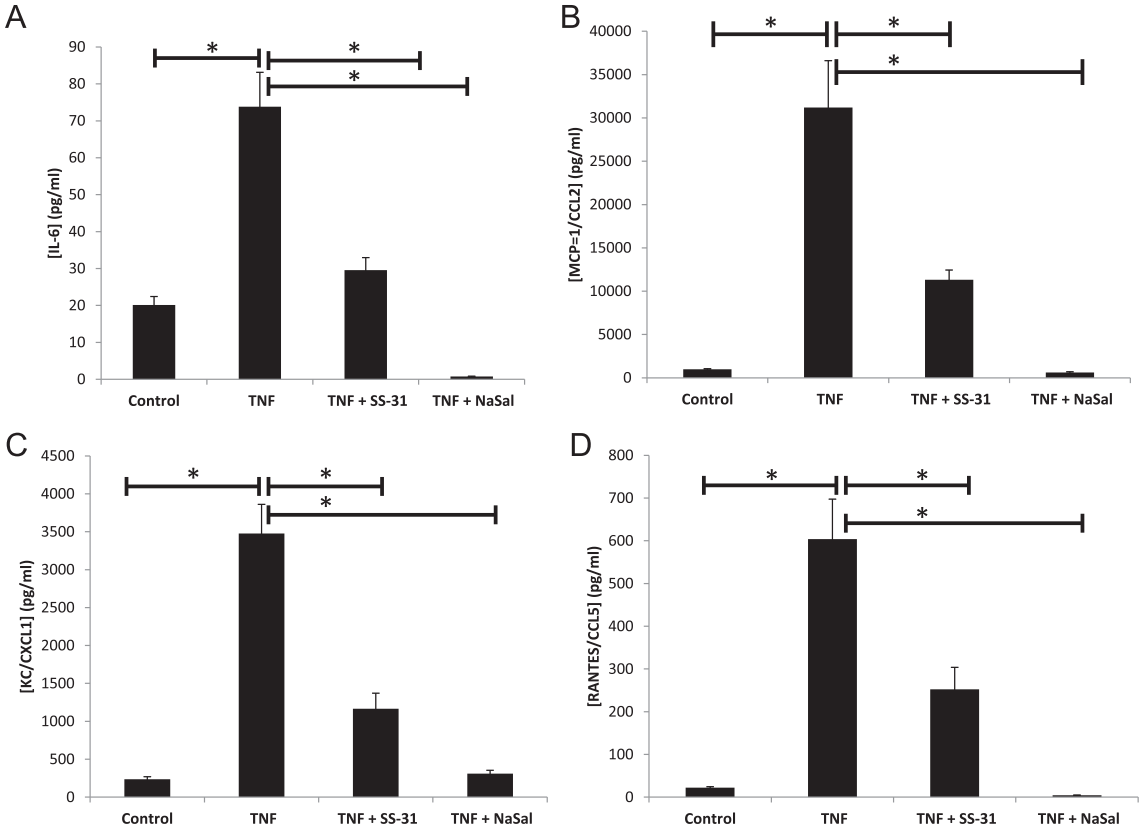

Fig. 3. Levels of (A) IL-6, (B) MCP-1/CCL2, (C) KC/CXCL-1 and (D) RANTES/CCL5 in media of C2C12 myotubes at 3 h following treatment with TNF-α (25 ng/ml) or following pre-treatment for 1 h with SS-31 (5 μM) or sodium salicylate (1 μM) followed by treatment with TNF-α for 3 h.

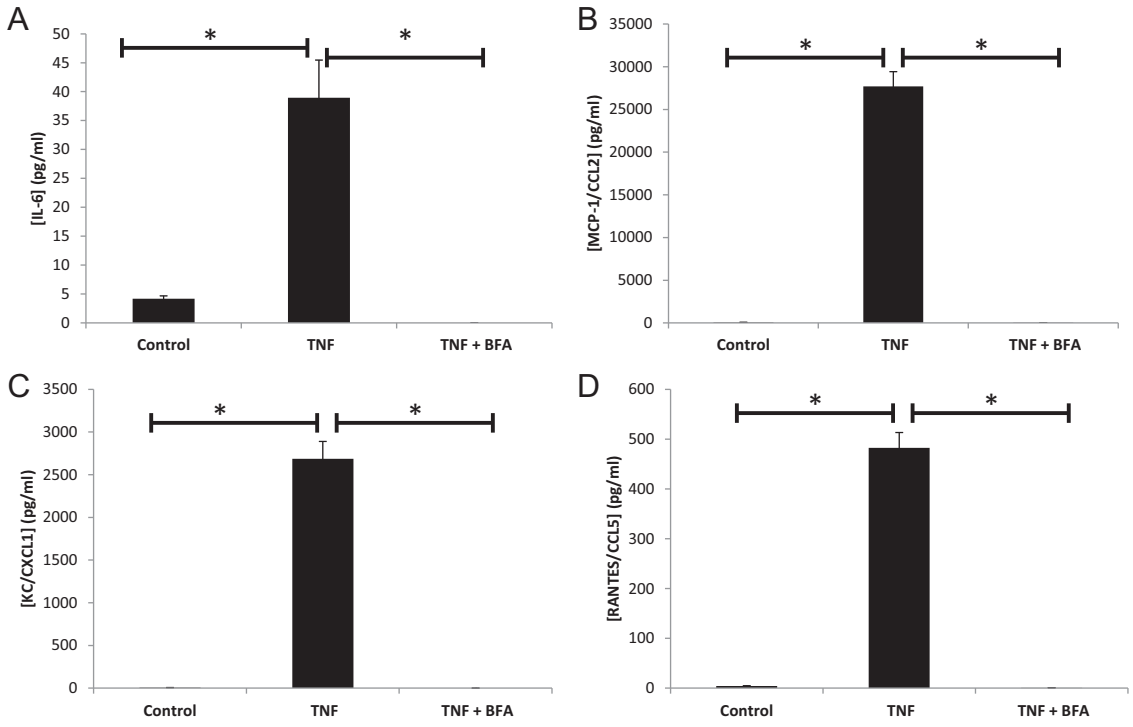

Fig. 4. Levels of (A) IL-6, (B) MCP-1/CCL2, (C) KC/CXCL-1 and (D) RANTES/CCL5 in media of C2C12 myotubes at 3 h following treatment with TNF-α (25 ng/ml), or pre-treated for 1 h with Brefeldin A (1 μg/ml) followed by TNF-α for 3 h. Data are represented as mean ± SEM ($n=5$–6 in each group), $^{*}p<0.05$.

aims of the current study were to determine the role that ROS generation, in particular mitochondria-derived superoxide, plays in the production of myokines by C2C12 myotubes following treatment with TNF-α and to examine whether NF-kB activation mediated this process. Changes in cytokine profiles in the local environment may be particularly important to skeletal muscle, where they may have effects on satellite cells, neurons and other cells. Since skeletal muscle is the largest organ system and protein store in the human body, skeletal muscle may also be a significant source of cytokine generation in particular situations. Thus, determining the mechanisms responsible for myokine release from muscle may have important implications for the role of muscle in systemic inflammation, with the potential to develop novel therapeutic interventions to modulate this release.

We hypothesised that treatment of muscle cells with TNF-α would result in the golgi-mediated release of a number of myokines and that this process is likely mediated, at least in part, by mitochondria-derived superoxide leading to activation of the canonical pathway of the redox-sensitive transcription factor NF-kB. We further hypothesised that modulation of mitochondria-generated superoxide would reduce activation of NF-kB and reduce the TNF-α mediated release of myokines.

Treatment of muscle cells with TNF-α resulted in the golgi-mediated release of myokines. Large gene-array screening has identified a plethora of inflammatory genes expressed in skeletal muscle [1,34]. Upregulation of CCL2, IL-6, CXCL-1 and CCL5 gene expression in response to TNF-α in this study support prior findings [1]. Our data also demonstrate a significant increase in the

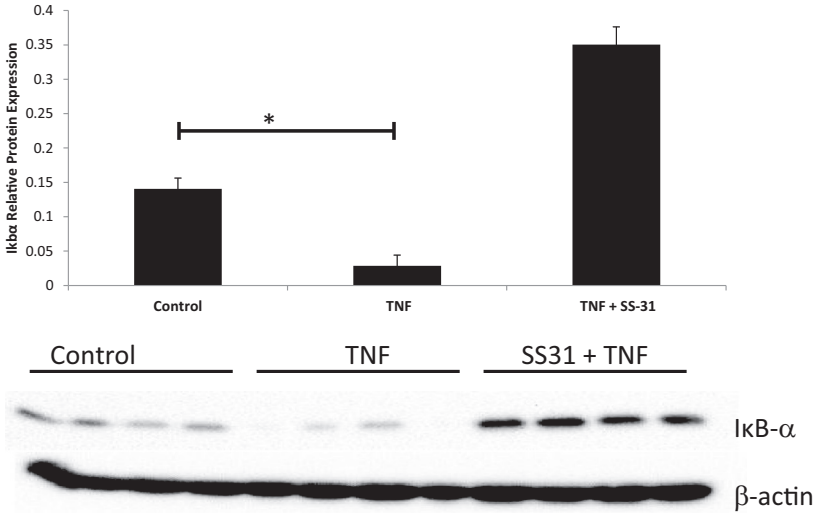

Fig. 5. Densitometry and representative Western blot image showing levels of IκBα and β-actin (housekeeper) in C2C12 myotubes treated with TNF-α (25 ng/ml) or pre-treated with SS-31 peptide) followed by TNF-α for 3 h, ($n=4$).

release of IL-6, CCL2, CCL5 and CXCL-1 from C2C12 myotubes at three hours following TNF-α treatment, in a golgi-mediated process, demonstrating that skeletal muscle can release a diverse profile of pro-inflammatory cytokines in response to TNF-α exposure. These results suggest that these muscle-derived cytokines may contribute significantly to the established systemic inflammatory state in circumstances where TNF-α is elevated. The precise physiological roles of myokines are poorly understood. Particularly, the evidence surrounding IL-6 is somewhat controversial. Exercise studies have clearly shown that skeletal muscle is a significant source of IL-6 and that muscle-derived IL-6 may have beneficial anti-inflammatory effects [4]. This anti-inflammatory function of IL-6 was observed following infusion of recombinant IL-6 in humans, which suppressed endotoxin-induced elevations in TNF-α [35]. In contrast, elevated circulating levels of IL-6 have been reported to cause skeletal muscle atrophy, primarily by disruption of protein synthesis pathways due to down-regulation of ribosomal s6 kinase expression [36]. Similarly, IL-6 inhibition using monoclonal antibodies was seen to improve survival in a rodent sepsis model [37]. Although these findings appear contradictory, it has been suggested that the most beneficial effect of IL-6 is through acute elevations in this cytokine (via exercise), compared with the deleterious effects of chronic increases [4], highlighting the importance of considering the degree to which muscle contributes to overall circulating levels of IL-6 in chronic pathological situations (e.g. critical illness and ageing) relative to acute situations (e.g. periods of short lived exercise).

Our observation of increased CCL2 and MCP-1 release from myotubes in response to TNF-α treatment supports previous observations [38]. The functions of CCL2 are reported as rather diverse, elevated levels have been detected in skeletal muscle in idiopathic myopathies [39] and in dystrophies [40]. There is also a correlation between serum levels of CCL2 and age-related muscle weakness [41]. In contrast, a murine CCL2 knockout model showed impaired muscle regeneration following ischaemia–reperfusion injury, inferring that this protein may have an important role in skeletal muscle repair processes [42]. Mechanistic-based analysis has suggested an important beneficial role for CCL2 [42]. Often in the case of skeletal muscle, cytokines are associated with dysfunction. There thus appears to be a "trade-off" between beneficial acute effects of myokines and deleterious effects of their chronic exposure. In a similar manner to CCL2, our observed release of CCL5/RANTES from myotubes following exposure to TNF-α supports previous study of the muscle secretome [38] and data from studies in inflammatory myopathies [39]. Additionally, macrophage-derived CCL5 in cardiotoxin injury has been reported to provoke T-cell infiltration into skeletal muscle and to impair regeneration [43].

CXCL-1/KC is a potent neutrophil chemoattractant and as far as we are aware, the observation of elevated CXCL-1 release from C2C12 myotubes is a novel finding since previous studies have focused on gene expression [44]. Previous studies have demonstrated that TNF-α exposure induces immune cell infiltration into skeletal muscle in vivo and our findings that muscle secretes CCL2/CCL5/CXCL-1 in a redox-dependent manner, provide further insight into the mechanistic control of inflammation-induced muscle dysfunction [45].

C2C12 myotubes show increased mitochondrial matrix superoxide generation and activation of NF-kB following treatment with TNF-α. The mechanisms involved in myokine regulation and secretion by muscle are poorly understood, however, it has been hypothesised that ROS may play a key role in these processes [24]. Studies demonstrated that IL-6 release from L6 myotubes was attenuated following intervention with N-acetylcysteine (NAC) treatment, thus demonstrating a role for ROS generation in the cytokine release process [25]. Studies in C2C12 myotubes have indicated that TNF-α induces direct changes in mitochondria-derived free radical species [23]. Based on this we focused our investigation on mitochondrial superoxide. Cells were loaded with MitoSOX Red. Control muscle cells showed no significant changes in fluorescence over the time course of the study. However, cells incubated in the presence of TNF-α showed a significant increase in 2-hydroxyethidium (2-OH-E^+) fluorescence, indicating an increase in superoxide production within the mitochondrial matrix. Thus, data clearly suggest that mitochondrial superoxide production is associated with TNF-α-induced myokine release from skeletal muscle cells. Previous studies have demonstrated that this association is likely to be focused on the redox-sensitive transcription factor NF-kB [21,46]. Thus, we examined degradation of IκBα as an index of activation of the NF-kB pathway [18]. IκBα levels in C2C12 myotubes were decreased following treatment with TNF-α (Fig. 4); an observation commensurate with the degradation of IκBα by the ubiquitin-proteasome upon stimulation and activation of the NF-kB pathway [47]. Previous studies have indicated ROS involvement downstream of TNF-α, whereby catalase prevented TNF-induced NF-kB activation in muscle cells [21]. Moreover, inhibition of complex I resulted in suppressed TNF-induced activation of NF-kB [46].

SS-31 mediated attenuation of superoxide production, NF-kB activation and myokine release from C2C12 myotubes in response to TNF-α. SS-31 peptide is a mitochondrial targeted antioxidant peptide that accumulates on the inner mitochondrial membrane. SS-31 has been proposed to scavenge superoxide [48,49], although this function of SS-31 is controversial, with more recent evidence that SS-31 preserves mitochondrial function by preserving mitochondrial bioenergetics [50]. Treatment of muscle cells with TNF-α resulted in increased 2-OH-E^+ fluorescence and this increase was reduced following treatment with SS-31 although whether this is a direct effect of SS-31 or an indirect effect of SS-31 on optimising mitochondrial function remains unclear.

Observations support previous findings that ROS may mediate the TNF-α-induced NF-κB activation in skeletal muscle [21]. Moreover, cells pre-treated with sodium salicylate, an inhibitor of NF-κB activation [17] (possibly by ROS scavenging mechanisms) demonstrated a significant attenuation in the levels of cytokines released in response to TNF-α treatment. These data suggest that the SS-31 attenuated myokine release from muscle could, at least in part, be mediated by NF-κB although the observation that synthesis of all cytokines did not appear to be affected by this treatment is somewhat confusing, potentially suggesting that a threshold level may be needed before myokines are released by muscle cells. Data support previous studies which used broad spectrum antioxidants, which demonstrated that ROS generation plays a role in cytokine release by muscle cells [25]. The results presented here suggest that mitochondrial superoxide (and potentially subsequent H_2O_2) may be a key ROS contributing to TNF-α-induced myokine release. For example, if chronic exposure to IL-6 is the main driver of the deleterious effects of IL-6 on muscle, the use of specific targeted antioxidants may be a viable approach to attenuate chronically elevated IL-6 levels, thus preserving muscle mass and function.

5. Conclusions

We have demonstrated that treatment of skeletal muscle cells with TNF-α induces an increase in ROS production, of which mitochondrial superoxide is a significant component. We have also identified that the cytokines IL-6, CXCL-1, CCL2 and CCL5 are released from muscle in response to treatment with TNF-α. The TNF-α-induced increased release of these myokines is mediated, at least in part, by mitochondrial superoxide and the redox-sensitive

transcription factor NF-κB. Our study results potentially provide mechanistic insights into cytokine production and release by skeletal muscle cells.

Acknowledgements

The authors thank the Medical Research Council for their generous financial support and Dr M.B Reid (University of Florida, College of Health and Human Performance, USA) for the kind gift of the SS-31 peptide.

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