Muscle atrophy in patients with Type 2 Diabetes Mellitus: roles of inflammatory pathways, physical activity and exercise

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ABSTRACT

Muscle atrophy is caused by an imbalance in contractile protein synthesis and degradation which can be triggered by various conditions including Type 2 Diabetes Mellitus (T2DM). Reduced muscle quality in patients with T2DM adversely affects muscle function, the capacity to perform activities of daily living, quality of life and ultimately may increase the risk of premature mortality. Systemic inflammation initiated by obesity and prolonged overnutrition not only contributes to insulin resistance typical of T2DM, but also promotes muscle atrophy via decreased muscle protein synthesis and increased ubiquitin-proteasome, lysosomal-proteasome and caspase 3-mediated protein degradation. Emerging evidence suggests that the inflammation-sensitive Nuclear Factor κ B (NF-κB) and Signal Transducer and Activator of Transcription 3 (STAT3) pathways may contribute to muscle atrophy in T2DM. In contrast, exercise appears to be an effective tool in promoting muscle hypertrophy, in part due to its effect on systemic and local (skeletal muscle) inflammation. The current review discusses the role inflammation plays in muscle atrophy in T2DM and the role of exercise training in minimising the effect of inflammatory markers on skeletal muscle. We also report original data from a cohort of obese patients with T2DM compared to age-matched controls and demonstrate that patients with T2DM have 60% higher skeletal muscle expression of the atrophy transcription factor FoxO1. This review concludes that inflammatory pathways in muscle, in particular, NF-κB, potentially contribute to T2DM-mediated muscle atrophy. Further in-vivo and longitudinal human research is required to better understand the role of inflammation in T2DM-mediated atrophy and the anti-inflammatory effect of exercise training under these conditions.

Key words: Skeletal muscle, inflammation, cytokines, training

ATROPHIC SIGNALLING IN SKELETAL MUSCLE

Muscle atrophy occurs in response to many insults, including prolonged disuse, ageing and chronic disease such as Type 2 Diabetes Mellitus (T2DM) (30, 73, 92). Muscle atrophy is the result of a negative balance between the rate of contractile protein synthesis and degradation. In catabolic conditions, muscle atrophy in combination with inactivity can decrease the capacity to perform activities of daily living, quality of life and subsequently increase mortality (84, 146). The ubiquitin-proteasome, autophagy-lysosome and caspase-3-mediated proteolytic pathways are responsible for protein degradation in muscle and thus contribute to muscle atrophy (Figure 1) (73, 110). In healthy muscle, the degradation of damaged or unfolded proteins is vital for the maintenance of cellular homeostasis (73, 77). In atrophic conditions such as disuse or diabetes, however, prolonged increased activity of these pathways increases the rate of contractile protein degradation, ultimately leading to muscle atrophy (30, 44, 73). In addition, decreased protein synthesis is apparent in T2DM and disuse, primarily through decreased activation of the mammalian target of rapamyacin (mTOR) pathway (Figure 1) (13, 34).

A variety of genes, collectively termed “atrogenes”, are involved in muscle atrophy (74, 111). Perhaps the most prominent of these muscle atrogenes are two E3 ubiquitin ligases, muscle RING finger 1 (MuRF1, or TRIM63) and Atrogin-1 (also known as MAFbx or FBXO32) (17, 48). These

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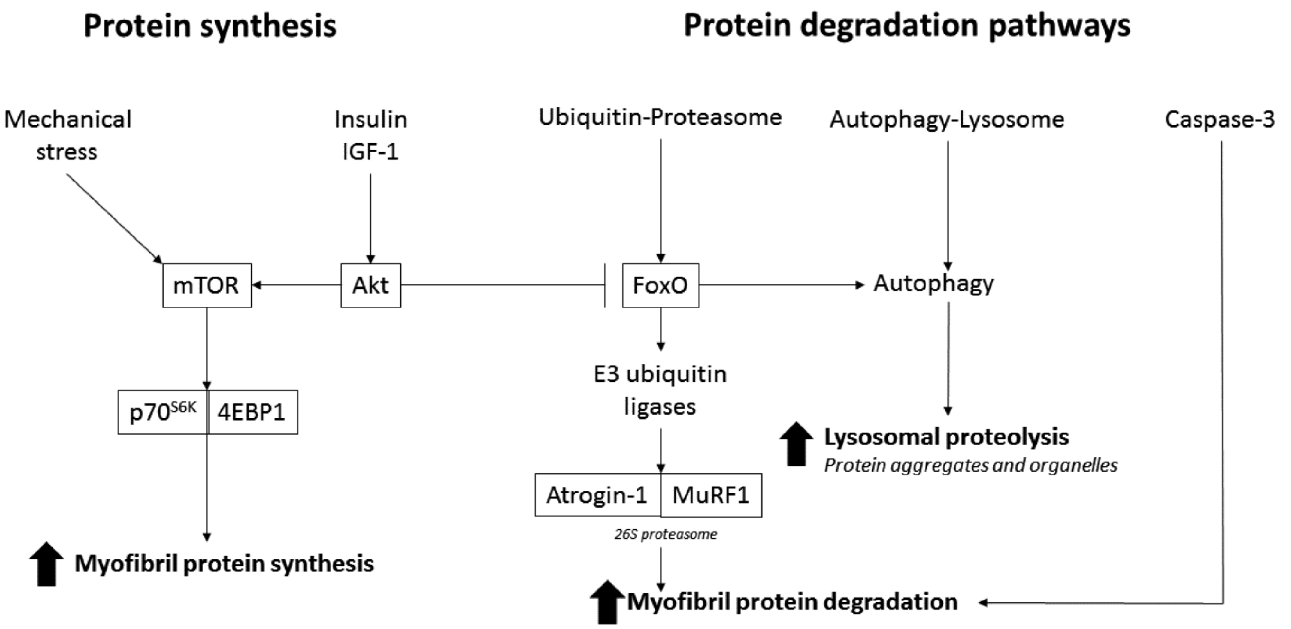

Figure 1: Protein synthesis and degradation pathways in skeletal muscle. Arrows represent activation, capped lines represent inhibition. Abbreviations: mTOR, mechanistic target of rapamycin; $p70^{S6k}$, $p70^{S6}$ kinase; IGF-1, insulin-like growth factor 1; FoxO, forkhead box O transcription factor; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1.

atrogenes are key components of the ubiquitin-proteasome system and are activated by the atrophy-related transcription factors, forkhead box O family transcription factors 1 and 3a (FoxO 1 and 3a) (85, 111, 144). In mice, global deletion of either Atrogin-1 or MuRF1 attenuated denervation-mediated atrophy (17), whereas Atrogin-1 and MuRF1 protein and mRNA were increased in hind-limb unloading (17), dexamethasone treated myotubes (145), and cancer cachexia (74). It is not yet clear, however, whether MuRF1 or Atrogin-1 are chronically upregulated in humans with catabolic conditions (38, 140). The activation of FoxOs, Atrogin-1 and MuRF1 may be an earlier and potentially transient maladaptation in some atrophic conditions. In streptozotocin (STZ)-induced type 1 diabetes, upregulation of Atrogin-1 and MuRF-1 mRNA is apparent only up to 3 weeks post injection in mice and rats (28, 36, 72). These findings suggest that the timing of the experiment is crucial for identifying atrophic markers and may explain disparities observed between studies. Similarly, short durations of disuse in humans (<10 days) resulted in elevated Atrogin-1 and MuRF1 mRNA (1, 22, 34, 122), whereas no change was seen after 2 weeks (1, 19, 34). Taken together, these results suggest that whilst the acute regulation of FoxO1 and 3a, Atrogin-1 and MuRF1 *in vivo* and *in vitro* is relatively well understood in atrophic conditions, the time-course of maladaptations to these important atrogenes in humans with catabolic conditions is not completely understood. This is likely due to the complex interactions between disease/condition duration, medication usage, physical activity levels, and muscle atrophy.

T2DM AS AN INFLAMMATORY DISEASE

Insulin resistance is defined by a reduction or inability of insulin stimulated glucose uptake in insulin target tissues (35). Insulin resistance in skeletal muscle, which is seen in T2DM and obesity, has substantial adverse effects on glucose metabolism as it is a major site of glucose uptake and disposal in response to insulin (16, 42). Insulin resistance may be caused by several mechanisms, including chronically increased production of reactive oxygen species and mitochondrial dysfunction (60, 108, 117), endoplasmic reticulum (ER) stress (40), lipotoxicity, and glucotoxicity (37). The mechanisms by which these factors lead to the development of T2DM may be in part mediated by the activation of inflammatory pathways, or exacerbated by inflammation (40, 59, 117). Indeed, higher circulating C-reactive protein (CRP), and various pro-inflammatory cytokines such as tumour necrosis factor α (TNF α) and interleukin 6 (IL-6) are observed in patients with T2DM (55, 102, 118). Furthermore, in muscle from obese T2DM patients, a range of pro-inflammatory pathways are upregulated (Figure 2), including chemokine (c-c motif) ligand (CCL2) (100), signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signalling 3 (SOCS3), and nuclear factor κ B (NF-κB) (87, 107, 119).

The role of cytokines in insulin resistance

T2DM promotes increased levels of pro-inflammatory cytokines such as TNFα and IL-6, which may be involved in the development of insulin resistance in skeletal muscle (41, 52, 114). Increased systemic levels of TNFα in T2DM reduces the level of inhibitor of NF-κB (IKB), that subsequently upregulates NF-κB and the c-Jun N-terminal kinases (JNK) pathways in muscle, resulting in insulin resistance through the inhibition of insulin receptor substrate 1 (IRS-1; Figure 2) (7, 52, 103). While increased TNFα has been shown to promote muscle insulin resistance acutely in some studies in humans and *in vitro* (33, 103), several studies contest the functional importance of TNFα independently on insulin resistance as pharmaceutical blockade of TNFα did not alter glycaemic control in humans (15, 39, 83). Another pro-inflammatory cytokine, IL-6, is elevated in obese individuals (40). Activation of the IL-6 receptor in muscle can activate the janus kinase (JAK)/STAT3 pathways that inhibits IRS-1 and subse-

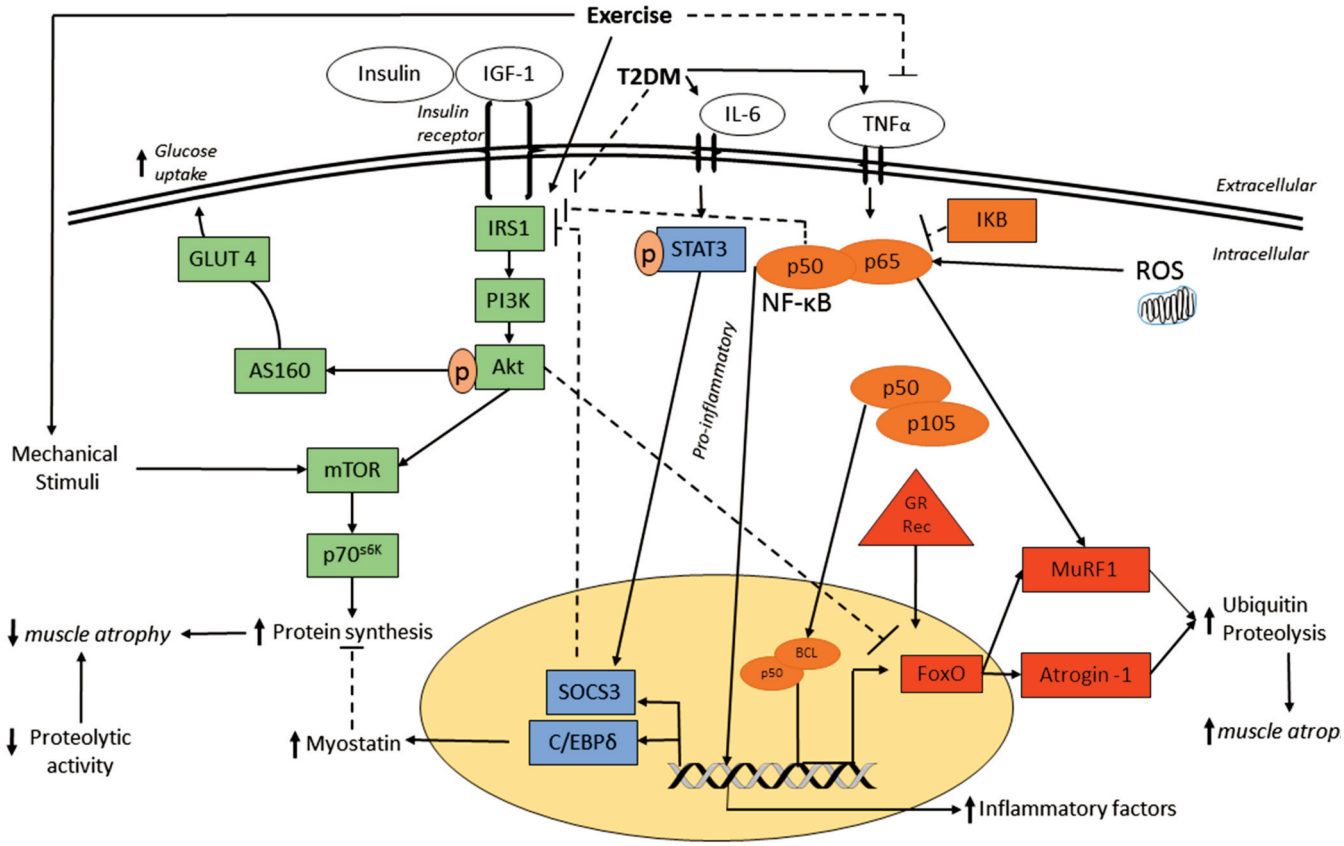

Figure 2: Insulin and inflammatory signalling and their potential signalling in protein synthesis and degradation in Type 2 Diabetes Mellitus (T2DM) and exercise training. Solid lines denote activation, dotted lines represent inhibitory effect. Some of these pathways, such as the p50-p105-BCL pathways are untested in T2DM. Abbreviations: IRS1, Insulin receptor substrate 1; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; SOCS3, Suppressor of cytokine signalling 3; C/EBPδ, CCAAT-enhancer-binding protein δ; STAT3, Signal transducer and activator of transcription 3; NF-κB, Nuclear factor κ B; IKB, Inhibitor of NF-κB; ROS, reactive oxygen species; GR Rec, Glucocorticoid receptor.

quent insulin-mediated glucose uptake through SOCS3 (Figure 2) (68). Initial studies investigating the role of IL-6 indicated that it reduced whole body insulin sensitivity and impaired muscle glucose uptake via reduced activity of the IRS-1 and Phosphatidylinositide 3-Kinase (PI3K) pathway (14, 67). The role of IL-6, however, remains unclear as several studies have reported there is no connection between IL-6 and insulin resistance in skeletal muscle (25, 68, 141). Furthermore, humans infused with IL-6 had increased muscle glucose uptake, possibly via activation of AMPK (25), a finding replicated in rodent muscle (141). The divergent and tissue specific actions of IL-6 might reflect secondary inflammatory signalling effects that are tissue-specific (141). The controversies surrounding whether IL-6 or TNFα are involved in the mechanism of insulin resistance in muscle suggests that changes in the level of individual cytokines may not be sufficient to cause insulin resistance. Further, secondary tissue-specific inflammatory signalling in response to cytokines along with the duration of elevations in systemic cytokines are important factors to be considered.

THE LINK BETWEEN INFLAMMATION AND MUSCLE ATROPHY IN T2DM

Patients with T2DM exhibit muscle atrophy that is initially mild in middle age (12, 124), and becomes more substantial with older age and diabetic neuropathy (6, 69, 75, 99). This loss of muscle leads to decreased strength, functional capacity and ultimately increased mortality in patients with T2DM (26, 75, 105). The following sections discuss the potential atrophic pathways in the muscle of patients with T2DM that are dysregulated via inflammatory processes, and conclude with novel data obtained from a small cohort of older age, obese patients with T2DM and age-matched controls

Insulin resistance promotes atrophy signalling in T2DM

Insulin resistance that is at least partially derived from systemic inflammation in T2DM and obesity is a key contributor to muscle atrophy signalling. The specific activity of Akt kinase in response to insulin was reduced by 34% in patients with T2DM compared to healthy controls (70). This impairment of the PI3K-Akt pathway has been implicated in decreasing both insulin mediated glucose uptake and protein synthesis in rodents and patients with T2DM (13, 18, 70, 121). The primary regulator of protein synthesis in skeletal muscle is the activation of mammalian target of rapamycin (mTOR), which is activated by Akt via insulin or insulin-like growth factor 1 (IGF1) and mechanical stimuli (18, 50, 131). However, the Akt-mTOR pathway also interacts with the ubiquitin-proteasome and autophagy-lysosome pathways (18, 85, 110, 111). Reduced activation of Akt decreases the phosphorylation of the FoxO transcription factors, which leads to their nuclear translocation and subsequent increase in the transcription of MuRF1 and Atrogin-1 (Figure 2) (9, 47, 70, 111). In non-diabetic haemodialysis patients, insulin resistance was

associated with an increased rate of muscle protein degradation (115). Further, obese *db/db* mice exhibit muscle atrophy, up to 43% increase in the rate of protein degradation, and insulin resistance compared to lean controls (95, 128). Administration of the insulin-sensitising drug, Rosiglitazone, to obese *db/db* mice recovered the maladaptations to the insulin signalling cascade and the ubiquitin-proteasome system, but only partly reversed the difference in muscle cross sectional area compared to controls (128). Rosiglitazone alleviates insulin resistance via several pathways, including increased circulating adiponectin (137, 138), and decreased levels of IL-6 and TNFα (71, 89). The reduction of these pro-inflammatory cytokines (IL-6 and TNFα) may attenuate muscle atrophy signalling through mechanisms independent of insulin signalling, such as reduced activation of the pro-inflammatory transcription factor NF-κB, which is discussed in later in this review (62).

Non-esterified fatty acids (NEFA) cause insulin resistance in muscle through elevations in intramuscular diacylglycerol (DAG) and ceramides (2, 57, 91, 123), which can lead to insulin resistance via upregulation of the NF-κB inflammatory pathway through activation of the JNK pathway (3, 56, 103, 132). The role of DAG and ceramides in promoting insulin resistance through the NF-κB and JNK pathways in muscle has been reliant on cell culture or animal models of T2DM and obesity, and although research in humans with T2DM and obesity is sparse, it appears to support the role of NF-κB and JNK in NEFA mediated insulin resistance (82). NEFA also promote muscle atrophy-related signalling and protein degradation *in vitro* through impaired activation of the PI3K-Akt pathway (21, 133). In cultured myotubes, Akt phosphorylation was reduced with incubation of 500 μM palmitate (NEFA), a concentration similar to that found in the plasma of patients with T2DM (112). The reduced Akt phosphorylation coincided with increased FoxO3a nuclear localisation, increased Atrogin-1 mRNA, and an increase in the rate of cellular protein degradation (21, 133). Further, the upregulation of FoxO3a in palmitate-treated cells increased the autophagy markers BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and microtubule-associated proteins 1A/1B light chain 3B (LC3a/b), suggesting that palmitate alone upregulates both the ubiquitin-proteasome and lysosome-autophagy systems through the impairment of Akt signalling. Only high levels of NEFA, like palmitate, induce atrophic signalling, whereas incubation of cells with non-saturated fats, such as docosahexaenoic acid and linoleic acid, prevent NEFA-induced muscle atrophy signalling (21, 27, 133). It is important to note that NEFA-induced muscle atrophy research is a relatively new area of research, and the direct role of palmitate-induced changes to the rate of protein synthesis, inflammatory pathways, autophagy, and induction of ER stress in regard to muscle atrophy signalling requires further investigation.

Whilst impaired insulin signalling in muscle is an important contributor to both decreased protein synthesis, via the Akt-mTOR pathway and activation of proteolysis, reduced insulin action alone may not be sufficient to cause muscle atrophy. Adrenalectomy prevented the increase in protein degradation caused by an acute STZ injection in rodents despite downregulation of the PI3K-Akt pathway (61). Although rodent models of diabetes, such as *db/db* mice, have substantial insulin resistance and muscle atrophy (128), other diabetic rodent models such as TallyHo mice become insulin resistant without muscle atrophy (95). This differential response between the *db/db* and TallyHo models may have occurred due to the higher circulating glucocorticoids and inflammatory cytokines in obese *db/db* mice; although this explanation is speculative and needs to be investigated further. Thus, whilst inflammation is a key mediator of insulin resistance which subsequently impairs protein synthesis and degradation signalling, T2DM induced atrophy is likely to involve other contributing signalling factors. Such factors could include increased circulating glucocorticoids, cytokines, NEFA, and upregulation of tissue-specific inflammatory pathways (23, 61, 62, 127, 143).

Pro-inflammatory muscle pathways and atrophy signalling in T2DM

In addition to their role in insulin resistance, increased circulating pro-inflammatory cytokines and NEFA directly upregulate several inflammatory pathways, such as the NF-κB and STAT3 pathways (Figure 2), leading to increased activation of the ubiquitin proteasome system (23, 96, 143). NF-κB is a protein complex comprising a family of proteins which share the Rel homology domain, allowing for nuclear translocation, DNA binding, binding to other NF-κB subunits and interaction with the inhibitor of NF-κB, IKB (10). Whilst NF-κB exists as mono- and hetero-dimer proteins, the p50/p65 heterodimer of NF-κB is the most important for transcription of canonical target genes (10). However, an alternative p105-p50-B-cell lymphoma 3-encoded protein (BCL3) transcription pathway has also recently been described that contributes to disuse atrophy, although its role in T2DM is unknown (Figure 2) (62). NF-κB can be activated through various pathways that are targeted by pro-inflammatory cytokines, including TNFα, and by circulating NEFA through the toll-like receptor 4 (TLR4) (40, 113). Whilst elevated TNFα by itself is not sufficient to cause muscle atrophy (90, 106), upregulation of NF-κB can cause muscle atrophy in rodents (23, 127). Mice overexpressing the inhibitor of NF-κB Kinase β (IKKβ) had a 15-fold increase in NF-κB, which reduced muscle fibre cross sectional area by 50-65%, depending on the muscle group (23). In other studies, transgenic overexpression of dominant negative IKKβ/α in rat muscle caused a 70% reduction in disuse-initiated muscle atrophy, a response that was presumed to be due to inhibition of NF-κB (127). Importantly, NF-κB can increase the degradation of specific muscle proteins via increasing expression of the E3 ubiquitin ligase MuRF1 (134), suggesting that the atrophic effects of increased NF-κB activity are not solely mediated by insulin resistance. Whilst NF-κB is increased in muscle during atrophic conditions in humans (94), including T2DM (125), its direct role in T2DM related atrophy is relatively unknown. In cancer cachexia, atrophy was attenuated with overexpression of dominant negative IKKβ, independent of the canonical p65 NF-κB pathway, suggesting that IKKβ may act through the NF-κB p50-p105-BCL3 pathway in cachexia-induced atrophy (32). Considering the elevated systemic inflammation and activation of the NF-κB pathway in T2DM skeletal muscle (40, 125), the NF-κB pathways are a promising, but relatively unexplored, area of T2DM mediated muscle atrophy.

STAT3 is a signalling protein that is activated by pro-inflammatory cytokines such as IL-6. Recently it was found to impair protein synthesis signalling in muscle in chronic kidney disease (CKD), cancer cachexia and STZ-induced diabetes in mice (116, 143). STAT3 is implicated in insulin resistance (Figure 2) (87), but only recently has the mechanism directly linking STAT3 and atrophy been described (143). In mice with CKD, genetic knockout of STAT3, or small molecular inhibition of STAT3, attenuated muscle atrophy in the gastrocnemius and tibialis anterior muscles (143). The mechanism of STAT3-dependent atrophy was elucidated *in vitro*, where phosphorylation of STAT3 upregulated myostatin transcription via CAAT/enhancer-binding protein δ (C/EBP δ), a finding confirmed using C/EBP δ knockout mice with CKD. Thus, activation of STAT3 via inflammatory factors such as IL-6 can reduce protein synthesis via the induction of insulin resistance through SOCS3 (142), and increased transcription of myostatin (143). However, the importance of this newly discovered STAT3-CEBP δ-myostatin pathway in T2DM needs to be investigated.

EXERCISE, INFLAMMATION AND ATROPHY SIGNALLING

Exercise and inflammation in T2DM

Evidence suggests chronic exercise, particularly endurance training or combined endurance and resistance training, in patients with T2DM can lower both systemic and muscle-based inflammation (11, 54, 66), although not all studies report consistent findings (104, 135, 148). A comprehensive study by Balducci et al., (11) investigated a range of systemic inflammatory markers over the course of 12 months in T2DM participants completing either low intensity endurance exercise, moderate-high intensity endurance exercise, or combined resistance and endurance training. While CRP decreased in all of the exercise groups, only the combined training group had lower circulating levels of both TNFα and IL-6 after 12 months, whereas the high intensity endurance group also had lower circulating IL-6 (11). In another study, frequent moderate intensity endurance exercise (4 times per week for 6 months) reduced CRP (66). Importantly, changes in IL-6, CRP and TNFα occurred independently of alterations in fat mass (11, 66, 139), implying that the reduced systemic inflammation was directly due to exercise rather than loss of adipose tissue. This may indicate that improvements in fitness through exercise, rather than changes in adiposity per se, decrease the levels of systemic inflammatory markers. Several groups reported no change to CRP or IL-6 with either resistance training or with moderate intensity endurance exercise (104, 135, 148). This could be due to a range of different factors, including insufficient program duration, exercise mode, frequency, intensity, and relatively small sample sizes. Further studies are needed to determine which exercise mode, intensity and duration is needed to influence pro-inflammatory markers.

In lean and obese non-diabetic patients, acute endurance exercise transiently increased muscle NF-κB DNA binding activity, whereas no change was found after exercise in patients with T2DM (125). This may, in part, be due to the already high basal levels of NF-κB activity in T2DM (125). Studies investigating the anti-inflammatory effects of chronic exercise (exercise training) in patients with T2DM are sparse. There is, however, evidence that chronic exercise attenuates muscle-based inflammation in T2DM (119). The inhibitor of NF-κB, IKB, was lower in T2DM muscle compared to controls and was increased in response to eight weeks of endurance training (119). In addition, muscle TNFα was also lower in muscle of T2DM patients after eight weeks of moderate intensity (70% of $\dot{V}O_{2peak}$) endurance training (119), suggesting that endurance exercise has anti-inflammatory properties in skeletal muscle in T2DM. However, a separate study in humans with T2DM found that a 16 week resistance training program caused no change in muscle-based markers of inflammation, including TNFα (51). Altogether, the data to date indicate that endurance training can reduce inflammation in T2DM, whilst the effect of resistance training is still unclear.

Exercise attenuates muscle atrophy

Exercise, particularly resistance training, is an effective method to promote muscle hypertrophy and attenuate muscle atrophy during various atrophic conditions (4, 5, 130). Indeed, 6-12 weeks of resistance training (three times per week, 70-80% of 1RM) increased knee extensor muscle size, and improved strength in patients with T2DM (20, 46, 58, 64). mTOR is the primary signalling pathway by which exercise training stimulates muscle hypertrophy and protein synthesis (50, 131). Mechanical stimuli, such as resistance training, have the potential to activate muscle mTOR complex 1 (mTORC1) through phosphorylation and subsequent lysosomal exclusion of the mTORC1 repressor tuberous sclerosis complex 2 (63, 131). Further, the extracellular-signal-regulated kinases 1/2 (ERK1/2) and the PI3K-Akt-mTOR pathways also increase mTORC1 activity (18, 50). The upregulation of mTORC1 phosphorylates several proteins vital for protein synthesis and hypertrophy, such as ribosomal S6 kinase 1 ($p70^{S6K1}$) and 4E-BP1 (50). Indeed, patients with T2DM exhibit a reduced capacity for protein synthesis, perhaps due to a reduced 4E-BP1 phosphorylation in muscle in response to protein feeding and insulin (101, 120). In an animal model, obese sarcopenic Zucker rats exhibit reduced contractile protein synthesis (15% of the protein synthesis rate of lean controls) in response to exercise compared to lean controls (93). The anabolic resistance to insulin and protein feeding in combination with increased ubiquitin-proteasome activation may contribute to the lower muscle mass with older age in T2DM (13). Resistance training is an effective therapy against anabolic resistance in T2DM, as mTOR can be activated independently of insulin or insulin-like growth factor -1 (IGF-1) with resistance training (8, 43, 49, 50, 131), and resistance training can increase *vastus lateralis* Type I and II muscle fibre CSA by 18-21% in T2DM patients (20, 46, 58, 64). Whether resistance training can attenuate muscle atrophy through reducing muscle-based inflammation is unclear, but presents one potential mechanism which requires further investigation.

In addition to the direct effect of mTORC1 activation on protein synthesis, upregulation of mTORC2 by training may also attenuate muscle atrophy. mTORC2 activates Akt, causing downregulation of the ubiquitin-proteasome system (53, 147).

In support of this premise, eight weeks of resistance training decreased muscle FoxO1 while increasing both Akt and mTOR phosphorylation in healthy humans, whilst detraining decreased Akt phosphorylation and increased FoxO1 (76). Further, muscle Atrogin-1 mRNA in muscle was reduced 48 hours after a single bout of resistance exercise (86). The decrease in Atrogin-1 mRNA reported by Mascher et al., (86) was accompanied by increased MuRF1 mRNA immediately post-exercise (86) and in a separate study, MuRF1 mRNA was increased after a bout of intense interval exercise (8). The divergent signalling response with the E3 ubiquitin ligases suggests exercise mode and intensity are important factors in the activation of the ubiquitin-proteasome system after exercise; with intense exercise potentially temporarily upregulating atrogene signalling, whilst resistance training may decrease ubiquitin proteasome signalling (8, 86). These divergent data further suggest that in healthy populations acute activation of the ubiquitin-proteasome system in response to exercise may be required for removal of damaged proteins and is unlikely to have a prominent role on muscle atrophy or hypertrophy, likely due to the substantial concurrent increase in protein synthesis (8, 43, 49). In inflammatory conditions, such as T2DM, however, the downregulation of the ubiquitin-proteasome system with training, potentially via improvements in inflammatory pathways, may be of greater importance. For instance, long term resistance training (12 months), increased thigh CSA muscle mass by 4.5% in older-aged patients with T2DM, and the increase in muscle mass was associated with a lower circulating CRP (88). A limitation of the study was that muscle samples were not taken, preventing the evaluation of muscle inflammation. To our knowledge, no *in vivo* evidence investigating the effects of T2DM and resistance training on both muscle atrophy and muscle-based inflammation exists; thus, the potential anti-inflammatory role of exercise training and its effect on the ubiquitin-proteasome system are still not understood in T2DM.

Exercise training increases insulin sensitivity via the IRS1-PI3K-Akt pathway in T2DM, which consequently may upregulate the mTOR pathway and protein synthesis (18, 31, 129, 131). As systemic inflammation substantially contributes to insulin resistance, the improvements in systemic and local (muscle) inflammation by exercise training may upregulate protein synthesis and reduce the ubiquitin-proteasome protein degradation via upregulation of the IRS1-PI3K-Akt pathway (18, 33, 40, 50, 111). As described previously, the NF-κB pathway is capable of directly upregulating atrophy signalling, and NF-κB is emerging as a potential inflammatory pathway which can promote atrophy signalling in T2DM (23, 134). As such, the NF-κB pathway may also be important in training adaptations to T2DM muscle, as NF-κB signalling is upregulated in humans with T2DM and decreased with training (52, 119). However, no studies that investigated NF-κB in muscle in humans with T2DM measured markers of the ubiquitin-proteasome system at baseline or after training.

CONCLUSIONS AND FUTURE DIRECTIONS

T2DM is characterised by systemic, low-grade inflammation that impairs insulin sensitivity and upregulates muscle atrophy signalling. Inflammation-induced insulin resistance decreases protein synthesis through the PI3K-Akt pathway, and upregulates the ubiquitin-proteasome system via FoxO family proteins and their downstream E3 ubiquitin ligases. There is emerging evidence that inflammation may also directly promote atrophy signalling through pathways such as NF-κB and STAT3. However, studies in T2DM muscle investigating inflammatory and atrophy pathways need to be performed. Exercise training, particularly resistance training, has proven particularly valuable in promoting increased muscle mass through mTOR signalling and potentially reducing inflammation in T2DM; although to what extent improvements in inflammatory pathways contribute to attenuating muscle atrophy signalling or improving hypertrophy is yet to be elucidated.

Future studies should explore the mechanism/s behind the effects of T2DM (and in particular hyperinsulinemia) on muscle atrophy as well as the mechanism by which exercise training can minimise or prevent the effects of diabetes on skeletal muscle. The NF-κB pathway is emerging as an important contributor to atrophy signalling in muscle; however the exact mechanisms behind this cause and effect are still unclear. It is possible that the newly discovered p50-p105 and BCL3-NF-κB pathway, which has not been investigated in T2DM-mediated muscle atrophy, is a promising area of future investigation. The recently described STAT3-C-EBPδ-myostatin pathway also requires investigation in models of T2DM such as high fat feeding, and interventional studies in humans. The muscle atrophy in T2DM is most severe with advanced age. Hence, muscle signalling involved in other atrophic conditions prevalent in T2DM, such as physical inactivity and sarcopenia, and their potential interaction with T2DM mediated atrophy is a clinically important area of future research. Most importantly, longitudinal interventional studies examining muscle cross sectional area, muscle atrophy signalling and muscle inflammatory pathways are required in human T2DM populations, ideally also with structured training and aging populations.

ORIGINAL RESEARCH

Muscle inflammation and atrophic signalling in humans with T2DM

The role of inflammation in muscle degradation pathways in humans remains largely unexplored. Patients with T2DM exhibit increased systemic inflammation (40), as evident through the chronic upregulation of several inflammatory pathways and markers, including CCL2, NF-κB and STAT3, which are associated with insulin resistance and/or muscle atrophy signalling *in vivo* and *in vitro* (23, 65, 87, 109, 143). The association between muscle inflammatory markers and the ubiquitin-proteasome system, however, has not been investigated in humans with T2DM. Therefore, we investigated markers of muscle inflammation (CCL2, NF-κB p65 subunit, STAT3 and SOCS3) and ubiquitin-proteasome signalling (FoxO1, Atrogin-1 and MuRF1) in *vastus lateralis* muscle of older age obese patients with T2DM and age-matched controls. We hypothesized that T2DM patients would have increased inflammatory markers and upregulation of the ubiquitin-proteasome system compared to the controls.

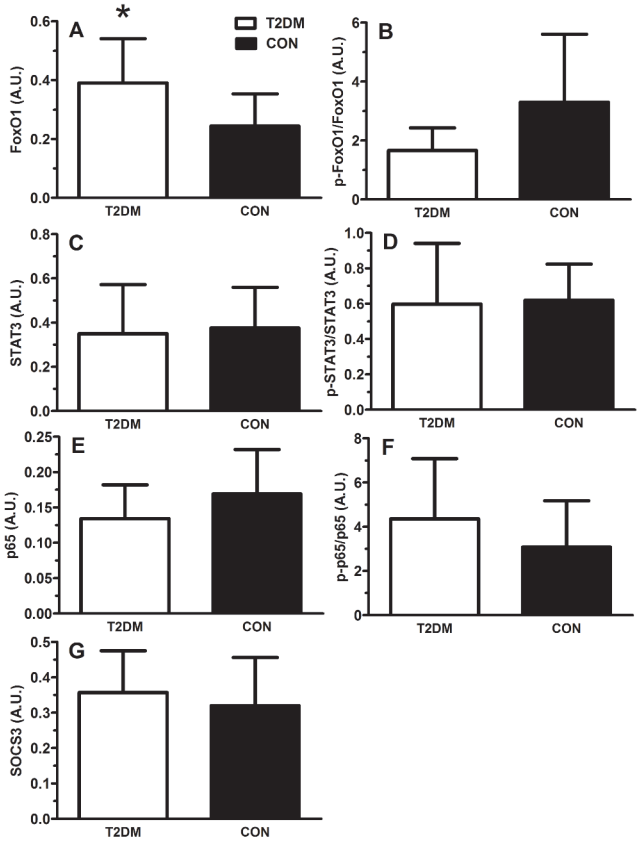

Figure 3: Skeletal muscle protein content of FoxO1 (A), p-FoxO1 relative to FoxO1 (B), STAT3 (C), p-STAT3 relative to STAT3 (D), p65 (E), p-p65 relative to p65 (F), and SOCS3 (G). All data was normalised to GAPDH abundance. Hollow bars denote participants with type 2 diabetes mellitus (T2DM), and filled bars represent age matched controls (CON). * different to CON ($p < 0.05$) with BMI as a covariate. For T2DM n = 12, and in CON n = 9. Data is presented as mean ± SD.

METHODS

Overall, 12 sedentary obese T2DM patients (T2DM; 5 females, 7 males, Age: 63.5 ± 13.8, BMI: 39.0 ± 5.5 kg.m^{-2}, mean ± SD) and 9 age matched, sedentary controls without insulin resistance (CON; 6 females, 3 males, Age: 67. 4 ± 8.0, BMI: 28.0 ± 5.9 kg.m^{-2}) participated in the study. A *vastus lateralis* muscle biopsy was performed for the assessment of markers of muscle inflammation and atrophy (CCL2, FoxO1, SOCS3, STAT3, p65 subunit of NF-κB, MuRF1 and Atrogin-1). Subject characteristics, including medications, are described in the Supplementary Methods.

RESULTS

Skeletal muscle FoxO1 protein abundance was 60% higher in T2DM ($p = 0.02$, Figure 3) compared to CON. There were no differences in protein abundance for STAT3, p65, SOCS3, p-STAT3/STAT3 and p-p65/p65 (Figure 4), or with mRNA expression of *MURF1, ATROGIN-1, CCL2, SOCS3, FOXO1* and *FOXO3* between groups (Table 1). In the pooled data, p-FoxO1/FoxO1 was negatively correlated to BMI ($r = -0.50$, $p = 0.02$, Figure 4), p65 was inversely related to BMI ($r = -0.50$, $p = 0.02$, Figure 4) and fasting glucose correlated with BMI ($r = 0.63$, $p = 0.01$). In regards to associations to physical fitness, there was a negative correlation between $\dot{V}O_{2peak}$ and the ratio of muscle p-STAT3/STAT3 in T2DM ($r = -0.60$, $p = 0.04$, Figure 5), but no other significant correlations to any other muscle inflammation or atrophy markers (*CCL2*, STAT3, p65 and FoxO1).

DISCUSSION OF ORIGINAL DATA

Our data indicate that atrophy signalling transcription factor FoxO1 (total protein) is increased in skeletal muscle in obese older-aged patients with T2DM. This is consistent with upregulation of the ubiquitin-proteasome proteolytic system despite finding no concomitant differences in several markers of muscle inflammation compared to age-matched controls. In T2DM, Akt activation is reduced (70), leading to increased FoxO transcription and upregulation of the E3 ubiquitin ligases MuRF1 and Atrogin-1 (111). Hence, in the absence of any difference in muscle inflammatory markers, our findings of increased FoxO1 reflect an increased catabolic state T2DM muscle, which may be in part caused by decreased Akt phosphorylation. Whilst we did not detect any differences in *ATROGIN-1* and *MURF1* mRNA expression, the expression of these proteins is transient and does not remain elevated during conditions of prolonged disuse- or glucocorticoid-induced atrophy (1, 24, 29). The ratio of p-FoxO1/FoxO1 was inverse-

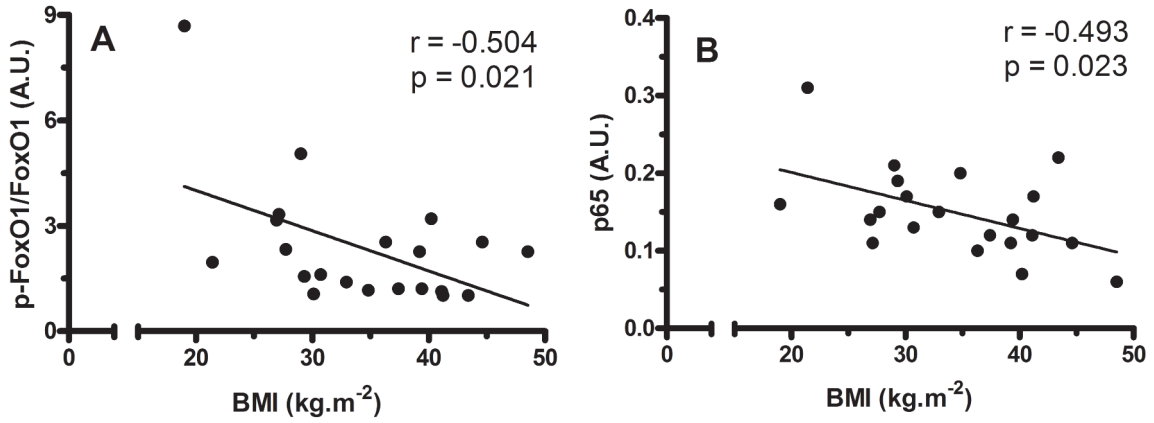

Figure 4: Correlations between p-FoxO1 relative to FoxO1 and BMI (A), and between p65 and BMI in all participants pooled (B).

Table 1: Gene expression of *MURF1, ATROGIN-1, CCL2, SOCS3, FOXO1* and *FOXO3* in T2DM and CON. Data represents n = 11 for T2DM and n = 8 for CON. Data is presented as mean ± SD. All data was normalised to *TBP*.

mRNA (A.U.)	**T2DM**	**CON**	**p-value**
MURF1	59.6 ± 57.3	45.1 ± 14.8	0.84
ATROGIN-1	134.2 ± 73.0	85.1 ± 34.5	0.11
CCL2	4.0 ± 5.3	2.0 ± 0.9	0.90
SOCS3	5.6 ± 6.8	6.3 ± 5.9	0.72
FOXO1	15.0 ± 12.4	12.9 ± 7.2	0.61
FOXO3	51.1 ± 59.9	36.1 ± 13.4	0.99

ly associated with BMI, suggesting that increased adiposity is related to an increase in FoxO1 activation; phosphorylation of FoxO1 inhibits FoxO nuclear localisation and transcription (17). Hence, the inverse association between p-FoxO1 and BMI may have been be due to obesity-induced inflammation and/or insulin resistance.

Despite the increased FoxO1 in T2DM muscle and the inverse relationship between p-FoxO1 and BMI, the p65 subunit of NF-κB was negatively correlated with BMI in the pooled data. Combined with the lack of difference in muscle inflammation in our T2DM patients, the findings from our study are incongruent with previous studies reporting obesity and T2DM promoting muscle-based inflammation signalling (87, 107, 119). It is important to note, however, that this study was cross sectional, and the sample size was relatively small. Thus, our data suggest that the relationship between inflammation and atrophy signalling is complex, and multiple factors are required to be taken into consideration. For example, the development of muscle inflammation may have been transient in these participants, altered by pharmaceutical treatment and other lifestyle factors, and complicated by the potential existing systemic inflammation in the age-matched control group (97, 98). Further, other inflammatory pathways not analysed in our study may have contributed to the higher FoxO1 in T2DM, such as through NF-κB via the p50-p105-BCL3 pathway, as is seen in disuse and cancer cachexia (32, 62). Finally, emerging evidence indicates that microRNA is important in proteolytic signalling; for example microRNA 486 inhibits FoxO1 expression (136). The inconsistencies between our findings in muscle inflammation and the few previous studies conducted in human skeletal muscle (87, 107, 119) indicate that further research is needed to elucidate the complex signalling between inflammatory and atrophic pathways in human T2DM muscle, and the extrinsic factors which may affect these signalling pathways.

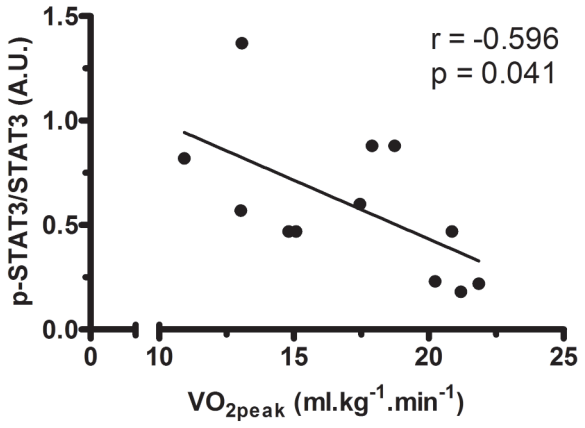

Figure 5: Correlation between p-STAT3 relative to STAT3 and $\dot{V}O_{2peak}$ in patients with type 2 diabetes mellitus.

In regards to the role of aerobic fitness in muscle inflammation and atrophy signalling in T2DM, we report a negative correlation between $\dot{V}O_{2peak}$ and the ratio of muscle p-STAT3/STAT3 ($r = -0.60$, $p = 0.04$, Figure 5); suggesting that lower aerobic fitness is associated with increased activation of inflammatory pathways in skeletal muscle of T2DM patients. This is consistent with previous research which reported both endurance and combined exercise training reduced systemic and muscle-based inflammation in T2DM (11, 119). However, the effects of exercise intensity, mode and frequency on systemic inflammation, muscle STAT3 signalling and other muscle-based inflammation with T2DM requires substantially more investigation *in-vivo*, particularly in humans.

CONCLUSIONS FROM ORIGINAL DATA

Our findings suggest that patients with T2DM exhibit upregulation of FoxO1 in skeletal muscle, suggesting greater muscle catabolism in muscle of patients with T2DM. In contrast to previous studies, concurrent upregulation of several inflammatory pathways including NF-κB p65, STAT3 and CCL2 was not observed, suggesting a complex relationship between muscle proteolytic and inflammatory pathways in humans that requires further investigation. Finally, we also found a negative relationship between $\dot{V}O_{2peak}$ and muscle p-STAT3/STAT3, suggesting that endurance exercise may be a useful intervention to reduce muscle inflammation in T2DM.

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SUPPLEMENTAL METHODS

Twelve patients diagnosed with type 2 diabetes mellitus (T2DM; 5 females, 7 males, Age: 63.5 ± 13.8, BMI: 39.0 ± 5.5 $kg.m^{-2}$, mean ± SD) and nine age matched non-diabetic controls (CON; 6 females, 3 males, Age: 67. 4 ± 8.0, BMI: 28.0 ± 5.9 $kg.m^{-2}$) participated. In the T2DM group partici-

pants were taking a range of medications, including oral hypoglycaemic agents (n = 8), insulin, (n = 5), statins (n = 7), anti-coagulants (n = 2), ACE inhibitors (n = 8), beta-blockers (n = 2), Calcium channel blockers (n = 3), diuretics (n = 1), corticosteroid inhaler (n = 1), dopamine agonist (n = 1), proton pump inhibitors (n = 2), xanthine oxidase inhibitors (n = 2), antidepressants (n = 1), norethisterone (n = 1), calcium supplementation (n = 2), fish oil supplementation (n = 1), vitamin B12 supplementation (n = 1) and anticonvulsants (Topiramate, n = 1). In the CON group, the medications taken included statins (n =2), anti-depressants (n = 1), non-steroidal anti-inflammatory medications (n = 1), anti-coagulants (n = 1), proton pump inhibitors (n = 1) and thyroid hormone replacement therapy (n = 1). In both groups, participants were over 18 years of age, and the following exclusion criteria were adhered to: known coronary artery disease or evidence of ischemia on baseline stress echocardiography, significant (moderate or severe) valvular heart disease medication changes over the past 3 months, unstable diabetes as evidenced by hypoglycaemic events in the past week or HbA1c >9.0%, and persistent or permanent atrial fibrillation. Weight ($p = 0.001$), BMI ($p = 0.001$) and fasting blood glucose ($p = 0.001$) were higher in T2DM, whilst total cholesterol ($p = 0.001$), LDL ($p = 0.001$) and HDL ($p = 0.004$) were lower in T2DM (Supplemental Table 1). In the T2DM, body fat percentage was 41.8 ±6.1%, lean mass 57.1 ± 9.4 kg, and HbA1c 7.3 ± 2.7%.

Supplemental table 1: Participant characteristics of the Type 2 diabetes mellitus group (T2DM) and age matched controls (CON).* different to CON ($p < 0.05$). For T2DM n = 12, for CON n = 9, except fasting glucose and blood lipid measurements where n = 10 for T2DM. SBP = systolic blood pressure, DBP = diastolic blood pressure. Values are represented as mean ± SD.

	T2DM	CON
Age (years)	63.5 ± 13.8	67.4 ± 8.0
Weight (kg)	104.5 ± 13.5*	74.1 ± 19.8
Height (cm)	164.5 ± 9.1	162.1 ±7.4
BMI ($kg.m^{-2}$)	39.0 ± 5.5*	28.0 ± 5.9
Fasting glucose ($mmol.L^{-1}$)	8.9 ± 2.4*	4.5 ± 1.4
HDL ($mmol.L^{-1}$)	1.1 ± 0.3*	1.7 ± 0.6
LDL ($mmol.L^{-1}$)	2.2 ± 0.8*	3.7 ± 0.6
Total cholesterol ($mmol.L^{-1}$)	3.9 ± 1.1*	6.0 ±0.6
Triglycerides ($mmol.L^{-1}$)	1.5 ± 0.9	1.2 ± 0.6
SBP (mmHg)	136 ± 22.9	110 ± 44.4
DBP (mmHg)	80.1 ± 17.3	66 ±25.2
$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	17.1 ± 3.7	n/a

Each participant received both verbal and written explanations of the study before giving informed consent and the protocol was approved by the Human Research Ethics Committees of both Victoria University and Austin Health. Only T2DM participants performed the symptom-limited $\dot{V}O_{2peak}$ test with a 12 lead ECG (Model X-Scribe Stress Test 114 System, Mortara Instrument Inc., WI, USA) on a Cybex MET 100 exercise cycle, and had a DXA scan (78, 79). The $\dot{V}O_{2peak}$ test protocol comprised of an initial intensity of 25 W, with increments of 20 W/min for men and 10 W/min for women (78, 80). Control participants did not perform the $\dot{V}O_{2peak}$ test or DXA scan. Within the next three weeks, all participants then completed: a fasting blood sample for blood glucose, HbA1c% (T2DM only), and blood lipid profile (45). Blood pressure was measured using a mercury sphygmomanometer, and a resting *vastus lateralis* muscle biopsy (80, 81) was performed, with a minimum of 48 hours between each testing session.

For immunoblot analysis, homogenization of approximately 20 mg of skeletal muscle was performed using 300 µl of lysis buffer (20 mM Tris/HCl, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Nonidet P40) including protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Australia) using the Precellys®24 tissue homgeniser (20 sec, setting 5500 rpm) (Sapphire Bioscience, NSW, Australia) and 1.0 mm zirconia/silica beads (Daintree Scientific, Tasmania, Australia). Protein content was determined using the Biorad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein (30 µg) was separated by 4-15% SDS-PAGE using Criterion TGX precast gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto PVDF membranes (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories, Hercules, CA) and blocked with 5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, Australia). Primary antibodies, diluted in blocking buffer were applied and incubated overnight at 4°C; p-STAT3 (Tyr705), STAT3, p-p65 NF-KB (ser536), p65 NF-KB, p-FoxO1 (ser256), FoxO1 (Cell Signaling Technology Inc., Danvers, MA, USA), SOCS3 (Santa Cruz Biotechnology, CA, USA) and GAPDH (Sigma-Aldrich, Australia). Membranes were exposed to anti-rabbit HRP-conjugated secondary antibodies (GE Healthcare, NSW, Australia) and visualized by enhanced chemiluminescence (Super Signal West Femto Maximum Sensitivity Substrate, Thermo-Fisher Scientific, VIC, Australia). Blot images were captured using the Chemidoc MP Imaging system (Bio-Rad Laboratories, Hercules, CA) and the density of the bands was quantified using Image Lab 4.1 software (Bio-Rad Laboratories, Hercules, CA). Membranes were stripped (Restore Western Blot Stripping Buffer, Thermo-Fisher Scientific, VIC, Australia) before being re-probed for total STAT3, p65 NF-KB, FoxO1 and GAPDH.

Total cellular RNA was extracted as performed as described previously (126). RNA was transcribed into cDNA using the SuperScript™VILO cDNA Synthesis Kit (Life Technologies, VIC, Australia). qPCR was performed using the Bio-Rad CFX384 PCR system (Bio-Rad Laboratories, NSW, Australia) and PCR performed in duplicate with reaction volumes of 10 µl, containing SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories), forward and reverse primers and cDNA template. Data were analyzed using a comparative quantification cycle (Cq) method where the amount of target relative to NF is given by $2^{-\Delta\Delta Cq}$. Primers were designed using NCBI Primer BLAST from gene sequences obtained from GenBank and listed below: *ATROGIN-1* forward: 5'CATCCATATGTACACTGGTCCAAAGA; *ATROGIN -1* reverse: 5' – TCCGATACACCCACATGT-

TAATG; *MURF-1* forward: 5' – GGCGTGGCTCTCATTC-CTT; *MURF-1* reverse: 5' – TCTCCAAGTTCTCCAGTG-GATT; *CCL2* forward: 5' – CGCCTCCAGCATGAAAGTCT; *CCL2* reverse: 5' – GGAATGAAGGTGGCTGCTATG; *SOCS3* forward: 5' – GACCAGCGCCACTTCTTCA: *SOCS3* reverse: 5' – CTGGATGCGCAGGTTCTTG; *FOXO3A* forward: 5' – TGCAAACCTGCCCGTCAT; *FOXO3A* reverse: 5' – CTAAGCTCCCATTGAACATGT; *FOXO1* forward: 5'-CCGAACAGGATGATCTTGGAG; *FOXO1* reverse 5'-GCGGGTACACCATAGAATGCA; *TBP* forward: 5'-CGAATATAATCCCAAGCGGTTT; *TBP* reverse 5'-CCGTGGTTCGTGGCTCTCT.

STATISTICS

All data are presented as means ± SD and statistical significance was accepted at $p < 0.05$. Data was analysed using either an analysis of covariance (ANCOVA), with BMI as a covariate due to the difference in BMI between groups, or via independent samples t-tests. Correlations were performed using Pearson product moment coefficient correlations.

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