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SYMPOSIUM REVIEW

Beyond atrophy: redox mechanisms of muscle dysfunction in chronic inflammatory disease

Michael B. Reid and Jennifer S. Moylan

Department of Physiology and Centre for Muscle Biology, University of Kentucky, Lexington, KY 40536, USA

Abstract Chronic inflammatory diseases such as heart failure, cancer and arthritis have secondary effects on skeletal muscle that cause weakness and exercise intolerance. These symptoms exacerbate illness and make death more likely. Weakness is not simply a matter of muscle atrophy. Functional studies show that contractile dysfunction, i.e. a reduction in specific force, makes an equally important contribution to overall weakness. The most clearly defined mediator of contractile dysfunction is tumour necrosis factor (TNF). TNF serum levels are elevated in chronic disease, correlate with muscle weakness, and are a predictor of morbidity and mortality. Research is beginning to unravel the mechanism by which TNF depresses specific force. TNF acts via the TNFR1 receptor subtype to depress force by increasing cytosolic oxidant activity. Oxidants depress myofibrillar function, decreasing specific force without altering calcium regulation or other aspects of myofibrillar mechanics. Beyond these concepts, the intracellular mechanisms that depress specific force remain undefined. We do not know the pathway by which receptor–ligand interaction stimulates oxidant production. Nor do we know the type(s) of oxidants stimulated by TNF, their intracellular source(s), or their molecular targets. Investigators in the field are pursuing these issues with the long-term goal of preserving muscle function in individuals afflicted by chronic disease.

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Corresponding author M. B. Reid: Department of Physiology, University of Kentucky, 800 Rose Street, MS508, Lexington, KY 40536-0298, USA. Email: michael.reid@uky.edu

Abbreviations CHF, chronic heart failure; cPLA$_2$, cytosolic phospholipase A$_2$; ERK1/2, extracellular regulatory kinases 1 and 2; NAC, *N*-acetylcysteine; NO, nitric oxide; nSMase, neutral sphingomyelinase; PLA$_2$, phospholipase A$_2$; PKCζ, protein kinase Cζ; ROS, reactive oxygen species; SMase, sphingomyelinase; TNF, tumour necrosis factor-α; TNFR1 and TNFR2, TNF receptor subtype 1 and 2.

Michael Reid is the Shih-Chun Wang Professor and Chair of the Department of Physiology at the University of Kentucky. Research interests include redox homeostasis in skeletal muscle, chronic inflammation-induced weakness, and interventions to preserve muscle function. His research team was the first to demonstrate that skeletal muscle produces reactive oxygen species, that NO is an endogenous modulator of muscle contraction, and that oxidative stress plays a causal role in human muscle fatigue. **Jennifer Moylan** is an Assistant Professor in the Department of Physiology at the University of Kentucky. She has a diverse research background, work ranging from mechanisms of chloroplast mRNA processing to innate immunity to skeletal muscle function. Current efforts include investigation of unique aspects of skeletal muscle biology that involve a convergence of lipid and redox signalling, focusing on identifying novel targets for interventions that preserve muscle function.

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This article focuses on muscle-derived oxidants as potential mediators of weakness in chronic inflammatory disease. The concept is supported by a large and disparate literature on human pathophysiology. For example, inflammation and oxidative stress are closely linked to muscle atrophy and weakness in conditions that include ageing, myotonic and Duchenne dystrophies, chronic obstructive pulmonary disease, kidney disease, rheumatoid arthritis, sepsis, cancer, type 2 diabetes, liver disease and chronic heart failure (CHF). These clinical associations argue for basic and translational research to define cellular mechanisms and identify effective therapies.

With many chronic inflammatory diseases, muscle weakness runs parallel with, and in part independently of, muscle atrophy. For example, ageing human populations experience a decline in muscle strength that outpaces the loss of muscle mass (Goodpaster *et al.* 2006; Hairi *et al.* 2010). Studies of patients with either severe (Levine *et al.* 2003) or mild (Ottenheijm *et al.* 2005) chronic obstructive pulmonary disease define the mechanisms of muscle weakness in more detail. Skinned diaphragm fibres from these patients have 25–35% reduced specific force that is independent of muscle mass. Patients with rheumatoid arthritis have significant muscle weakness. Many factors influence the reduction in strength (−25 to −50%, Stenstrom & Minor, 2003): loss of mass, joint deformity, pain. Intrinsic myofibrillar dysfunction has not been established in patients with rheumatoid arthritis (Helliwell & Jackson, 1994). However, mice with collagen-induced arthritis have impaired muscle function that may be linked to oxidative modification of myofibrillar proteins (Yamada *et al.* 2009). Studies of myofibrillar function in animal models (van Norren *et al.* 2009) or patients (Weber *et al.* 2009) with cancer cachexia are limited but contrast with other inflammatory diseases. In populations studied thus far, although absolute strength is reduced, force normalized for loss of muscle mass remains unchanged.

Our laboratory is pursuing this problem in the context of heart failure. Weakness of limb and respiratory muscles plagues individuals who suffer from chronic heart failure (Hammond *et al.* 1990; Evans *et al.* 1995; Carmo *et al.* 2001). Loss of muscle strength exacerbates an array of debilitating symptoms and affects patient survival. Muscle weakness is an independent predictor of survival (Meyer *et al.* 2001) that correlates with breathlessness (McParland *et al.* 1992; Mancini *et al.* 1994; Weiner *et al.* 1999) and exercise limitation (Chua *et al.* 1995; Meyer *et al.* 2001). Muscle weakness is not a simple function of atrophy. Force loss generally exceeds the loss of muscle mass. Specific force (force per cross-sectional area) is depressed in muscle fibres from patients with heart failure (Szentesi *et al.* 2005) and in animal models of the disease (Supinski *et al.* 1994; Howell *et al.* 1995; Stassijns *et al.* 1999). This represents loss of function independent of muscle mass, i.e. contractile dysfunction.

How does inflammation in a remote organ such as the heart weaken skeletal muscle? The answer appears to be multifactorial with systemic inflammation being a major contributor (Gan *et al.* 2004; Yende *et al.* 2006). Circulating pro-inflammatory mediators – notably interleukin-6, C-reactive protein, sphingomyelinase (SMase), and tumour necrosis factor-α (TNF) – are elevated in patients with chronic cardiopulmonary disease and correlate with muscle weakness (Cicoira *et al.* 2001; Toth *et al.* 2006; Yende *et al.* 2006; Doehner *et al.* 2007). Among these humoral factors, TNF is most strongly implicated. TNF serum concentration is inversely related to muscle strength in patients with heart failure (Cicoira *et al.* 2001; Toth *et al.* 2006). Intravenous infusion of TNF decreases pressure generation by the canine diaphragm *in vivo*, a response evident within 3 h (Wilcox *et al.* 1994), and exposure to high concentrations of TNF can depress force of excised muscle *in vitro* (Wilcox *et al.* 1996). At the tissue level, muscle dysfunction appears to be mediated, at least in part, by oxidative stress. Clinical data show that CHF depresses the activities of major antioxidant enzymes and causes protein oxidation in human muscle (Linke *et al.* 2005; Vescovo *et al.* 2008). Laboratory studies confirm oxidative stress as a potential mediator, demonstrating that free radical production by muscle is elevated in experimental heart failure (Li *et al.* 2000; Supinski & Callahan, 2005; Coirault *et al.* 2007).

TNF and contractile dysfunction

TNF plays dual roles in skeletal muscle. Autocrine release of TNF is crucial for inducing myogenesis during both injury-induced regeneration (Chen *et al.* 2005; Liu *et al.* 2010) and mechanical stimulation of myogenesis (Chen *et al.* 2005; Zhan *et al.* 2007; Liu *et al.* 2010). The level of TNF increases in injured muscle through synthesis by myofibres and by release from infiltrating inflammatory cells (Collins & Grounds, 2001; Warren *et al.* 2002). Mechanical stimulation induces myofibre release of TNF by activation of TNF-converting enzyme and processing of membrane-bound pro-TNF (Zhan *et al.* 2007). TNF exerts its action through stimulation of p38 MAP kinase, phosphorylation of MEF2 (myocyte enhancer factor 2) transcription factors (de Angelis *et al.* 2005) and E47 (Lluis *et al.* 2005). Phosphorylation of E47 in turn stimulates MyoD transactivation (Puri *et al.* 2000). Thus TNF is an important mediator of muscle myogenesis in response to injury, disease, or training. In contrast, chronic exposure to TNF promotes muscle weakness (Li *et al.* 2000; Tisdale, 2008).

TNF-induced weakness has long been attributed to loss of muscle mass or cachexia and was originally designated ‘cachectin’ in recognition of its catabolic

action (Reid & Li, 2001). However, the weakness is not entirely attributable to loss of muscle mass. When force measurements are normalized to muscle mass, TNF depresses specific force of muscle in intact animals. We first observed this phenomenon in transgenic mice engineered for stable, cardiac-specific overexpression of TNF (Li *et al.* 2000) which modestly elevates circulating TNF levels (250–350 pg ml^{-1}). These animals are a genetic model of heart failure and we intended to study the changes associated with cardiac cachexia. Instead, adult TNF transgenic mice were phenotypically normal; animal growth, excised muscle weights, fibre bundle cross-sectional areas, and muscle fibre ultrastructure were indistinguishable from wild-type animals. However, functional studies identified a 40% decrement in the specific force of diaphragm fibre bundles. Thus, specific force was depressed by TNF serum levels that were too low to cause atrophy. This identified contractile dysfunction as a separate process from TNF-induced catabolism. Extrapolating to humans, these data predict that contractile dysfunction in patients with chronic heart failure may be more prevalent than appreciated and may precede cachexia.

TNF causes a similar loss of function in muscles of wild-type mice (Hardin *et al.* 2008). We determined this by injecting adult animals with recombinant TNF and harvesting muscle fibre bundles for functional assessment at various time points. As in transgenic animals, TNF depressed specific force of wild-type muscle across a broad range of stimulus frequencies. The force loss was comparable to the decrement observed in CHF patients (Fig. 1). It occurred within one hour and persisted for at least 48 h. This experimental model further enabled us to test receptor specificity of TNF effects. Respiratory and limb skeletal muscles express both the 55 kDa TNF receptor subtype 1 (TNFR1) and the 75 kDa subtype TNFR2 (De Bleecker *et al.* 1999). We tested TNF effects in mice that were genetically deficient in either subtype and found that TNFR1 deficiency abolished the response to TNF, preserving specific force, whereas TNFR2 deficiency did not (Fig. 1). Thus, TNF appears to act via TNFR1 to cause contractile dysfunction in intact animals.

Loss of specific force is not simply an indirect systemic effect of TNF, e.g. on hormonal status, vascular function, or nutrition. TNF can act directly on skeletal muscle to depress function. Incubation of wild-type muscle with recombinant TNF *in vitro* decreases specific force within hours, a response seen in both limb muscle (Reid *et al.* 2002) and respiratory muscle (Li *et al.* 2000; Reid *et al.* 2002). These observations confirm a direct effect of the cytokine on muscle (Wilcox *et al.* 1996; Alloatti *et al.* 2000; Li *et al.* 2000; Reid *et al.* 2002) and justify studies of the underlying cell biology.

Research using single muscle fibres show that TNF depresses specific force by altering myofibrillar function. In intact single fibres, we determined that TNF decreases specific force of tetanic contractions without altering calcium regulation (Reid *et al.* 2002). TNF had no effect on resting calcium, peak tetanic calcium, or the shape of tetanic calcium waveforms. These findings suggested that the site of TNF-induced dysfunction is downstream of the calcium transient, i.e. at the myofibrillar level. Studies of permeabilized muscle fibres confirmed this thesis (Hardin *et al.* 2008). Animals were treated with TNF or buffer for one hour. Diaphragms were harvested and single fibres were isolated and chemically permeabilized, enabling direct activation of myofilaments using exogenous calcium. Permeabilized fibres from TNF-treated animals showed depressed specific force (Fig. 1) over a wide range of calcium activation. Thus TNF induces modifications *in vivo* that are maintained in a membrane-free environment. The effect on specific force was highly selective. TNF did not alter other aspects of myofibrillar mechanics; the calcium concentration required for half-maximal activation, Hill coefficient, rate of tension recovery, and maximal shortening velocity were unaltered. These findings are consistent with troponin, myosin heavy chain, or tropomyosin as potential molecular targets of TNF/TNFR1 signalling.

Oxidative stress as a mediator

Free radicals and their redox derivatives modulate contractile function of skeletal muscle. Skeletal muscle fibres continually synthesize parent radicals in the two major redox cascades, nitric oxide (NO) derivatives and reactive oxygen species (ROS). Oxidant activity in skeletal muscle

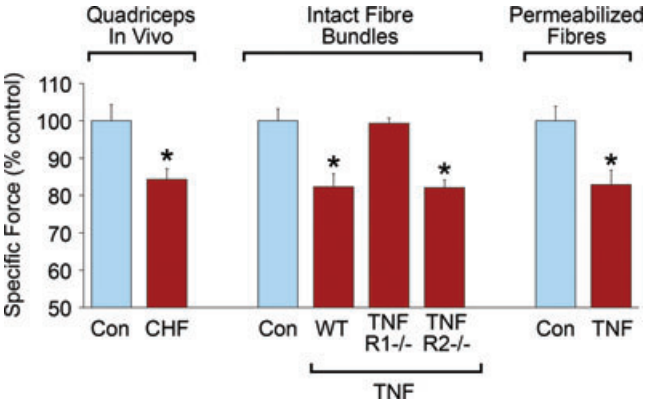

Figure 1. Heart failure and TNF depress specific force
Comparison of CHF-induced weakness in human quadriceps muscle during knee extension efforts (left) *versus* effects of *in vivo* TNF treatment on murine diaphragm fibre bundles from wild-type, TNFR1-deficient, and TNFR2-deficient animals (centre) and on permeabilized single fibres from murine diaphragm (right); data adapted from original reports (Harrington *et al.* 1997; Hardin *et al.* 2008).

is increased by a wide variety of conditions that promote weakness, fatigue, or both. It is likely that overproduction of muscle-derived oxidants mediate TNF effects on muscle function. Myofibrillar proteins are relatively sensitive to oxidative stress and are likely to be the main site of diaphragm dysfunction in chronic disease (Tikunov *et al.* 1996). Consistent with this view, specific force of intact muscle fibres is depressed by ROS or NO concentrations that are too low to alter tetanic calcium levels (Andrade *et al.* 1998*a*,*b*, 2001). Thus, ROS mimic the biological action of TNF, preferentially acting on targets downstream of the calcium transient, i.e. at the myofibrillar level.

Experimental evidence shows that muscle-derived oxidants are essential mediators of TNF/TNFR1-induced dysfunction. TNF increases cytosolic oxidant activity in skeletal muscle fibres. This is a robust companion to contractile dysfunction which parallels the loss of specific force caused by transgenic overexpression (Li *et al.* 2000), injection of the recombinant cytokine *in vivo* (Hardin *et al.* 2008), and direct exposure by *in vitro* incubation (Li *et al.* 2000). The latter example is illustrated in Fig. 2. Like contractile dysfunction, the rise in oxidant activity is also TNFR1-dependent; it is abolished by genetic deficiency in TNFR1 but not TNFR2 (Hardin *et al.* 2008). In skeletal muscle, large or persistent increases in cytosolic oxidant activity diminish specific force. In the case of TNF, oxidants have been identified as downstream effectors by the use of pharmacological antioxidants that interrupt oxidant signalling. *In vivo* experiments tested the effect of injecting animals with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; a water-soluble vitamin E derivative) prior to TNF. Trolox pretreatment markedly depressed oxidant activity and prevented contractile dysfunction in muscles of TNF-treated animals. Experiments *in vitro* have yielded similar results. Muscle fibre bundles preincubated with *N*-acetylcysteine (NAC; thiol antioxidant, supports glutathione resynthesis) maintained normal specific forces

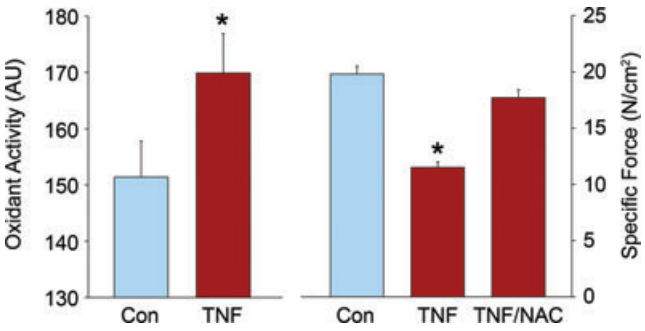

Figure 2. Muscle-derived oxidants mediate TNF action
The decrement in rise in oxidant activity of muscle fibres exposed to TNF *in vitro* (left) is paralleled by a fall in specific force that is abolished by pretreatment with the antioxidant NAC (right); data adapted from original report (Li *et al.* 2000).

despite direct TNF exposure. Interestingly, incubation with NAC for 30 min increased the specific force of muscles from TNF transgenic animals by almost one-half. Thus, despite life-long elevation of circulating TNF levels, a substantial portion of the contractile dysfunction was acutely reversible. This further supports the thesis that TNF depresses specific force via a post-translational mechanism.

Targets for mechanistic research

The cellular mechanism by which TNF depresses contractile function is a promising area for future research. It is clear that TNF initiates the process by activating TNFR1 (Hardin *et al.* 2008). But how does this increase oxidant activity in muscle fibres? The existing literature suggests a cascade of potential signalling events that are illustrated in Fig. 3. Early postreceptor signalling is probably mediated by one or more sphingomyelinase isoforms. Neutral (nSMase), acid (aSMase) and secretory (sSMase) sphingomyelinases have distinct biochemical properties, are regulated via unique mechanisms, and can have opposing effects on cell function (Wiegmann *et al.* 1994). TNFR1 links to nSMase activation via the adaptor proteins FAN (factor associated with neutral sphingomyelinase activation) (Adam-Klages *et al.* 1997) and RACK1 (receptor for activated C kinase-1) (Tcherkasowa *et al.* 2002) that bind a cytoplasmic signalling domain upstream of the death domain. nSMase hydrolyses sphingomyelin in the cell membrane to generate ceramide. Clinical data show that sphingomyelinase activity correlates with elevated TNF levels and diminished muscle strength in patients with chronic heart failure (Doehner *et al.* 2007), suggesting a role for ceramide signalling in inflammation-induced weakness. Consistent with this model, we recently discovered that ceramide depresses the contractile function of skeletal muscle (Ferreira *et al.* 2010).

Ceramide mediates the rise in oxidant production stimulated by TNF in other cell types (Suematsu *et al.* 2003), a response attributed to mitochondria in studies of cardiac myocytes (Suematsu *et al.* 2003). We postulate a homologous role for ceramide in skeletal muscle fibres. Ceramide-induced weakness is mediated by a rise in muscle-derived oxidant activity (Ferreira *et al.* 2010). The rise in oxidant activity may be triggered by direct interaction of ceramide with protein kinase Cζ (PKCζ). Ceramide binding activates PKCζ and stimulates translocation (Fox *et al.* 2007). In turn, PKCζ phosphorylates extracellular regulatory kinases 1 and 2 (ERK1/2), increasing ERK1/2 activity and stimulating ERK1/2-dependent signal transduction (Schonwasser *et al.* 1998).

Phospholipase A_2 (PLA_2) is the putative link between ERK1/2 and oxidant production. Our studies (Gong *et al.* 2006) and those of other investigators (Zuo *et al.* 2004) indicate that PLA_2 regulates diaphragm oxidant production in an isoform-specific manner. Murine muscle expresses three isoforms of PLA_2: the 85 kDa cytosolic $cPLA_2$ (Group IV) isoform, 85 kDa calcium-independent $iPLA_2$ (Group VI), and 14 kDa secretory $sPLA_2$ (Group VII) (Schaloske & Dennis, 2006). TNFR1 signalling activates $cPLA_2$ which synthesizes arachidonic acid from membrane phospholipids and appears to be essential for TNF-stimulated oxidant production (Woo *et al.* 2000). ERK1/2 phosphorylates $cPLA_2$ (Lin *et al.* 1993), increasing $cPLA_2$ activity and stimulating arachidonic acid release. These findings identify $cPLA_2$ as a potential target of TNFR1/ERK1/2 signalling and a potential source of arachidonic acid to stimulate oxidant production.

These data do not identify the source or composition of TNF-stimulated oxidants. Skeletal muscle continually generates NO derivatives. These may derive from at least two constitutively-expressed NO synthase (NOS) isoforms. A muscle-specific neuronal NOS isoform (nNOSμ) localizes to the dystrophin complex in fast-type fibres whereas the endothelial isoform (eNOS) may be associated with mitochondria. Muscle also generates ROS which are detectable in the cytosol and extracellular space. Putative sources of ROS within muscle fibres include the mitochondrial electron transport chain, a sarcolemmal NAD(P)H oxidase complex, and xanthine oxidase.

TNF/TNFR1 signalling can increase production of either NO or ROS, responses that appear to be cell type specific. Our data do not yet identify the dominant mechanism in skeletal muscle. Live-cell measurements of oxidant activity were made using 2,3-dichlorofluorescin diacetate (DCFH-DA), a fluorescence probe that detects both NO derivatives and ROS, and antioxidant interventions (Trolox, NAC) were selected to buffer both cascades. Nor does the functional response to TNF help

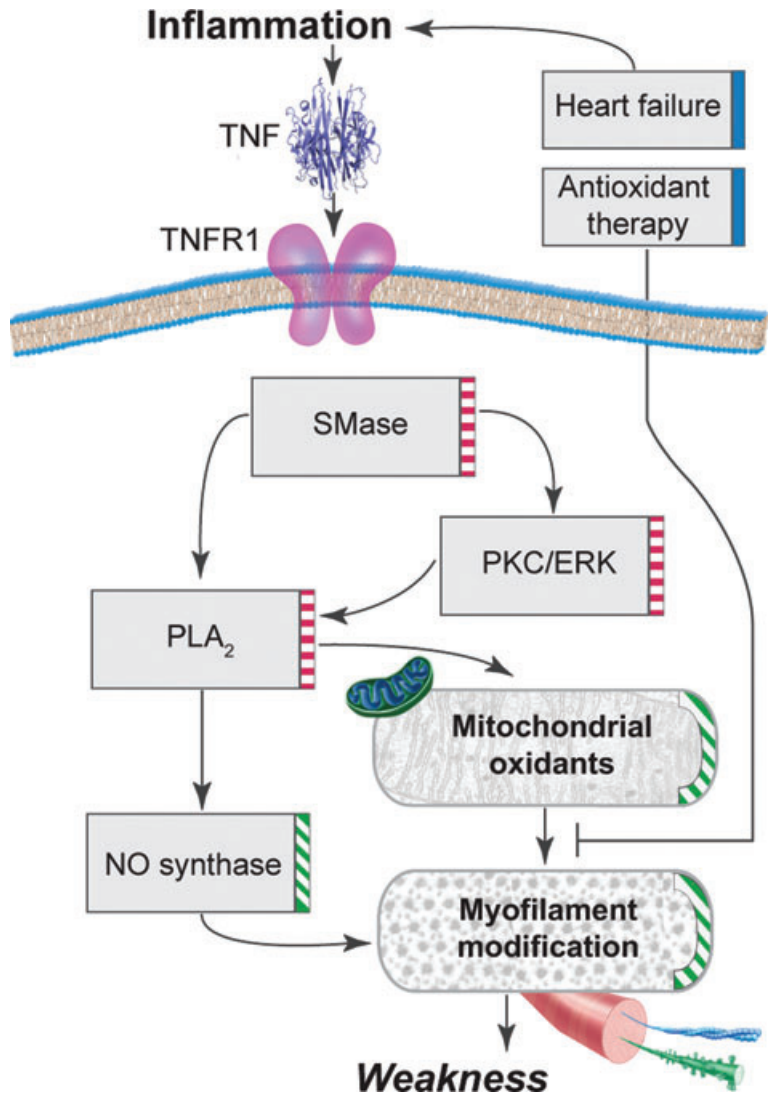

Figure 3. Model of signalling events that mediate heart failure-induced weakness
Model shows hypothetical mechanism by which chronic heart failure could depress specific force of skeletal muscle. Boxes below the sarcolemma (horizontal band) depict intracellular events that regulate TNFR1/oxidant signalling (right edge, red dashed bar) and oxidative inhibition of myofilament function (green hatched bar). Boxes above sarcolemma represent extracellular events that modulate the process (blue bar). TNF, tumour necrosis factor; TNFR1, TNF receptor subtype 1; SMase, sphingomyelinase; PKC, phosphokinase C; ERK, extracellular regulatory kinase; PLA_2, phospholipase A_2; NO, nitric oxide.

discriminate between NO and ROS. Like TNF, direct exposure to either NO or ROS decreases specific force of intact muscle fibres via effects on myofibrillar proteins. Thus, the redox cascade that mediates TNF-induced weakness remains an open question.

The mechanism by which oxidant activity depresses myofibrillar function is particularly interesting. Acute TNF administration to mice depresses specific force by 30–40% within 60 min (Hardin *et al.* 2008) while the weakness caused by long-term TNF overexpression is partially reversed by brief incubation in an antioxidant solution (Li *et al.* 2000). The rapid nature of these responses suggests that TNF depresses specific force via a post-translational mechanism. The mechanism may involve direct reaction of TNF-stimulated oxidants with myofilament proteins; for example, the myosin head (Burke *et al.* 1976), tropomyosin (Williams & Swenson, 1982), troponin C (Putkey *et al.* 1993) and actin (Liu *et al.* 1990) have regulatory sulfhydryls that are sensitive to oxidation and could alter myofilament interactions. Alternatively, we and our colleagues have proposed that redox control may occur upstream of the myofibrils, e.g. redox-sensitive kinases or phosphatases that alter the phosphorylation state of myofibrillar proteins and thereby influence force (Andrade *et al.* 2001). Other post-translational mechanisms may include carbonylation (Coirault *et al.* 2007), ubiquitination (Dalla Libera *et al.* 2005; van Hees *et al.* 2007), and myosin degradation (Tikunov *et al.* 1996; van Hees *et al.* 2007).

Summary and conclusion

Chronic inflammatory diseases decrease specific force of skeletal muscle. This contractile dysfunction appears to be mediated via oxidant effects on myofilament proteins. TNF is strongly implicated as an important molecule in the process. Beyond these basic concepts, the intracellular mechanisms that depress specific force remain largely undefined. We do not know the pathway by which receptor–ligand interaction stimulates oxidant production. Nor do we know the type(s) of oxidants stimulated by TNF, their intracellular sources, or the myofilament proteins that are affected. Research is needed to define the cellular mechanism and identify novel therapies. Skeletal muscle weakness is a major clinical problem. It promotes exercise intolerance and breathlessness that plague individuals with heart failure, cancer, arthritis and other chronic diseases. It complicates chronic disease, diminishes the quality of life, and makes death more likely. Effective strategies to preserve skeletal muscle function would have broad clinical significance. Discovery and implementation of such therapies is a long-term goal for scientists in this field of muscle biology.

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