Respiratory and Limb Muscle Weakness Induced by Tumor Necrosis Factor-α

Involvement of Muscle Myofilaments

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The respiratory and limb skeletal muscles become weakened in sepsis, congestive heart failure, and other inflammatory diseases. A potential mediator of muscle weakness is tumor necrosis factor (TNF)-α, a cytokine that can stimulate muscle wasting and also can induce contractile dysfunction without overt catabolism. This study addressed the latter process. Murine diaphragm and limb muscle (flexor digitorum brevis [FDB]) preparations were used to determine the relative sensitivities of these muscles to TNF-α. Intact muscle fibers were isolated from FDB and microinjected with indo-1 to measure changes in sarcoplasmic calcium regulation. We found that TNF-α depressed tetanic force of the diaphragm and FDB to comparable degrees across a range of stimulus frequencies. In isolated muscle fibers, TNF-α decreased tetanic force without altering tetrasic calcium transients or resting calcium levels. We conclude that (1) TNF-α compromises contractile function of diaphragm and limb muscle similarly, and (2) TNF-α decreases force by blunting the response of muscle myofilaments to calcium activation.

Keywords: respiratory muscles; muscle, skeletal; cytokines; tumor necrosis factor; muscle contraction

Weakness of respiratory and limb skeletal muscles frequently afflicts patients in the intensive care unit and patients with chronic cardiopulmonary disease. It is generally believed that diseases of the lungs, heart, and other organs exert remote effects on respiratory and limb muscles via inflammatory mediators released into the circulation. Chronic inflammatory disease can induce progressive loss of muscle mass with a corresponding decrement in force (1–6), changes thought to be mediated by catabolic cytokines, altered hormonal status, nutritional imbalance, and physical inactivity. Inflammatory disease may also cause weakness without muscle wasting (7–11), indicating a loss of contractile regulation. The mechanisms responsible for this contractile dysfunction are less well understood.

A decade ago, Wilcox and associates (12) showed that activated monocytes secrete soluble factors that inhibit diaphragm force production. Among these, tumor necrosis factor (TNF)-α has emerged as a potential mediator of contractile dysfunction. Circulating TNF-α levels are elevated in patients with chronic obstructive lung disease (13), heart failure (14), sepsis (15), transplant rejection (16, 17) and other inflammatory diseases that cause secondary muscle weakness (18–20). Circulating blood levels as high as 3 to 6 ng/ml (21, 22) have been measured in such patients. Functional studies indicate that TNF-α can induce contractile dysfunction. Systemic administration of exogenous TNF-α to dogs causes a rapid reduction in diaphragm force production in vivo (23). Transgenic overexpression of TNF-α by cardiac myocytes causes circulating TNF-α levels to rise in mice, decreasing diaphragm force to half-normal despite normal muscle mass and ultrastructure (24). Similar contractile losses can be replicated in vitro by exposing isolated muscle preparations to recombinant TNF-α (24–27).

The latter findings indicate that TNF-α acts directly on muscle fibers to disrupt excitation-contraction coupling, broadly defined by Catterall (28) as, “...the process of coupling chemical and electrical signals at the cell surface to the intracellular release of calcium and ultimate contraction of muscle fibers.” The most likely cause of dysfunction is a loss of calcium regulation. As reviewed by Chakraborti and colleagues (29), TNF-α alters cytoplasmic calcium in a variety of nonmuscle cell types via effects on intracellular calcium stores. In adult cardiac myocytes, TNF-α decreases peak sarcoplasmic calcium levels during the systolic contraction sequence, thereby diminishing calcium activation of cardiac myofilaments and exerting a negative inotropic effect (30). It is reasonable to suppose that a homologous mechanism pertains in skeletal muscle. TNF-α is reported to depolarize the resting membrane potential of skeletal muscle fibers (31), an action that tends to decrease voltage-dependent calcium release. This said, TNF-α effects on calcium regulation have not been tested in skeletal muscle.

The present study was designed to test two hypotheses: (1) Diaphragm and limb muscle differ in their sensitivities to TNF-α. In separate studies, TNF-α has been shown to depress force of rodent diaphragm fiber bundles (24–26) or extensor digitorum longus muscles (27). However, the reports of Alloati and coworkers (27) and Li and associates (24) both suggested that diaphragm and limb muscles differ significantly in their sensitivities to TNF-α. In the current study, we tested this postulate by directly comparing TNF-α effects on contractile function of murine diaphragm and flexor digitorum brevis (FDB), a hindlimb muscle. The latter was used because FDB is primarily composed of fast fibers (32), like mouse diaphragm (33), and because muscle fibers isolated from FDB could be used to test our second hypothesis. (2) TNF-α decreases skeletal muscle force by inhibiting calcium transients. We tested our second postulate by use of intact muscle fibers isolated from FDB. Fibers were microinjected with the calcium dye indo-1, enabling measurement of sarcoplasmic calcium concentration under resting conditions and during tetanic contractions. Data were collected from individual fibers before and after TNF-α exposure. FDB fibers were used because they are short, enabling calcium dye to be loaded using a single injection site, because FDB lacks excessive extracellular connective tissue that can impede fiber isolation, and because we have experience with this preparation (32, 34–39).
METHODS

Animal Use

Experimental protocols were approved by the local ethics committee. Animals were housed and procedures were conducted in accordance with animal welfare policies of the United States Public Health Service and the Swedish National Board for Laboratory Animals.

Excised Muscle Preparations

Adult male NMRI mice were killed by cervical dislocation and diaphragm and FDB muscles were excised. One fiber bundle was isolated from each hemidiaphragm. FDB muscles were studied intact.

Single Fiber Preparation

Intact single fibers were isolated from FDB muscle and studied according to Lännergren and Westerblad (37).

Measurement of Internal Calcium Concentration

We measured internal calcium concentration ([Ca\textsuperscript{2+}]) using methods described previously (35). The calcium indicator indo-1 (Molecular Probes, Eugene, OR) was microinjected into each fiber and fluorescence was measured using a commercial system (Photon Technology International, Photo Med, Wedel, Germany). Excitation light was set to 360 nm; light emitted at 405 nm and 495 nm was measured. The ratio of light emitted at 405 nm to that emitted at 495 nm was translated to [Ca\textsuperscript{2+}]. In subsequent analyses, resting [Ca\textsuperscript{2+}] was assessed as the average signal obtained 100 milliseconds before stimulation; tetanic [Ca\textsuperscript{2+}] and force were determined by averaging signals from the final 100 milliseconds of stimulation.

Experimental Protocols

TNF-α effects on diaphragm fiber bundles and FDB muscles were tested as described previously (24). Diaphragm and FDB preparations from a single animal were simultaneously incubated in oxygenated Tyrode’s solution (in mM: NaCl 121, KCl 5, CaCl\textsubscript{2} 1.8, MgCl\textsubscript{2} 0.5, NaH\textsubscript{2}PO\textsubscript{4} 0.4, NaHCO\textsubscript{3} 24, glucose 5.5; fetal calf serum of approximately 0.2%; pH 7.4; 25°C). Solutions bathing one diaphragm fiber bundle and one FDB muscle contained recombinant murine TNF-α 500 ng/ml (Boehringer Mannheim, Grenzach-Wyhlen, Germany; Alexis, Lausen, Switzerland). Time-matched contralateral control muscles were incubated in buffer alone. After 4 hours, each muscle was removed to a stimulation chamber containing TNF-α-free buffer (25°C). Length was adjusted to optimize twitch force. We then measured isometric forces evoked by direct stimulation at 1 (evokes twitch contraction), 10, 20, 30, 40, 50, 60, 80, 100, 120, and 150 Hz using supramaximal voltage, 0.5 millisecond pulses, and trains of 500 milliseconds (diaphragm) or 1,000 milliseconds (FDB). Force was recorded for later analysis.

Single fibers were mounted to optimize tetanic force and microinjected with indo-1. After equilibration, baseline function was established by duplicate 80 Hz stimulations delivered 15 minutes apart. TNF-α was then added to the superfusate and experimental fibers were stimulated at 80 Hz every 15 minutes for 45–225 minutes. Control fibers underwent an identical protocol without TNF-α. Force and [Ca\textsuperscript{2+}] signals were recorded for later analysis.

Statistical Analysis

To assess differences between two conditions, data sets were tested for normality and equality of variance and were then evaluated using Student’s t test (TNF-α effects among muscles) or paired t test (TNF-α effects within individual fibers) (40). Force–frequency relationships were assessed by use of two-way repeated measures analysis of variance (41). Rates of change of individual variables (resting [Ca\textsuperscript{2+}], tetanic force, tetanic [Ca\textsuperscript{2+}]) were determined by use of linear regression (40).

RESULTS

TNF-α Effects on Force of Diaphragm Versus Limb Muscle

TNF-α induced contractile dysfunction in both muscles that we tested. Maximum tetanic force developed by diaphragm fiber bundles treated with TNF-α (183 ± 5.3 kPa) was less than the maximum force of time- and stimulus-matched controls from the contralateral hemidiaphragm (215 ± 13 kPa; p < 0.05). TNF-α also decreased twitch force in diaphragm preparations (62 ± 2.6 kPa versus 76 ± 5.4; p < 0.04). Examined more broadly, TNF-α depressed force across the entire range of diaphragm activation. Figure 1 depicts the resulting shift of the force-frequency relationship (p < 0.0001). FDB muscles exhibited a similar response. TNF-α tended to depress maximal tetanic force (439 ± 41 kPa treated versus 534 ± 39 control), a difference that was not resolvable by t test (p < 0.09) due to substantial variability in absolute forces among muscles. TNF-α effects were evident when examined across the full range of muscle activation. As shown in Figure 2, TNF-α depressed FDB force at stimulation frequencies from 20 to 150 Hz (p < 0.01 by repeated measures analysis). This decrement was similar in magnitude to that observed in diaphragm. Unlike diaphragm, however, TNF-α did not alter tetanic force at 10 Hz in FDB. Nor was twitch force altered (63 ± 7 kPa versus 63 ± 8 kPa).

Stability of Isolated Single Fibers

Stability was assessed by monitoring untreated (control) fibers under experimental conditions for 4 hours. At the outset, maximal forces developed in response to 100 Hz stimulation averaged 331 ± 40 kPa, well within the normal range for this preparation (32, 34–39). For the remainder of the protocol, the tetanic force elicited by near-maximal (80 Hz) stimulation was used as an index of functional status. Data in Figure 3 show that the rate of force decline was minimal over this time. Regression analyses of data from individual fibers indicated an average rate of 1.67 ± 0.3%/hour, substantially less than the 5–10%/hour reported for isolated muscles or fiber bundles studied in vitro (42–44).

TNF-α Effects on Force in Single Fibers

Prior to TNF-α exposure, the functional properties of experimental fibers were indistinguishable from those measured in control fibers (see previous section). Maximal tetanic force averaged 340 ± 30 kPa (n = 5) and tetanic force declined at a mean rate of −2.04 ± 1.35%/hour. Exposure to TNF-α accelerated the fall of tetanic force in each fiber tested. This effect is shown in Figure 4. On average, TNF-α exposure doubled the rate of force decline to −4.33 ± 1.3%/hour (p < 0.01). This corresponds to an overall drop of 17% (~60 kPa) over 4 hours, corroborating the total decrements seen in diaphragm

![Figure 1.](image)
(-15%) and FDB muscles (-18%) over the same period. Figure 5 illustrates the effect of TNF-α on force development during individual tetanic contractions. Tracings from a control fiber depict the force waveform developed at the outset of the protocol and the modest decrement seen after 4 hours. Adjacent tracings from an experimental fiber illustrate the exaggerated decrement caused by TNF-α. Aside from lower force, there was no obvious effect of TNF-α on the shape of the tetanus. Neither the rate of rise of force nor the rate of relaxation was altered. This was a consistent observation among treated fibers (data not shown).

**TNF-α Effects on [Ca²⁺], Regulation**

At the beginning of experimental protocols, resting [Ca²⁺] was comparable in control (n = 3) and experimental (n = 5) fibers, averaging 88.0 ± 9.6 nm overall. Control fibers exhibited a slow increase in resting [Ca²⁺], that progressed at an average rate of 6.0 ± 1.0 nm/hour (p < 0.001). TNF-α did not alter the regulation of resting [Ca²⁺]; the rate of increase in experimental fibers (8.2 ± 2.7 nm/hour) was not different from control (p > 0.58). The stability of calcium activation during tetanic stimulation was assessed using control fibers. Regression analysis detected no systematic change in tetanic [Ca²⁺], transients during 4 hours incubation in TNF-α – free buffer (overall equation = 1.265 nm – [0.0920 nm/minute × minutes]; p > 0.43). TNF-α effects on force do not reflect a loss of calcium activation. We assessed the rate of change of tetanic [Ca²⁺] in TNF-α – treated fibers by regression analysis. Tetanic [Ca²⁺] did not decrease in response to TNF-α. If anything, tetanic [Ca²⁺] tended to increase slowly over time (0.011 ± 0.061%/minute), a trend that was not significantly different from zero (p > 0.15). Figure 6 illustrates the raw data used to assess [Ca²⁺] regulation. Tracings from a control fiber depict resting [Ca²⁺], and the details of a tetanic transient, including the rapid rise of [Ca²⁺] at the onset of stimulation, pulsatile transients during stimulation, and the rapid recovery of [Ca²⁺] when stimulation was terminated. Neither peak tetanic [Ca²⁺] nor the shape of the tetanic [Ca²⁺] waveform were altered by prolonged incubation. Similarly, tracings from an experimental fiber illustrate the insensitivity of [Ca²⁺] transients to TNF-α exposure, as no change was observed.

**DISCUSSION**

Skeletal muscle weakness is a common complication of inflammatory disease. Clinical reports show that respiratory and limb muscles develop less force than normal in patients with chronic obstructive pulmonary disease (6), chronic heart failure (11), sepsis (45), transplant rejection (5), cancer (46), and other chronic inflammatory processes (2). Similar loss of diaphragm function is seen in animal models of inflammatory disease, for example, septic peritonitis (47), endotoxin administration (48), or lipopolysaccharide injection (49). Muscle weakness predisposes affected individuals to ventilatory insufficiency and diminished exercise capacity. Loss of function is commonly attributed to loss of muscle mass, since muscle wasting and cachexia are familiar complications of chronic inflammation (50).

Less obvious is the loss of force that can occur independent of muscle size. This phenomenon has been reported in a variety of inflammatory diseases (7–10) but is best documented in the context of heart failure. Harrington and colleagues (11) documented such losses by measuring cross-sectional area and

**Figure 2.** TNF-α effects on force production by FDB. Less force was developed by muscles treated with TNF-α (open symbols) than by paired controls (closed symbols) at frequencies of 20–150 Hz (p < 0.01). Means shown ± SEM; n = 9/group.

**Figure 3.** Stability of contractile function in intact single fibers. Forces developed during periodic 80 Hz stimulation are shown for a 4-hour period. Open symbols (circle, triangle, inverted triangle) designate data from individual control fibers (n = 3). Line depicts regression fitted to combined data; slope = -1.55%/hour; p < 0.001.

**Figure 4.** TNF-α stimulates loss of force in single fibers. Values depict mean rates of force decline (%/hour) measured by regression analysis using data from individual experimental fibers (n = 5) before and after exposure to TNF-α 500 ng/ml. TNF-α accelerated the loss of force in each of five fibers studied (p < 0.02).

**Figure 5.** Depression of tetanic force transients by TNF-α. Individual tracings obtained from intact single fibers stimulated at 80 Hz before (solid line) and after (dotted line) 4-hour incubation in either buffer alone (left panel, control fiber) or in TNF-α 500 ng/ml (right panel, experimental fiber).
TNF-α inhibits the force of contraction in both cardiac and skeletal muscle. In isolated cardiac myocytes, Yokoyama and associates (30) showed that direct TNF-α exposure has a negative inotropic effect that is caused by a reduction in peak [Ca\(^{2+}\)] during the systolic contraction sequence. We expected the mechanism of [Ca\(^{2+}\)] action to be similar in skeletal muscle fibers. Electrophysiologic data indirectly supported this expectation. Wilcox and colleagues (23) reported that intravenous administration of TNF-α decreases compound action potentials in the canine diaphragm whereas Tracey and coworkers (31) showed that direct TNF-α exposure could depolarize the skeletal muscle sarcolemma. These observations suggested that TNF-α might depress voltage-dependent calcium release. But on the contrary, TNF-α did not alter tetanic [Ca\(^{2+}\)] transients. Nor were resting [Ca\(^{2+}\)] levels altered in inactive muscle fibers. The fact that force production fell with no change in [Ca\(^{2+}\)] transients indicates that TNF-α must disrupt contractile function downstream of the calcium signal (i.e., at the myofilament level).

How does TNF-α binding to its sarcolemmal receptor cause myofilament dysfunction? What transduces the signal? Isolated reports suggest that prostaglandins (12) or platelet activating factor (27) might play a role. A larger body of data implicates muscle-derived oxidants, reactive oxygen species (ROS) or nitric oxide (NO) derivatives, as second messengers. Increased ROS or NO production modulates inflammatory dysfunction in skeletal muscle (57). Recent illustrations include studies of muscle injury following resistive loading (58), septic peritonitis (47), lipopolysaccharide-induced dysfunction (49), and strenuous muscle contraction (59). TNF-α levels are elevated in such conditions and are likely to promote ROS or NO production. TNF-α has been shown to increase oxidant levels in muscle fibers (24) and extracellular release of NO derivatives (27). More importantly, TNF-α-induced weakness can be partially prevented by inhibitors of muscle-derived oxidants (24) or NO production (27). This resembles the capacity of antioxidants and NO synthase inhibitors to preserve muscle function in inflammatory processes such as resistive loading (58), peritonitis (47), and sepsis (60).

TNF-α effects on contractile regulation are similar to the effects of direct ROS or NO exposure. As observed with TNF-α, exogenous ROS and NO donors decrease tetanic force in intact fibers without altering tetanic [Ca\(^{2+}\)] transients (34, 35, 39). This robust finding indicates myofilament proteins are more sensitive to redox modulation than the proteins that regulate voltage-dependent calcium release (i.e., ion channels and transporters of the sarcolemma and sarcoplasmic reticulum [SR]). It is intriguing that contractile losses caused by TNF-α or exogenous oxidants are partially or wholly reversible by reduced thiol donors (24, 35, 39). Which myofilament proteins are most likely to undergo reversible oxidative inhibition? The most obvious candidate is myosin ATPase which has well-characterized sulfhydryl moieties that inhibit cross-bridge function upon oxidation (61). Troponin C is another candidate protein that exhibits oxidative inhibition (62). Alternatively, oxidative signaling might indirectly inhibit myofilament function via second messenger systems (e.g., redox-sensitive kinases or phosphatases that modulate the phosphorylation state of myofilament proteins). In contrast to the present study, prolonged or intensive oxidative stress can alter voltage-dependent calcium release (35). Such changes may reflect loss of SR regulation (63) or disruption of sarcolemmal continuity, as occurs in sepsis (45). The present data provide no evidence that TNF-α, alone, induces such changes, but neither do they rule it out.
Muscle weakness

Limitations of the Experimental Design

First, stability is a perennial concern with isolated muscle preparations (64). In studies of diaphragm fiber bundles and FDB muscles, time-dependent changes were controlled by use of time- and stimulus-matched controls. In studies of intact fibers, the time required for isolation, mounting, and dye injection make paired comparisons impractical. Instead, we assessed stability by measuring the time-course of contractile changes in control fibers. Force and calcium regulation were remarkably stable in the absence of TNF-α, documenting the usefulness of this preparation for prolonged protocols. This stability enabled experimental fibers to be used as their own controls in our subsequent studies of TNF-α effects on calcium regulation.

Second, a pharmacologic TNF-α concentration was used to overcome problems associated with in vitro experimentation. These include: (1) TNF-α adhesion to vessel and tubing surfaces, (2) rapid loss of TNF-α activity in physiologic buffers, (3) exaggerated diffusion distances within tissue, and (4) relatively short exposure times. Our approach was adapted from the literature (24–26) as a means of producing contractile dysfunction in mouse diaphragm. They further showed that eight weeks exposure to circulating TNF-α is associated with disease activity and the degree of anemia in patients with rheumatoid arthritis: interrelations with cellular sources and patterns of soluble immune mediator expression. Clin Infect Dis 1994;18:S147–S153.

Similarly, our current studies are designed to detect changes that may occur when intact muscle is exposed to lower TNF-α concentrations for longer periods of time.

Third, the present study was conducted at room temperature. This enhances the stability of isolated muscle preparations (64) but inhibits contractile changes mediated by endogenous free radicals (65). Accordingly, TNF-α effects on force were smaller in the current study than in studies conducted at 37°C (24).

Fourth, our in vitro experiments do not account for systemic effects of TNF-α that indirectly exaggerate skeletal muscle weakness in vivo, such as altered hormonal status (66) and reduced food consumption (67).

Conclusion

We reject both of our original hypotheses. TNF-α exerts similar effects on respiratory and limb skeletal muscles to inhibit contractile function via effects on the myofilaments. Calcium homeostasis is not appreciably altered. This mechanism enables TNF-α to cause muscle weakness in the absence of overt protein loss. Thus, in individuals with inflammatory disease, loss of muscle mass and contractile dysfunction appear to represent two separate processes with additive effects on muscle weakness. Elevated levels of circulating TNF-α are traditionally thought to mediate muscle catabolism. Our current data add to a growing body of evidence that TNF-α also induces contractile dysfunction.

References


