TNF/TNFR1 signaling mediates doxorubicin-induced diaphragm weakness

Laura A. A. Gilliam, Jennifer S. Moylan, Leonardo F. Ferreira, and Michael B. Reid

Department of Physiology and Center for Muscle Biology, University of Kentucky, Lexington, Kentucky

Submitted 3 August 2010; accepted in final form 16 November 2010

Gilliam LA, Moylan JS, Ferreira LF, Reid MB. TNF/TNFR1 signaling mediates doxorubicin-induced diaphragm weakness. Am J Physiol Lung Cell Mol Physiol 300: L225–L231, 2011. First published November 19, 2010; doi:10.1152/ajplung.00264.2010—Doxorubicin, a common chemotherapeutic agent, causes respiratory muscle weakness in both patients and rodents. Tumor necrosis factor-α (TNF), a proinflammatory cytokine that depresses diaphragm force, is elevated following doxorubicin chemotherapy. TNF-induced diaphragm weakness is mediated through TNF type 1 receptor (TNFR1). These findings lead us to hypothesize that TNF/TNFR1 signaling mediates doxorubicin-induced diaphragm muscle weakness. We tested this hypothesis by treating C57BL/6 mice with a clinical dose of doxorubicin (20 mg/kg) via intravenous injection. Three days later, we measured contractile properties of muscle fiber bundles isolated from the diaphragm. We tested the involvement of TNF/TNFR1 signaling using pharmaceutical and genetic interventions. Etanercept, a soluble TNF receptor, and TNFR1 deficiency protected against the depression in diaphragm-specific force caused by doxorubicin. Doxorubicin stimulated an increase in TNFR1 mRNA and protein (P < 0.05) in the diaphragm, along with colocalization of TNFR1 to the plasma membrane. These results suggest that doxorubicin increases diaphragm sensitivity to TNF by upregulating TNFR1, thereby causing respiratory muscle weakness.

CHEMOTHERAPY; INFLAMMATION; SKELETAL MUSCLE; CANCER

SYMPTOMS OF RESPIRATORY MUSCLE insufficiency are evident in cancer patients undergoing chemotherapy. Patients experience decreased maximal inspiratory pressures, an indicator of respiratory muscle weakness (53), along with dyspnea (27) and exercise intolerance (16). Over half of patients undergoing doxorubicin chemotherapy report dyspnea, closely associated with impaired physical performance (38). Doxorubicin may contribute to these symptoms by depressing the function of respiratory muscles. Doxorubicin administered in vivo depresses specific force of murine diaphragm (20), the primary muscle of inspiration. This study addresses the cellular mechanism by which doxorubicin depresses diaphragm-specific force.

A candidate mechanism for doxorubicin-induced diaphragm weakness involves TNFα (TNF), a proinflammatory cytokine, and the TNF receptor subtype 1 (TNFR1). Doxorubicin stimulates TNF expression by immune cells (56) and cardiac muscle (46) and increases serum TNF levels in both humans and rodents (45, 52). Most cellular responses to TNF are the result of TNFR1 activation, localizing TNFR1 to the plasma membrane and amplifying downstream signaling (59). In diaphragm, TNF acts via TNFR1 to depress specific force (24).

Integrating these facts, we hypothesized that diaphragm weakness stimulated by doxorubicin is mediated via TNF/TNFR1 signaling. We tested this hypothesis using a murine model of doxorubicin chemotherapy (10, 20, 52). TNF/TNFR1 signaling was interrupted using the anti-TNF drug etanercept, a soluble TNF receptor, and genetically engineered mice deficient in TNFR1 (TNFR1<sup>-/-</sup>). Mice received a single intravenous injection of doxorubicin. After 72 h, diaphragm fiber bundles were excised and contractile function was measured ex vivo.

MATERIALS AND METHODS

Materials. Doxorubicin was purchased from Bedford Laboratories (Bedford, OH). Etanercept was purchased from Immunex (Thousand Oaks, CA). (+)-Tubocurarine chloride hydrate (25 µM) was purchased from Sigma (St. Louis, MO). Mouse TNFα DuoSet ELISA kit was purchased from R&D Systems (Minneapolis, MN). The ELAST ELISA Amplification System was purchased from PerkinElmer LAS, (Waltham, MA). Primers for GAPDH (acc. no. NM_008084.2; forward and reverse 5'-CATGCGCTTCCGTGT-TCCTA-3', 5'-GCGGACGCTAGTACA-3'), TNF (acc. no. NM_013693.2; 5'-TCACTGACACACATACAGA-3', 5'-GACAT-TCGAGGCTCCAGTGA-3'), and TNFR1 (acc. no. NM_011609.4; 5'-TCCGTTGCAAATGTACA-3', 5'-GGCAACAGCCGGAATAC-3') were purchased from Invitrogen (Carlsbad, CA). The TNF antibody was purchased from Chemicon (Millipore, Bedford, MA). The TNFR1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The annexin II antibody was purchased from ECM Biosciences (Versailles, KY). Fluorescence-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Animal care. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Studies were conducted at the University of Kentucky using 6- to 8-wk-old male C57BL/6 mice (Harlan, Indianapolis, IN) and TNFR1 receptor-deficient mice (TNFR1<sup>-/-</sup>); B6.129-Tnfrsf1a<sup>tm1Mak</sup>; The Jackson Laboratory, Bar Harbor, ME) with background strain C57BL/6 as wild types. Animals were maintained in the Division of Laboratory Animal Resources facility on a 12:12-h dark:light cycle and provided food and water ad libitum.

Drug administration. Mice were given an intravenous injection of doxorubicin (20 mg/kg). This dose is equivalent to 60 mg/m<sup>2</sup> based on the conversion factor established by Freireich (17), which is derived from the relationship between body weight and surface area of the animal. This falls within the clinical dosing regimen for treatment of hematological malignancies (60–75 mg/m<sup>2</sup>) (22). Control animals received the same volume of vehicle (PBS). The diaphragm was excised for analysis at 24 h time points following a single injection (24, 48, and 72 h). For etanercept experiments, there were four experimental groups: vehicle, vehicle + etanercept, doxorubicin, and doxorubicin + etanercept. Mice were given two subcutaneous injections of etanercept (5 mg/kg): 24 h pre- and 36 h post-doxorubicin injection. For all endpoints, no statistical difference existed between the vehicle and vehicle + etanercept groups. Those groups were combined into one control group for further statistical analyses.

Contractile function. Experiments were performed as described previously (19). In brief, mice were anesthetized with isoflurane and euthanized by cervical dislocation. The diaphragm was excised and placed in Krebs-Ringer solution (in mM: 137 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>) equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>.
CO₂ (pH ~7.4). A fiber bundle with its associated rib and central tendon was isolated from the costal diaphragm. The muscle was attached to a force transducer (BG Series 100g; Kulite, Leonia, NJ) using 4–0 silk suture. The force transducer was mounted on a micrometer used to adjust muscle length. The muscle was placed in a temperature-controlled organ bath between platinum wire stimulating electrodes and stimulated to contract isometrically using electrical field stimulation (Grass S48; Quincy, MA). The output of the force transducer was recorded using an oscilloscope (546601B; Hewlett-Packard, Palo Alto, CA) and computer software (Axoscope 9.2; Molecular Devices, Sunnyvale, CA). In each experiment, the muscle was adjusted to the length where twitch force was maximal (optimal length, Lₒ) at room temperature, and Lₒ was measured using an electronic caliper (CD-6/H11033; Mitutoyo America, Aurora, IL). The bath temperature was then increased to 37°C, followed by an equilibration period of 30 min. For direct doxorubicin exposure, muscles were incubated in 2 μg/ml (2 μM) doxorubicin for 1 h. One minute before stimulation, 25 μM (+)-tubocurarine chloride hydrate was added to the organ bath. The force-frequency relationship was determined using contractions evoked at 2-min intervals using stimulus frequencies of 1 (twitch stimulus), 15, 30, 50, 80, 120, 150, 250, and 300 Hz. Pulse and train durations were 0.3 and 250 ms. Time-to-peak twitch force (TPT) and twitch half-relaxation time (1/2 RT) were also measured. Following each experiment, the muscle was removed, blotted dry, and weighed. Cross-sectional area was determined as described by Close (11). Specific forces were expressed as N/cm².

ELISA. TNF was measured in serum using a mouse-TNFα DuoSet ELISA kit according to the manufacturer’s recommendations with the following modifications: 1) serum samples were incubated with the capture antibody at room temperature, 2) biotinylated detection antibody was incubated for 2 h at 37°C, and 3) streptavidin-horseradish peroxidase (HRP) was incubated for 20 min at 37°C. The HRP signal was amplified using the ELAST ELISA Amplification System according to the manufacturer’s recommendations. A microplate reader (Spectramax M2, Molecular Devices) was used to detect optical density of the colorimetric signal. The concentration of TNF was calculated using a standard curve (recombinant mouse TNF

Western blot analysis. Diaphragm muscles were homogenized in 2× lysis buffer (20 mM Tris, pH 7.2, 2% SDS) and then diluted 1:1 in 2× sample loading buffer (120 mM Tris, pH 7.5, 200 mM DTT, 20% glycerol, 4% SDS, and 0.002% bromphenol blue). Proteins were fractionated on 15% SDS-polyacrylamide gels (Criterion precast gels; Bio-Rad, Hercules, CA) and transferred to reduced-fluorescence PVDF membrane (Immobilon-FL; Millipore, Bedford, MA). Membranes with transferred proteins were blocked for 1 h at room temperature in Odyssey blocking buffer (LI-COR, Lincoln, NE). Membranes were incubated with primary antibodies overnight at room temperature in Odyssey blocking buffer mixed 1:1 with PBS plus 0.2% Tween, followed by four 5-min washes. Membranes were incubated with fluorescence-conjugated secondary antibodies in Od-
Doxorubicin depresses diaphragm force (Fig. 2), confirming our previous results (20). To test TNF as a mediator of doxorubicin action, mice were injected with etanercept in combination with doxorubicin. Doxorubicin causes a loss in body weight (20), which is not protected with etanercept treatment (Fig. 2A). This loss in weight did not affect the physical dimensions of diaphragm fiber bundles. We saw no differences between groups in fiber bundle weight (doxorubicin 2.5 ± 0.2 mg, doxorubicin + etanercept 2.7 ± 0.1 mg, control 2.9 ± 0.2 mg, P > 0.3) or cross-sectional area (doxorubicin 0.26 ± 0.04 mm², doxorubicin + etanercept 0.29 ± 0.01 mm², control 0.31 ± 0.02 mm², P > 0.2). Lₒ was not different between groups (P > 0.3). The depression in diaphragm-specific force caused by doxorubicin was abolished by etanercept treatment (Fig. 2B). The relative force-frequency curve was shifted leftward (Fig. 2C). In both doxorubicin-treated groups the twitch:tetanus ratio was significantly increased (doxorubicin 0.37 ± 0.03, doxorubicin + etanercept 0.34 ± 0.01, control 0.27 ± 0.01, P < 0.01). TPT was not altered (doxorubicin 18 ± 1 ms, doxorubicin + etanercept 19 ± 1 ms, control 19 ± 1 ms, P > 0.5) nor was ½ RT changed (doxorubicin 18 ± 1 ms, doxorubicin + etanercept 16 ± 2 ms, control 16 ± 1 ms, P > 0.7).

RESULTS

Diaphragm bundles were exposed for 1 h to 2 μg/ml of doxorubicin, a similar concentration found in the serum of patients undergoing doxorubicin chemotherapy (49). We saw no differences in specific force (Fig. 1) following direct doxorubicin exposure.
The protective effect of etanercept, a soluble TNF receptor, suggested circulating TNF might mediate the doxorubicin-induced dysfunction. Circulating TNF was measured using a standard ELISA kit, with a tyramide amplification system that enabled detection of TNF at levels <15 pg/ml. The linear regression slope of the standard curve was greater with amplification, suggesting greater sensitivity (amplified $5.2 \times 10^{-3}$, unamplified $2.0 \times 10^{-3}$). Despite amplification, serum TNF levels fell below the detection limit of our assay in both groups. Nor did doxorubicin alter TNF mRNA or protein levels in the diaphragm.

TNFR1 signaling mediates TNF-induced skeletal muscle weakness (24). Diaphragm TNFR1 mRNA levels were 50% greater than control 48 h after doxorubicin exposure (Fig. 4A). TNFR1 protein exists in three different isoforms (25), all of which were detected in the diaphragm. The full-length 55-kDa TNFR1 was decreased 48–72 h following doxorubicin administration, with no change in the soluble 28-kDa isoform (Fig. 4B). The 48-kDa TNFR1 isoform was increased at 72 h following doxorubicin (Fig. 4B).

The majority of TNFR1 resides in the golgi apparatus and is translocated to the plasma membrane upon stimulation by TNF (8, 33). We observed TNFR1 staining within diaphragm fibers and in close approximation to annexin II, a plasma membrane marker (Fig. 5). Figure 5 is a representative image of our observations in three animals, four diaphragm sections per animal. TNFR1 staining is less prominent in vehicle-treated animals.

TNFR1 deficiency did not protect against doxorubicin-induced loss of body weight (Fig. 6A). As in the etanercept experiments (above), doxorubicin did not affect the size of diaphragm fiber bundles from TNFR1−/− mice. Reductions in fiber bundle weight (doxorubicin 2.7 ± 0.4 mg vs. vehicle 3.4 ± 0.3, $P > 0.2$) and cross-sectional area (doxorubicin 0.28 ± 0.03 mm² vs. vehicle 0.35 ± 0.03, $P > 0.1$) were not significant. Nor was Lo different ($P > 0.9$).

TNFR1 deficiency abolished the depression in specific force caused by doxorubicin (Fig. 6B) but the leftward shift in the relative force-frequency curve persisted (Fig. 6C). An increase in the twitch:tetanus ratio approached statistical significance (doxorubicin 0.32 ± 0.02 vs. vehicle 0.27 ± 0.02, $P > 0.06$), but we observed no change in TPT (doxorubicin 19 ± 1 ms vs. vehicle 20 ± 2, $P > 0.6$) or ½ RT (doxorubicin 15 ± 1 ms vs. vehicle 17 ± 1, $P > 0.3$).

**DISCUSSION**

These studies demonstrate that TNF/TNFR1 signaling mediates diaphragm weakness induced by doxorubicin. Etanercept, a soluble TNF receptor, prevented the depression in force caused by doxorubicin. We detected no changes in circulating or muscle-derived TNF. Rather, doxorubicin appears to stimulate expression and sarcolemmal localization of TNFR1. Genetic TNFR1 deficiency protected the diaphragm against doxorubicin-induced weakness, confirming an essential role for TNF/TNFR1 signaling.

**Direct effects of doxorubicin.** Circulating levels of doxorubicin are $\sim 1.25 \mu g/ml$ following an intravenous doxorubicin injection (12 mg/kg) (32). Extrapolating from our 20 mg/kg dose, circulating levels of doxorubicin in our model are ex-
Bonferroni test. 0.01 for overall difference by repeated-measures ANOVA; *H11021.

Muscle function (12, 42, 60). Exposure to TNF in vivo de-inflammation diseases and closely associated with the loss of rubicin injections (19, 52). TNF is implicated in numerous chemotherapy (45) and in healthy rodents that receive doxorubicin itself could alter muscle function. The 55-kDa full-length receptor is found on the diaphragm muscle fibers and thereby induce contractile dysfunction.

The data that implicate TNF are from our studies of etanercept, a soluble TNF receptor that blocks the functional activity of circulating TNF (23). Rheumatoid arthritis patients are prescribed etanercept to block chronic inflammation (2). Etanercept administration also attenuates the inflammatory response in other conditions, diminishing TNF in the circulation and mimicking TNFR1 deficiency (31, 40, 54). While serum TNF levels fell below the limits of our assay, diaphragm protection by etanercept suggests TNF is essential for doxorubicin-induced dysfunction.

Fig. 6. TNFR1 deficiency protects against doxorubicin-induced diaphragm dysfunction. A: body weight over 3 days following doxorubicin administration. Specific force (B) and relative force (C) measured 72 h following injection. Data are means ± SE; n = 8 (vehicle) or 7 (doxorubicin); for A and C, P < 0.01 for overall difference by repeated-measures ANOVA; *P < 0.01 by Bonferroni test.

expected to be 2 μg/ml, approximately two times the concentration of circulating doxorubicin in patients following chemotherapy (~1 μg/ml) (14, 49). We show that direct, short-term exposure to 2 μg/ml of doxorubicin in vitro does not alter the force of diaphragm fiber bundles. It is possible that with long-term exposure, doxorubicin itself could alter muscle function directly. However, an indirect mechanism appears more likely. Other studies (36, 46) and our current results suggest that doxorubicin causes muscle dysfunction through secondary mediators.

Roles of TNF and TNFR1. One potential mediator is TNF, which is elevated in cancer patients undergoing doxorubicin chemotherapy (45) and in healthy rodents that receive doxorubicin injections (19, 52). TNF is implicated in numerous inflammatory diseases and closely associated with the loss of muscle function (12, 42, 60). Exposure to TNF in vivo depresses skeletal muscle-specific force (1, 24), linking the inflammatory cytokine with skeletal muscle dysfunction.

In our study we found no changes in circulating or muscle-derived TNF following doxorubicin administration. This difference from other studies of healthy rodents is likely due to the different method of doxorubicin administration, intravenous vs. intraperitoneal injection. We have shown that intraperitoneal injection of doxorubicin causes an exacerbated inflammatory response, most likely drug-induced peritonitis. This localized inflammatory response is absent after intravenous injection of doxorubicin, although diaphragm weakness still persists (20).

The data that implicate TNF are from our studies of etanercept, a soluble TNF receptor that blocks the functional activity of circulating TNF (23). Rheumatoid arthritis patients are prescribed etanercept to block chronic inflammation (2). Etanercept administration also attenuates the inflammatory response in other conditions, diminishing TNF in the circulation and mimicking TNFR1 deficiency (31, 40, 54). While serum TNF levels fell below the limits of our assay, diaphragm protection by etanercept suggests TNF is essential for doxorubicin-induced dysfunction.

TNFR1 mediates TNF signaling in a vast majority of cells (59). For example, selective deletion of this receptor subtype protects against carrageenan-induced inflammation of the lungs (40), proinflammatory signaling in macrophages (13), and TNF-induced muscle weakness (24). Our current data show that TNFR1 deficiency also protects diaphragm against doxorubicin-induced weakness.

Cellular mechanism. Doxorubicin may weaken the diaphragm by altering TNFR1 expression. We found that doxorubicin increases TNFR1 mRNA, suggesting alterations of transcriptional regulation in response to doxorubicin. For example, members of the CCAAT/enhancer binding protein (C/EBP) family regulate the TNFR1 promoter (9) suggesting C/EBP proteins as potential gene regulators by which doxorubicin might increase TNFR1 expression. Alternatively, doxorubicin might increase TNFR1 mRNA by promoting mRNA stabilization.

Doxorubicin selectively altered tissue levels of all three TNFR1 isoforms. Doxorubicin increased the 48-kDa isoform, an exosome-associated TNFR1 receptor that promotes TNFR1 signaling among cells, thereby altering function (61). For example, circulating exosomes from patients in septic shock are presumed to contain the 48-kDa TNFR1 isoform and have been shown to depress contractile function of healthy cardiac muscle (5). Similarly, by stimulating the exosome-associated isoform, doxorubicin could promote TNFR1 signaling among diaphragm muscle fibers and thereby induce contractile dysfunction. The 55-kDa full-length receptor is found on the plasma membrane and undergoes proteolytic cleavage to form the 28-kDa soluble TNFR1 (25, 47). Doxorubicin stimulated loss of the full-length receptor from the diaphragm over time, suggesting cleavage and release of the soluble 28-kDa isoform. By binding to free TNF, soluble TNFR1 may neutralize the cytokine or may function as a carrier protein, increasing TNF half-life and biological activity (29, 40).

A second mechanism of doxorubicin action appears to be TNFR1 translocation. Under basal conditions, TNFR1 is housed in the golgi and trafficked to the plasma membrane in response to TNF stimulation (8, 25, 33). Similarly, doxorubicin
treatment increased TNFR1 expression in the diaphragm and stimulated translocation of TNFR1. Greater TNFR1 availability at the cell surface favors ligand binding, increasing diaphragm sensitivity to TNF, and promoting respiratory muscle weakness.

The intracellular mechanism by which TNFR1 activation impairs contractile function appears to involve redox signaling. TNF stimulates skeletal muscle to produce oxidants via a TNFR1-dependent mechanism (24). This increase in oxidants can cause contractile dysfunction by affecting myofibrillar proteins (3, 35) or calcium homeostasis (55, 58). Permeabilized muscle fibers from TNF-treated animals show a depression in calcium-activated force with no alterations in calcium sensitivity or cross-bridge cycling rate (24). Myofilament proteins are susceptible to oxidation, which can alter structure and impair contractile function (7, 26). These findings suggest oxidants downstream of TNFR1 signaling could alter the myofibrillar lattice, leading to contractile dysfunction.

Translational relevance. Dyspnea, or shortness of breath, is a sign of respiratory muscle insufficiency in humans (6, 48, 57) and a common symptom found in cancer patients undergoing chemotherapy (15, 53). Multiple factors, both drug and disease related, may contribute to dyspnea and related side effects resulting in serious clinical consequences. Patients may become too ill to receive chemotherapy, delaying clinic visits, decreasing treatment efficacy, and lessening the probability of a successful outcome (18, 30, 39, 50). These side effects also may contribute to the development of a TNFR1-dependent mechanism (24).

TNFSF15-dependent mechanisms (24). This increase in oxidants can cause contractile dysfunction by affecting myofibrillar proteins (3, 35) or calcium homeostasis (55, 58). Permeabilized muscle fibers from TNF-treated animals show a depression in calcium-activated force with no alterations in calcium sensitivity or cross-bridge cycling rate (24). Myofilament proteins are susceptible to oxidation, which can alter structure and impair contractile function (7, 26). These findings suggest oxidants downstream of TNFR1 signaling could alter the myofibrillar lattice, leading to contractile dysfunction.

Translational relevance. Dyspnea, or shortness of breath, is a sign of respiratory muscle insufficiency in humans (6, 48, 57) and a common symptom found in cancer patients undergoing chemotherapy (15, 53). Multiple factors, both drug and disease related, may contribute to dyspnea and related side effects resulting in serious clinical consequences. Patients may become too ill to receive chemotherapy, delaying clinic visits, decreasing treatment efficacy, and lessening the probability of a successful outcome (18, 30, 39, 50). These side effects also may contribute to the development of a TNFR1-dependent mechanism (24).

TNFSF15-dependent mechanisms (24). This increase in oxidants can cause contractile dysfunction by affecting myofibrillar proteins (3, 35) or calcium homeostasis (55, 58). Permeabilized muscle fibers from TNF-treated animals show a depression in calcium-activated force with no alterations in calcium sensitivity or cross-bridge cycling rate (24). Myofilament proteins are susceptible to oxidation, which can alter structure and impair contractile function (7, 26). These findings suggest oxidants downstream of TNFR1 signaling could alter the myofibrillar lattice, leading to contractile dysfunction.

Translational relevance. Dyspnea, or shortness of breath, is a sign of respiratory muscle insufficiency in humans (6, 48, 57) and a common symptom found in cancer patients undergoing chemotherapy (15, 53). Multiple factors, both drug and disease related, may contribute to dyspnea and related side effects resulting in serious clinical consequences. Patients may become too ill to receive chemotherapy, delaying clinic visits, decreasing treatment efficacy, and lessening the probability of a successful outcome (18, 30, 39, 50). These side effects also may contribute to the development of a TNFR1-dependent mechanism (24).

TNFSF15-dependent mechanisms (24). This increase in oxidants can cause contractile dysfunction by affecting myofibrillar proteins (3, 35) or calcium homeostasis (55, 58). Permeabilized muscle fibers from TNF-treated animals show a depression in calcium-activated force with no alterations in calcium sensitivity or cross-bridge cycling rate (24). Myofilament proteins are susceptible to oxidation, which can alter structure and impair contractile function (7, 26). These findings suggest oxidants downstream of TNFR1 signaling could alter the myofibrillar lattice, leading to contractile dysfunction.


