Tumor necrosis factor-α and malnutrition-induced inhibition of diaphragm fiber growth in young rats

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Submitted 1 March 2005; accepted in final form 29 June 2005


Lewis, Michael I., Xiaoyu Da, Hongmei Li, and Mario Fournier. Tumor necrosis factor-α and malnutrition-induced inhibition of diaphragm fiber growth in young rats. J Appl Physiol 99: 1649–1657, 2005—The aim of the present study was to test the hypothesis that tumor necrosis factor (TNF)-α has a significant pathophysiological role in malnutrition-induced inhibition of diaphragm muscle growth in young growing rats. Three groups of rats were studied: 1) control (CTL); 2) nutritional deprivation (ND; 50% of normal food intake for 7 days); and 3) ND + rat specific anti-TNF-α antibody. DIA fiber cross-sectional areas were determined. Serum and muscle TNF-α levels were measured by real-time PCR, ELISA, and immunohistochemistry. Body weights decreased 20% in ND rats and increased 46% in CTL animals. Anti-TNF-α had no effect on body weight or on DIA mass in ND animals. ND significantly reduced cross-sectional areas of all fiber types (33–46%). Anti-TNF-α failed to attenuate ND-induced inhibition of DIA fiber growth. Serum TNF-α levels increased 2.6-fold in ND animals, with levels suppressed to below CTL values with anti-TNF-α. DIA TNF-α mRNA and protein levels increased two- to threefold in ND rats. Anti-TNF-α antibodies suppressed muscle levels of the cytokine in ND animals to near CTL values. TNF-α immunoreactivity in all DIA fibers revealed similar directions of change in both ND groups. Direction and magnitude of change in DIA phosphorylated p38 MAPK (a likely second messenger of TNF-α) tracked those of TNF-α. Muscle levels of IGF-I mRNA and phosphorylated Akt were markedly reduced in ND animals with no change following anti-TNF-α therapy. Thus rat anti-TNF-α at a dose known to neutralize the cytokine failed to attenuate or reverse ND-induced inhibition of DIA fiber growth in our model.

MALNUTRITION CONTRIBUTES SIGNIFICANTLY to both morbidity and mortality in children and may develop acutely in hospitalized pediatric patients, particularly those with critical illness (18, 41). Our laboratory has previously reported that the diaphragms of young growing animals exhibit significantly reduced nutritional reserve (26). Although 90 h of complete food deprivation significantly reduced the cross-sectional areas (CSAs) of all diaphragm fiber types in young growing animals, no impact was noted in adult rats following an identical experimental protocol (26, 27). Similarly, Goodman et al. (15) reported negative influences on both arms of muscle protein turnover in young growing rats after short-term fasting, whereas no impact was observed in adult animals. Furthermore, Oster et al. (38) reported significantly greater depression of insulin-like growth factor (IGF)-I with malnutrition in young rats compared with adult animals. It would thus appear that the respiratory and limb muscles of young growing animals are unable to biochemically adapt to even short periods of malnutrition (15).

Basal serum levels of tumor necrosis factor (TNF)-α have been reported to be significantly elevated in malnourished compared with well-nourished children (14). This is of interest as TNF-α, a proinflammatory cytokine (formally called “cachectin”), has been implicated in a number of disease states associated with muscle wasting/cachexia (2, 8, 9, 36, 52). Although circulating blood monocytes may be a source of TNF-α in some of these states (8), local production by skeletal muscle fibers acting in an autocrine or paracrine fashion may also contribute (44). Furthermore, skeletal muscles have been demonstrated to express both TNF-α type 1 and 2 receptors (50), thus facilitating the action of both systemic and locally derived TNF-α. In preliminary studies, we demonstrated increased TNF-α immunoreactivity in atrophied rat diaphragm muscle fibers after a number of catabolic insults [i.e., nutritional deprivation (ND) and administration of corticosteroids; Lewis MI, unpublished data]. This together with reports of increased circulating levels of TNF-α in malnourished children prompted us to consider whether TNF-α exhibited a pathogenic role in the negative influences of malnutrition on the diaphragm of young growing rats.

A number of pathophysiological actions of TNF-α may contribute to muscle wasting. For example, TNF-α has been shown to decrease the fractional synthesis rate of both myofibrillar and sarcoplasmic proteins in the rat gastrocnemius (20) by impacting on translation initiation. This may, in part, reflect inhibition of muscle IGF-I expression by TNF-α (11). Studies in muscle cell cultures exposed to TNF-α reported degradation of muscle-specific proteins, which was shown to be mediated by the transcription factor nuclear factor-κB (NF-κB) via an increase in ubiquitin-conjugating activity (30, 31) promoting muscle protein breakdown via the ubiquitin-proteasome proteolytic system (29, 33). In addition, TNF-α has recently been shown to upregulate a muscle-specific E3 ligase via p38 MAPK (28). TNF-α may also downregulate the myogenesis factor MyoD in differentiated muscle cells (16) and activate DNA fragmentation with cancer cachexia (5).

The aim of the present study was to test the hypothesis that TNF-α has a significant pathophysiological role in malnutrition-induced inhibition of diaphragm muscle growth in young growing rats. We thus evaluated the contribution of TNF-α by administering rat-specific monoclonal anti-TNF-α antibodies to young, growing, nutritionally depleted animals.

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THE STUDIES WERE PERFORMED IN YOUNG MALE SPRAGUE-DAWLEY RATS (INITIAL BODY WEIGHT 105 ± 1.9 g). A PURIFIED POWDERED BALANCED RODENT DIET (AIN 93G, DYETS) WAS USED TO MANIPULATE THE DIET SO AS TO PROVIDE 50% OF NORMAL INTAKE OVER 7 DAYS IN SEVERAL GROUPS. THIS MODERATE LEVEL OF ND WAS PREVIOUSLY REPORTED BY US TO PRODUCE ATROPHY OF ALL DIAPHRAGM FIBER TYPES IN YOUNG RATS (22). WATER WAS FED TO ALL ANIMALS AD LIBITUM. IN CONTROL (CTRL) ANIMALS, THE DIET WAS PROVIDED AD LIBITUM. ALL ANIMALS WERE HOUSED INDIVIDUALLY IN A THERMALLY CONTROLLED ENVIRONMENT (22°C) WITH A DARK-LIGHT CYCLE OF 1:1.

RAT-SPECIFIC ANTI-TNF-α MONOClonAL ANTIBODIES (RRT108) WERE PROVIDED AS A GIFT BY CENTOCOR (RADNOR, PA). THESE ANTIBODIES WERE DILUTED WITH PBS AND ADMINISTERED BY INTRAPERITONEAL INJECTION EVERY 48 HOURS DURING THE EXPERIMENTAL PROTOCOL, STARTING AT TIME 0. THE DOSE OF 15 mg/kg WITH EACH INJECTION PRODUCES SIGNIFICANT PROLONGED NEUTRALIZATION OF ENDOGENOUS RAT TNF-α (REFS. 10, 47; SHEALY DJ, PERSONAL COMMUNICATION). ANIMALS NOT RECEIVING ANTI-RAT TNF-α INJECTIONS WERE GIVEN EQUIVALENT VOLUMES OF PBS ALONE INTRAPERITONEALLY.

THE ANIMAL GROUPS INCLUDED 1) CTRL (n = 6), 2) ND (n = 7), AND 3) ND + ANTI-TNF-α (n = 7). THE EXPERIMENTAL PROTOCOL WAS APPROVED BY THE CEDARS-SINAI MEDICAL CENTER/BURNS AND ALLEN RESEARCH INSTITUTE ANIMAL CARE AND USE COMMITTEE.

IMMUNOHISTOCHEMICAL ANALYSES

THE ENTIRE COSTAL REGIONS OF BOTH HEMIDIAPHRAGMS WERE EXCISED AND WEIGHTED. A PORTION OF THE RIGHT MIDCOSTAL DIAPHRAGM WAS USED FOR IMMUNOHISTOCHEMICAL STUDIES, whereas the remainder was used for protein analyses. THE MIDCOSTAL DIAPHRAGM SEGMENT WAS MOUNTED ON CORK AND STRETCHED TO THE ESTIMATED OPTIMAL LENGTH AND THEN RAPIDLY FROZEN IN ISOPENTANE, WHICH HAD BEEN COOLED TO ITS MELTING POINT BY LIQUID NITROGEN. SERIAL SECTIONS WERE CUT AT 10-UM THICKNESS USING A CRYOSTAT (REICHERT-JUNG, MODEL 2800E, NUSLEICH, GERMANY) KEPT AT -20°C.

FIBER PROPORTIONS. THE IDENTIFICATION OF VARIOUS MYOSIN HEAVY CHAIN (MHC) ISOFORMS IN THE RAT DIAPHRAGM WAS OBTAINED BY INDIRECT IMMUNOPEROXIDASE TECHNIQUE. SERIAL DIAPHRAGM MUSCLE CRYoseCTIONS WERE DRIED AT ROOM TEMPERATURE, FIXED IN COLD ACETONE FOR 5 MIN, WASHED WITH PBS FOR 5 MIN, AND INCUBATED IN NORMAL GOAT SERUM FOR 15 MIN AT ROOM TEMPERATURE. SECTIONS WERE INCUBATED FOR 2 h AT ROOM TEMPERATURE IN ONE THE FOLLOWING MOUSE ANTI-MHC MONOCLONAL ANTIBODIES (DILUTED IN PBS): BA-D5 (1:10) REACTING WITH MHCIA/IIB/IIC/G; SC-71 (1:10) REACTING WITH MHCfast 2A, AND BF-35 (1:20) REACTING WITH ALL MHCs EXCEPT MHCfast 2X (ANTIbODIES KINDLY PROVIDED BY REGENERON PHARMACEUTICALS, Tarrytown, NY) (46). SECTIONS WERE RINSED WITH PBS AND EXPOSED TO AN APPROPRIATE BIOTINYLATED SECONDARY ANTIBODY FOR 30 MIN AT ROOM TEMPERATURE. CTRL SECTIONS WERE EXPOSED TO SECONDARY ANTIBODIES ONLY. SECTIONS WERE RINSED WITH PBS AND EXPOSED TO THE AVIDIN-BIOTINYLATED ENZYME COMPLEX (ELITE PK-6100; VECTOR LABORATORIES, BURLINGAME, CA) REAGENT FOR 20 MIN AT ROOM TEMPERATURE. SECTIONS WERE RINSED AGAIN WITH PBS, AND VISUALIZATION WAS OBTAINED AFTER EXPOSURE TO THE PERoxidase SUBSTRATE 3-AMINo-9-ETHYLENEDIAMINE (AEC) Peroxidase Substrate Kit; VECTOR Laboratories) FOR 10 MIN. SECTIONS WERE WASHED FOR 5 MIN AND MOUNTED WITH Glycerin jelly mounting medium. BASED ON DIFFERENCES IN IMMUNOREACTIVITY FOR MHC, MUSCLE FIBERS CAN BE CLASSIFIED INTO SEVERAL TYPES, AS above.

CSAs. COSTAL DIAPHRAGM MUSCLE FIBER CSAs WERE DETERMINED FROM MICROSCOPIC IMAGES OF DIGITIZED MUSCLE SECTIONS BY USING A COMPUTER-BASED IMAGE-PROCESSING SYSTEM. THE LATTER IS COMPOSED OF A LEITZ LABORLUX MICROSCOPE S (LEICA, DEERFIELD, IL), CHARGE-COUPLED DEVICE VIDEO CAMERA SYSTEM (MODEL VI-470, OPTRONICS ENGINEERING, GOLETA, CA), HIGH-RESOLUTION TRINITRON COLOR VIDEO MONITOR (MODEL PVM-1343MD, SONY, ICHIOMIYA, JAPAN), 486 DX 50-MHZ PERSONAL COMPUTER WITH A TARGA® IMAGING BOARD (TRUEVISION, INDIANAPOLIS, IN), AND MOCHA IMAGE-ANALYSIS SOFTWARE (VERsION 1.20, JANDEL, SAN RAFAEL, CA). A MICROSCOPE STAGE MICROMETER WAS USED TO CALIBRATE THE IMAGING SYSTEM FOR MoraPHORIZATION. THE CSAs OF INDIVIDUAL FIBERS WERE DETERMINED FROM THE NUMBER OF PIXELS WITHIN OUTLINED FIBER BOUNDARIES.

TNF-α IMMUNOREACTIVITY. ADDITIONAL SERIAL SECTIONS OF THE COSTAL DIAPHRAGM WERE PREPARED AS DESCRIBED ABOVE AND INCUBATED FOR 2 h AT ROOM TEMPERATURE IN POLYCLONAL GOAT ANTIBODIES AGAINST RAT TNF-α (R&D SYSTEMS, MINNEAPOLIS, MN) DILUTED IN PBS (1:10). SUBSEQUENT PROCEDURES WERE SIMILAR TO THOSE DESCRIBED ABOVE FOR MHC. THE LEVEL OF IMMUNOREACTIVITY WAS QUANTIFIED DENSITOMETRICALLY WITHIN INDIVIDUAL FIBER TYPES USING THE CALIBRATED IMAGE ANALYSIS SYSTEM DESCRIBED ABOVE.

PROTEIN ANALYSES

MHC ISOFORMS. MYOFIBRIL EXTRACTION AND ELECTROPHORETIC (SDS-PAGE) IDENTIFICATION OF MHC ISOFORMS WERE DETERMINED AS DESCRIBED IN DETAIL IN A PREVIOUS PUBLICATION (23). THE SEPARATING GELS WERE STAINED WITH SILVER NITRATE (SILVER STAIN PLUS KIT; BIO-RAD, HERCULES, CA), AND DENSITOMETRIC MEASUREMENTS WERE PERFORMED ON DRIED GELS. AFTER BACKGROUND SUBTRACTION, THE RELATIVE CONTRIBUTION OF EACH BAND WITHIN A GEL WAS DETERMINED.

AKT AND p38 MAPK

PROTEIN EXTRACTION. SOLUBLE PROTEIN WAS EXTRACTED FROM 50-MG SAMPLES OF THE RIGHT COSTAL DIAPHRAGM IN A 1:10 RATIO OF COLD CELL LYSIS BUFFER (CELL SIGNALING TECHNOLOGIES, BEVERLY, MA) ACCORDING TO MANUFACTURER’S PROTOCOL. HOMOGENIZATION WAS PERFORMED WITH A POLYTRON HOMOGENIZER, AND HOMOGENATES WERE CENTRIFUGED AT 14,000 RPM. THE SUPERNATANT WAS ALIQUOTED IN MICROCENTRIFUGE TUBES. PROTEIN CONCENTRATION WAS DETERMINED USING A COMMERCIAL PROTEIN ASSAY KIT (BIO-RAD) BASED ON THE BRADFORD (4) METHOD AND MEASURED WITH A SPECTROPHOTOMETER (SMARTSPECT 3000, BIO-RAD).

SDS-PAGE AND WESTERN BLOTTING. SAMPLES WERE BOILED AND COOLED BEFORE BEING USED FOR ELECTROPHORESIS. PROTEIN EXTRACTS WERE LOADED ON 4–20% LINEAR GRADIENT GELS AND ELECTROPHORESIZED BY SDS-PAGE. PROTEINS WERE ELECTROPHORETICALLY TRANSFERRED TO NYLON MEMBRANES. BLOTS WERE INCUBATED WITH RABBIT POLYCLONAL PRIMARY ANTIBODIES AT 4°C OVERNIGHT, AND WASHED AND INCUBATED WITH AN APPROPRIATE SECONDARY ANTIBODY AT ROOM TEMPERATURE FOR 1 h. THE BLOTS WERE VISUALIZED FOLLOWING DEVELOPMENT WITH ENHANCED CHELUMINESCENCE REAGENTS (ENHANCED CHELUMINESCENCE STREPTAVIDIN-HORSESERDISH PEROXIDASE, AMERSHAM BIOSCIENCES, PISCATAWAY, NJ), ACCORDING TO MANUFACTURER’S PROTOCOL. BLOTS WERE EXPOSED TO X-RAY FILM IN A CARPET, THE FILMS WERE SCANNED, AND IDENTIFIED BANDS WERE ANALYZED BY DENSITOMETRY USING A KODAK ANALYSIS SYSTEM. SOME BLOTS WERE REUSED BY EXPOSING THEM TO STRIPPER BUFFER (RESTORE, PIERCE, ROCKFORD, IL) AND REPROBED WITH A DIFFERENT ANTIBODY.

PRIMARY ANTIBODIES. BLOTS WERE INCUBATED WITH THE FOLLOWING ANTIBODIES OBTAINED FROM CELL SIGNALING TECHNOLOGIES: TOTAL AKT, PHOSPHORYLATED AKT AT THE C-TERMINUS (SER473), TOTAL p38 MAPK, AND PHOSPHORYLATED (THR180/TYR182) p38 MAPK.

SERUM AND MUSCLE TNF-α. SERUM AND COSTAL DIAPHRAGM MUSCLE LEVELS OF TNF-α WERE DETERMINED USING A COMMERCIAL KIT (OPTIEIA; PHARMINGEN, SAN DIEGO, CA), WHICH UTILIZES AN ELISA SPECIFIC FOR ENDOGENOUS RAT TNF-α. MEASUREMENTS WERE PERFORMED ACCORDING TO MANUFACTURER’S PROTOCOL, AND TNF-α LEVELS WERE EXPRESSED AS PICOMETERS PER MILLILITER (SERUM) AND PICOMETERS PER MILLIGRAM PROTEIN (MUSCLE). DIAPHRAGM MUSCLE PROTEIN CONCENTRATIONS WERE DETERMINED AS DESCRIBED ABOVE AND MEASURED WITH A SPECTROPHOTOMETER.
IGF-I and TNF-α mRNA Analyses

Total RNA extraction. Total RNA was extracted from 50-mg samples of the left costal diaphragm with TRIzol reagent (Invitrogen, Carlsbad, CA), according to manufacturer’s protocol. Quality and concentrations of total RNA were determined with a spectrophotometer (SmartSpec 3000). Samples were stored at −80°C in RNase-free water until analysis. Two micrograms of total RNA were reverse transcribed using oligo-dT primers (Invitrogen) and Omniscript reverse transcription kit (Qiagen, Valencia, CA), and reactions yielded 20 μl of first-strand cDNA.

Oligonucleotides. The primers for TNF-α, IGF-I, and GAPDH were designed based on published rat cDNA sequences using Primer3 software. Primers sequences for TNF-α (Ref. 19; GenBank accession no. NM_017008) were the following: upstream (5′ to 3′) TCA TCT TCT CAA AA and downstream (5′ to 3′) AGG TAC AGC CAA TCT GCT AA. Primers sequences for IGF-I (Ref. 42; GenBank accession no. M01580) were the following: upstream (5′ to 3′) TCA TCT TCT CAA AA and downstream (5′ to 3′) TGG CTT CCG GAG CTG TGA TC and downstream (5′ to 3′) ATG ATG TTC TGG GCT GCC CCA C. The expected lengths of the RT-PCR products were 169 bp for TNF-α, 51 bp for IGF-I, and 316 bp for GAPDH. GAPDH is a valid housekeeping gene since it is not affected in catabolic states such as malnutrition and corticosteroid treatments.

Real-time PCR. Primer efficiency tests were performed to compare amplification efficiency between the target genes (TNF-α and IGF-I) and the endogenous control gene (GAPDH). A PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) with V1.76A software was used to set the reaction plate design to perform the real-time PCR. The following experimental conditions were set: 50°C for 2 min, 95°C for 10 min, 95°C for 30 s, 60°C for 1 min, and set for 40 cycles. For each well, 65 ng of cDNA template were used with the master mix (Applied Biosystems) containing SYBR Green in a volume of 25 μl.

Statistical Analysis

The distribution of data was tested for normality. Statistical analysis was performed using ANOVA (SigmaStat version 2.0, Jandel, Richmond, CA). If a significant interaction was found, post hoc analysis (Newman-Keuls test) was used to compare differences in independent groups. An α level of 0.05 was used to determine significance. Values are presented as means ± SE.

RESULTS

Body and Muscle Weights

In ND animals, body weight fell progressively by 20.0 ± 2.3% over 7 days, whereas CTL animals increased body weight by 45.9 ± 1.8% (Fig. 1). In ND animals given TNF-α, no impact on body weight was observed, with weight decreasing with a similar rate of decline as ND animals (reduced 17.6 ± 2.1% at day 7; Fig. 1). Costal diaphragm weight was reduced by 36.7% in ND animals compared with controls and by 34.2% in ND + anti-TNF-α-treated animals. Values are means ± SE. *Significant reduction in muscle weight after 7 days in both ND groups for all 4 muscles and no impact of anti-TNF-α treatment.

Diaphragm Fiber Proportions and CSAs

The proportions of type I diaphragm muscle fibers were unaffected by ND (Fig. 3A). However, ND resulted in decreased proportions of type IIA fibers and increased proportions of type IIX diaphragm fibers (P < 0.05; Fig. 3A). In ND + anti-TNF-α animals, all fiber proportions were similar to those noted in CTL animals (Fig. 3A).
ND produced significant reduction in the CSAs of type I (~33%; \(P < 0.001\)), IIA (~43%; \(P < 0.001\)), and IIX (~46%; \(P < 0.001\)) diaphragm fibers compared with CTL rats (Fig. 3B). Similar levels of atrophy were observed in all diaphragm fiber types in ND and anti-TNF-\(\alpha\) rats compared with CTL animals (\(P < 0.001\); Fig. 3B). No differences in the CSAs of types I, IIA, and IIX fibers were noted between ND and ND + anti-TNF-\(\alpha\) groups (Fig. 3B).

**MHC Isoforms**

No differences were observed between the groups with regard to the relative proportions of MHC isoforms in the diaphragm muscle (Fig. 4).

**TNF-\(\alpha\) Studies**

*Serum TNF-\(\alpha\).* Mean serum TNF-\(\alpha\) levels were increased 2.6-fold in ND animals compared with CTL (\(P < 0.05\); Fig. 5A). In ND + anti-TNF-\(\alpha\) animals, the mean serum TNF-\(\alpha\) levels were suppressed to below CTL levels (i.e., 58% of CTL; \(P < 0.05\); Fig. 5A).

*Muscle TNF-\(\alpha\).* There was a twofold increase in TNF-\(\alpha\) concentration (pg/mg protein) in the diaphragm muscle of ND animals compared with the CTL group (\(P < 0.05\); Fig. 5B). Similar to serum studies, the provision of anti-TNF-\(\alpha\) antibodies suppressed muscle levels of the cytokine in ND animals to near CTL values (Fig. 5B).

**TNF-\(\alpha\) immunoreactivity in the diaphragm.** Immunohistochemical studies were performed to localize the enhanced expression of TNF-\(\alpha\) in ND animals within specific diaphragm fiber types. Figure 6 depicts digital photomicrographs showing increased intensity of TNF-\(\alpha\) immunoreactivity within all diaphragm fibers in an ND animal compared with CTL and ND + anti-TNF-\(\alpha\) groups. Mean gray-level intensities were

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**Fig. 3.** Diaphragm muscle fiber-type proportions (A) and mean fiber cross-sectional areas (CSAs; B) in CTL, ND, and ND + anti-TNF-\(\alpha\)-treated animals. Values are means \(\pm\) SE. Note that ND decreased type IIA and increased type IIX fiber proportions. Fiber proportions in anti-TNF-\(\alpha\)-treated animals were similar to control. *Significant reduction in muscle CSAs for all fiber types was observed in both ND groups with no impact of anti-TNF-\(\alpha\) treatment.

**Fig. 4.** Proportions of myosin heavy chain (MHC) isoforms 1/b/slow, and fast 2A, 2X, and 2B expressed in the diaphragm of CTL, ND, and ND + anti-TNF-\(\alpha\)-treated animals. Values are means \(\pm\) SE. Note that no differences were observed among the groups.

**Fig. 5.** Mean serum levels (A) and diaphragm muscle concentrations (B) of TNF-\(\alpha\) in CTL, ND, and ND + anti-TNF-\(\alpha\)-treated animals. Values are means \(\pm\) SE. *Significant increases in both serum and muscle TNF-\(\alpha\) in ND animals. + Anti-TNF-\(\alpha\) reduced these levels to CTL values in ND animals.
significantly increased in all fiber types in ND animals compared with the CTL group (type I: 1.3-fold; \( P \leq 0.05 \); type IIA: 1.4-fold; \( P < 0.05 \); type IIX: 1.7-fold; \( P < 0.01 \); Fig. 6). The provision of anti-TNF-\( \alpha \) antibodies to ND animals suppressed muscle immunoreactivity for TNF-\( \alpha \) to levels near CTL values (Fig. 6).

**Diaphragm muscle TNF-\( \alpha \) mRNA.** There was a threefold significant increase in the abundance of TNF-\( \alpha \) mRNA in the diaphragm of ND rats compared with CTL animals (\( P < 0.01 \); Fig. 7A). The provision of anti-TNF-\( \alpha \) antibodies to ND animals decreased the abundance of diaphragm TNF-\( \alpha \) mRNA levels by 41% (\( P < 0.05 \); Fig. 7A) to levels not significantly different from CTL animals (\( P = 0.09 \); Fig. 7A).

**Diaphragm IGF-I mRNA**

The abundance of IGF-I mRNA in the diaphragm of ND animals decreased by 70% compared with CTL rats (\( P < 0.0001 \); Fig. 7B). The provision of anti-TNF-\( \alpha \) antibodies to ND animals had no impact on IGF-I mRNA levels (Fig. 7B).

**Diaphragm Akt and p38 MAPK**

Western blot analyses showed no differences in the levels of total Akt in the diaphragm across all groups (Fig. 8A). By contrast, the abundance of phosphorylated (Ser\(^{473}\)) Akt was reduced by 72% in ND animals (\( P < 0.01 \); Fig. 8B), whereas it was reduced by 43% with the provision of anti-TNF-\( \alpha \) antibodies to ND animals (\( P < 0.05 \); Fig. 8B). This level, however, was still significantly greater than that found in nontreated ND animals (\( P < 0.05 \); Fig. 8B).

There were no significant differences in the levels of total p38 MAPK in the diaphragm across all groups (Fig. 9A). However, the abundance of phosphorylated (Thr\(^{180}/\text{Tyr}^{182}\)) p38 MAPK was increased fourfold in ND rats (\( P < 0.01 \); Fig. 9B). Of interest, the provision of anti-TNF-\( \alpha \) antibodies to ND
animals reduced the level of phosphorylated p38 MAPK back to CTL levels ($P < 0.01$; Fig. 9B).

**DISCUSSION**

Diaphragm muscle TNF-α mRNA abundance as well as serum and muscle levels of TNF-α were significantly elevated in our model of moderate unstressed malnutrition in young growing animals in which significant inhibition of skeletal muscle growth was demonstrated. The provision of rat-specific TNF-α antibodies, although suppressing diaphragm muscle TNF-α mRNA, serum TNF-α, diaphragm muscle levels of TNF-α, and p38 MAPK (a likely second messenger for TNF-α) to CTL levels, failed to attenuate or improve reduced diaphragm fiber CSAs induced by the nutritional insult. This may, in part, be due to the failure of TNF-α antibodies to significantly attenuate the marked reduction in the abundance IGF-I mRNA and phosphorylated Akt with ND.

**Critique of Methods**

Although it would appear an attractive hypothesis that increased serum and diaphragm muscle expression of TNF-α with a cachectic insult are, at least in part, related, proof of concept required cause and effect to be demonstrated experimentally. To this end, we proposed that neutralization of TNF-α effects systemically and locally should aid in proof of concept, particularly if the impact of TNF-α in promoting inhibition of muscle fiber growth in the malnourished state was substantial.

The rat-specific TNF-α antibodies are highly effective. In a WEH1 tumor cell cytotoxicity assay, $\sim 100 \mu g/ml$ of the antibody completely neutralized 10 pg/ml of rat TNF, whereas 4 mg/kg improved survival by 70% in rats given lethal doses of TNF-α (Shealy DJ, personal communication). Furthermore, 19 mg/kg of anti-TNF-α administered to polyarthritic mice prevented body weight loss, suppressed TNF-α and other proinflammatory cytokines, and promoted healing of joint damage (47), whereas 12.5 mg/kg of anti-TNF-α suppressed the induction and development of experimental myasthenia gravis in rats (10). Finally, administration of anti-TNF-α to aged male Sprague-Dawley rats substantially reversed insulin resistance in limb muscles (3).

The rat anti-TNF-α antibodies administered in the present study have a half-life of 3.5 days following a dose of 3 mg/kg. With the 15 mg/kg administered every 48 h, the estimated serum concentrations of the antibodies would be expected to rise to 300–500 μg/ml from the 100–150 μg/ml achieved after the first 5–6 h following administration (Shealy DJ, personal communication). Thus the dosage regimen used in the present study would be expected to be highly efficacious in neutralizing endogenous TNF-α. Indeed, in the present study, the...
antibody suppressed serum and diaphragm levels of TNF-α mRNA and protein (Western blot and immunohistochemistry) as well as the second messenger p38 MAPK. Furthermore, anti-TNF-α treatment normalized the reduced proportion of type IIA diaphragm fiber in ND animals, suggesting a separate signaling pathway mediating muscle fiber phenotype driven by TNF-α. Although it is known that calcineurin/NF-κB interaction can influence myosin phenotype (e.g., Ref. 48) and that TNF-α can activate NF-κB (1, 30), such mechanistic links between TNF-α and muscle phenotype are purely speculative. Although of interest, further investigation regarding such speculation is beyond the scope of this study.

The serum level of TNF-α in the present study was similar to that reported in rats (114 ± 12 pg/ml) by using a rat-specific ELISA kit (32) and in mice (~150 pg/ml) (47). Further suppression of serum TNF-α using anti-TNF-α antibodies in polyarthritic mice was similar to our values both in absolute terms and in direction of change (47). There is a paucity of data on protein levels of TNF-α in muscle tissue of rodents. In a single study (21), gastrocnemius muscle levels in male rats were about one-half the values obtained in the diaphragm of our CTL rats, which increased twofold 2 h after the administration of endotoxin. Limb muscles may express the cytokine differently, since we have also observed ~35% lower levels of TNF-α in the extensor digitorum longus compared with the diaphragm of rats (Fournier M, unpublished data).

Role of TNF-α in Cachexia

TNF may contribute to muscle cachexia (2, 5, 8, 9, 36, 52) systemically and locally by several distinct mechanisms. These include the induction of anorexia and reduced nutritional intake and stimulation of and/or synergy with several other proinflammatory cytokines with catabolic properties (e.g., IL-1β; Ref. 39). Direct effects of TNF-α include promoting impaired protein turnover in skeletal muscle including the diaphragm. For example, administration of TNF-α to rats for 5 days produced a significant decline in the synthesis of myofibrillar proteins in the diaphragm (MHC, myosin light chains, and G-actin) (6). Impaired fractional synthesis of myofibrillar proteins following TNF-α administration results from impaired translation initiation producing decreased mRNA translational efficiency (20). Although TNF-α has been reported to suppress local muscle expression of IGF-I (11), suppression therapy failed to alter markedly reduced expression of IGF-I mRNA in the diaphragm. TNF-α may also exert direct effects on muscle protein turnover by impacting on protein degradation by influencing the ubiquitin-proteasome pathway, which is generally regarded as the most important of the proteolytic systems for muscle proteins (35). It is of interest, therefore, that anti-TNF treatment of tumor-bearing rats abolished the significant increase in muscle ubiquitin gene expression (33). Li and Reid (30) recently demonstrated that TNF-α-induced protein degradation in differentiated muscle cell cultures was mediated by the transcription factor NF-κB and further that ubiquitin-conjugating activity is augmented by TNF-α/NF-κB signaling in skeletal muscle (29). More recently, it has been reported that TNF-α acts via a p38 MAPK to stimulate the expression of a muscle-specific E3 ligase (atrogin 1/MAFbx), which could contribute to muscle proteolysis as part of the ubiquitin-proteasome pathway (28). In the present study, despite suppression of p38 MAPK to CTL levels, no impact on malnutrition-induced inhibition of diaphragm muscle growth was observed. Although TNF-α can also promote apoptosis and impact on myogenesis pathways (5, 16), the pathogenetic contribution of these influences on muscle wasting clinically is not well defined.

Suppression of TNF-α Activity: Lack of Effect on Muscle Fiber Size

This paper highlights the importance of confirmatory study to provide proof of concept. The key question that arises, however, is why impressive suppression of TNF-α activity failed to attenuate malnutrition-induced inhibition of diaphragm muscle growth. This likely is due to the multiplicity of pathogenetic mechanisms leading to malnutrition-induced growth failure in our model.

Nutritional regulation of IGF-I is well described, with decreased circulating (liver source) and muscle expression of IGF-I exquisitely linked to significant caloric and/or protein deprivation (37, 51, 53). Reduced expression of IGF-I would be expected to curtail muscle protein synthesis by impacting on several important signal transduction pathways downstream of the IGF-I receptor that mediate the translation of muscle proteins (20, 24). In the present study, the significant reduction in diaphragm abundance of IGF-I mRNA with ND was unaffected by anti-TNF-α treatment. This would be expected to reduce protein expression of the growth factor, and indeed our laboratory has recently reported significant reductions in diaphragm muscle IGF-I protein levels in adult rats subjected to ND (25). Reduced IGF-I would negatively impact on both muscle protein synthetic and degradation pathways. Key signaling pathways downstream of the IGF-I receptor mediating muscle protein synthesis would be negatively impacted (24). Furthermore, dephosphorylation of Akt1 due to reduced influence of IGF-I can lead to activation of Foxo (forkhead-O) transcription factors with subsequent transcription of muscle-specific E3 ligases and other atrophy-related genes to promote muscle protein degradation. In the present study, ND resulted in marked suppression of phosphorylated Akt. Anti-TNF-α antibodies failed to significantly reverse the dephosphorylation of Akt. Thus the local muscle metabolic milieu in ND animals following anti-TNF-α treatment remained highly susceptible to disordered muscle protein turnover. Reduced serum levels of insulin with ND could also exhibit similar effects (13, 43, 45, 49). Furthermore, increased endogenous production of corticosteroids in animal models of undernutrition has been demonstrated (17). The latter could contribute to disordered muscle protein turnover, particularly muscle proteolysis. Finally, the systemic and muscle levels of TNF-α, although significantly elevated, may not have been sufficient to promote muscle wasting. For example, in patients presenting with severe sepsis, levels of TNF-α were close to 8,000 pg/ml (7). Lastly, TNF-α may act in concert with other cytokines in promoting atrophy (e.g., IL-1β, IL-6; Ref. 54), and so neutralizing TNF-α only may have had little effect.

In summary, a moderately severe paradigm of ND in young rapidly growing animals produced significantly elevated serum and diaphragm muscle levels of TNF-α and inhibition of diaphragm fiber growth of all fiber types. Neutralization of endogenous TNF-α failed to attenuate ND-induced inhibition.
of muscle growth. Thus TNF-α in this model of malnutrition had little or no pathogenetic role.

ACKNOWLEDGMENTS

We gratefully appreciate the outstanding assistance and critical reviews of the manuscript and study design by Drs. David J. Shealy and Michael B. Reid.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-071227, University of California-Tobacco-Related Disease Research Program Grants 7RT-0114 and 7RT-0161, and Centocor.

REFERENCES


