A proinflammatory tumor that activates protein degradation sensitizes rats to catabolic effects of endotoxin

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MUSCLE WASTING IN ADVANCED CANCER is associated with impaired functioning, intolerance to chemotherapy, and mortality (30, 37). Numerous factors may have a role in the development of muscle wasting; however, the end result is a shift in the catabolic-anabolic balance that promotes the breakdown of body protein (1).

The Yoshida ascites hepatoma (YAH) 130 in rats is a tumor with rapid growth and progressive development of cachexia. YAH induces a loss of skeletal mass through an enhanced rate of muscle protein degradation involving the activation of the intracellular ATP-ubiquitin-dependent proteolytic pathway (3). This pathway involves three enzymes that ubiquitinate proteins for degradation by the 26S proteasome (36). Ubiquitin-activating enzyme (E1) activates and transfers ubiquitin to an ubiquitin-conjugating enzyme (E2), which co-operates with an ubiquitin protein ligase (E3) to conjugate ubiquitin to the substrate. E3 is responsible for recognizing and targeting proteins for degradation. Several types of E3s have been identified, and at least three have been found to have increased expression in states of muscle atrophy, including YAH (22). Of these ligases, muscle-specific RING Finger 1 (MuRF-1) and atrogin-1/MAFbx have higher upregulation in muscle atrophy, suggesting a role in disease-associated muscle wasting (21).

In tumor models, including YAH, proinflammatory cytokines are clearly established to be mediators of protein catabolism. Levels of tumor necrosis factor-α (TNF-α) are increased in physiological fluids (35), and a causal role in elevated catabolism is demonstrated through the use of highly specific interventions, such as anti-TNF-α antibodies, soluble TNF-α receptors, and drugs that block TNF-α production, such as pentoxifylline (8, 9, 23). There is some evidence that the TNF-α response to endotoxin is exaggerated in tumor-bearing rats compared with non-tumor-bearing rats (7). Related to this finding, studies using endotoxin have demonstrated an increase in its lethality in rats bearing the Ward colon tumor (17) and mice bearing the Lewis lung carcinoma (26).

The presence of activated protein degradation and an enhanced sensitivity of tumor-bearing animals to proinflammatory signals would be expected to result in an enhanced catabolic response in tumor-bearing animals. Here, we hypothesized that in the YAH, bacterial endotoxin would elicit a disproportionately large activation of muscle protein catabolism compared with non-tumor-bearing animals. To test this hypothesis, we used a low-dose endotoxin to determine the impact of a mild catabolic stimulus on muscle wasting, nitrogen balance, and skeletal muscle protein degradation in rats bearing the YAH and non-tumor-bearing controls. Low doses of endotoxin (400 μg/kg body wt) produce a mild response that includes fever, metabolic response, and cytokine receptor gene expression in muscle (16, 38).

MATERIALS AND METHODS

Study Design

All studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and were approved by the institutional Animal Policy and Welfare Committee. Male Sprague-Dawley rats (n = 28; Charles River, St. Constant, QC, Canada) were used as hosts for the YAH. Rats were housed in individual wire metabolite cages throughout the study period. The room was temperature and humidity controlled with a 12:12-h light-dark cycle. All rats consumed a diet of ground laboratory chow (LabDiet 5001; PMI Nutrition International, St. Louis, MO) that met all rodent nutrient requirements (28). Weight and feed intake were measured daily.

Rats were randomly allocated to one of four treatment groups after a 1-wk adaptation to environment and diet. Treatment variables were tumor and low-dose endotoxin (LPS). Tumor cells, maintained in

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liquid nitrogen, were initially implanted into nonstudy rats (n = 2). Ascites fluid was harvested after 5 days of tumor growth and injected immediately into study rats (n = 14). YAH (50 µl ip) was implanted on study day 0. After 4 days of tumor growth, treatment with saline or LPS (400 µg of Escherichia coli O55:B5/kg sc) was given. Twenty-four hours later, rats were killed by CO2 asphyxiation. Urine and feces were collected on study days 3 and 4 for determination of nitrogen (N) balance. Muscles (gastrocnemius and soleus) were dissected, weighed, and frozen in liquid N. Gastrocnemius muscle was used for expression of components of the ATP-ubiquitin proteolytic pathway. Epitrochlearis muscle was dissected and incubated for determination of protein synthetic and degradation rates.

Analysis

N balance. Urine and feces were collected for two separate 24-h periods before and after LPS/saline treatment. Feces were dried at 60°C for 48 h and ground. Total urine and feces collected in each period were weighed. N content of urine, feces, and diet was determined using the macro-Kjeldahl method (5). N balance was calculated by subtracting the N output (total urinary N plus total fecal N) from dietary N intake (feed intake × N content of feed). Absorbed N was calculated for each rat by subtracting fecal N content from N intake. The difference between absorbed N and urinary N was used to determine the amount of absorbed N retained and was expressed as a percentage.

Muscle incubations. Muscle incubations and analysis were similar to methods described previously (3, 34). The epitrochlearis is a small, thin muscle, making it ideal for muscle incubations. After dissection of both epitrochlearis muscles, each was placed in a tube containing 3 ml of medium. All chemicals listed below are from Sigma (St. Louis, MO) unless otherwise noted. The medium was composed of Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4.8 mM KCl, 1.25 mM MgSO4, 25 mM NaHCO3, 1.2 4mM NaHPO4, 1.0 mM CaCl2, 2 mM HEPES, pH 7.4), glucose (8 mM), insulin (0.01 U/ml), bovine serum albumin (0.1% w/vol), leucine (0.17 mM), isoleucine (0.1 mM), and valine (0.2 mM). Muscles were initially incubated for 30 min at 37°C with 95% O2/5% CO2. Muscles were then transferred to another tube containing 3 ml of medium, with the addition of either [3H]phenylalanine (0.5 µCi/ml) and phenylalanine (1 mM) or cycloheximide (0.5 mM) followed by incubation for 2 h under the same conditions as described above. At the end of the incubation, muscles were transferred to tubes containing 2% perchloric acid (PCA), and 0.5 ml of 16% PCA was added to the medium to make a 2% PCA solution. Samples were frozen at –80°C.

In vitro muscle protein synthesis and degradation. Muscle protein synthesis was determined using [3H]phenylalanine incorporation into muscle protein. Muscles incubated in [3H]phenylalanine and phenylalanine were homogenized in 3 ml of 5% trichloroacetic acid (TCA) and centrifuged for 20 min. The supernatant containing the intracellular soluble fraction was removed, and after the pellet was washed three times in 5% TCA the protein was dissolved overnight at room temperature in 1.5 ml of Soluene-350 (40–60% toluene, 30–40% dimethyl dialkyl quaternion ammonium hydroxide, and 5–10% methanol; Packard Instruments, Meriden, CT). Disintegrations per min (dpm) in the intracellular and protein fractions were measured using a β-counter (Beckman LS 5801; Beckman Instruments, Irvine, CA). Intracellular phenylalanine concentration was measured using high-performance liquid chromatography (HPLC) with precolumn o-phthalaldehyde (OPA) derivatization (52). Intracellular specific activity (dpm/mg·mmol Phe-1,3 ml−1) was used as the precursor pool for muscle protein synthesis. Muscle protein concentration (mg/mg muscle) was measured using LECO FP-428 Nitrogen/Protein determinant (Leco, St. Joseph, MI), and protein mass (protein concentration × muscle mass) was used to calculate the specific activity of muscle protein (dpm/mg protein). Protein synthesis rate was calculated as nanomole phenylalanine incorporated into protein/milligram protein over the 2 h.

Muscle protein degradation was measured using the amount of phenylalanine released from muscle when protein synthesis was blocked by cycloheximide. The phenylalanine concentration of the medium was determined using HPLC and OPA derivatization. Degradation rate was expressed as nanomole phenylalanine released/milligram protein over the 2 h.

ATP-ubiquitin-dependent proteolytic system mRNA and protein expression. Levels of mRNA for components of the ATP-ubiquitin-dependent proteolytic system (ubiquitin, E2,4k, MuRF-1, and atrogin-1/MAFbx) were measured using Northern blot analysis on RNA extracted from frozen gastrocnemius muscles using the guanidinium isothiocyanate-CsCl method. Electrophoresis of muscle RNA on 1% agarose-containing formaldehyde was followed by transfer onto nylon membranes and cross-linked to the membranes by ultraviolet light. Membranes were hybridized with cDNA probes encoding for ubiquitin, E2,4k, MuRF-1, atrogin-1/MAFbx, or 18s rRNA. Quantification was by densitometric scanning of autoradiographs or by phosphorimager analysis, and values for ubiquitin system transcripts were normalized to the 18s rRNA to correct for differences in sample loading and transfer to membrane.

Protein levels of atrogin-1/MAFbx were measured by Western blotting. Gastrocnemius muscles were homogenized by using a Polytron in PBS containing 1% NP-40 and protease inhibitors. After centrifugation at 10,000 g, proteins from the soluble fraction were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with the primary atrogin-1/MAFbx antibody (gift of Regeneron Pharmaceuticals, Tarrytown, NY), followed by a secondary antibody for 1 h. Phosphorimager analysis was used for quantification, and values were normalized to GAPDH to correct for differences in sample loading and transfer to membrane.

Statistical Analysis

Statistical Analysis System (SAS, version 8.2; SAS Institute, Cary, NC) was used for statistical analysis. Data are expressed as means ± SE. Data were log-transformed if not normally distributed, and multiple variances were used if group variances were not homogenous. The effects of tumor before the saline/LPS administration were determined using one-way ANOVA in the SAS mixed procedure (weight, intake, and N balance, day 3). For the remaining analysis, treatment (tumor, LPS) and interaction effects were determined using two-way ANOVA in the SAS mixed procedure. Differences among treatment groups were identified using t-test. Statistical significance was considered at P < 0.05.

RESULTS

Effects of the Yoshida Hepatoma

Body weight (Table 1) was not different between control and tumor-bearing animals at the start of the study, but the growth
Table 2. Muscle weights 24 h after LPS or saline administration in tumor-bearing rats and non-tumor-bearing controls

<table>
<thead>
<tr>
<th></th>
<th>CON + LPS</th>
<th>CON + LPS</th>
<th>YAH</th>
<th>YAH + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius, mg</td>
<td>1,533±2*</td>
<td>1,498±4*</td>
<td>1,335±20*</td>
<td>1,331±50*</td>
</tr>
<tr>
<td>Soleus, mg</td>
<td>106±2.4</td>
<td>107±2.4</td>
<td>98±1.57</td>
<td>95±4.4</td>
</tr>
<tr>
<td>Epitrochlearis, mg</td>
<td>31.8±1.2*</td>
<td>30.8±2.0*</td>
<td>28.9±0.8*</td>
<td>27.1±1.3*</td>
</tr>
<tr>
<td>Protein Mass, mg</td>
<td>8.5±0.3*</td>
<td>8.5±0.3*</td>
<td>7.4±0.5*</td>
<td>7.4±0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Significant main effects (ANOVA, P < 0.05): 1tumor effect; 2LPS effect; 3interaction (tumor × LPS). Groups with same letter are not significantly different (P < 0.05).

Table 3. Nitrogen balance before and after LPS or saline administration in tumor-bearing and non-tumor-bearing controls

<table>
<thead>
<tr>
<th></th>
<th>CON + LPS</th>
<th>CON + LPS</th>
<th>YAH</th>
<th>YAH + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N intake, mg/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>1,197±36*</td>
<td>1,235±60*</td>
<td>1,110±30*</td>
<td>964±110*</td>
</tr>
<tr>
<td>Day 4</td>
<td>1,241±66*</td>
<td>1,083±91*</td>
<td>746±108*</td>
<td>520±91*</td>
</tr>
<tr>
<td>Δ</td>
<td>44±58*</td>
<td>45±56*</td>
<td>44±53*</td>
<td>44±54*</td>
</tr>
<tr>
<td>Urinary N, mg/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>381±13*</td>
<td>388±40*</td>
<td>288±20*</td>
<td>251±19*</td>
</tr>
<tr>
<td>Day 4</td>
<td>402±16*</td>
<td>397±32*</td>
<td>234±17*</td>
<td>251±37*</td>
</tr>
<tr>
<td>Δ</td>
<td>81±10*</td>
<td>10±24*</td>
<td>-54±24*</td>
<td>0±42*</td>
</tr>
<tr>
<td>Fecal N, mg/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>241±13*</td>
<td>280±21*</td>
<td>278±17*</td>
<td>220±19*</td>
</tr>
<tr>
<td>Day 4</td>
<td>304±18*</td>
<td>271±20*</td>
<td>224±16*</td>
<td>199±33*</td>
</tr>
<tr>
<td>Δ</td>
<td>63±10*</td>
<td>-9±27*</td>
<td>-54±20*</td>
<td>-21±26*</td>
</tr>
<tr>
<td>N balance, mg/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>575±21*</td>
<td>566±23*</td>
<td>544±28*</td>
<td>493±84*</td>
</tr>
<tr>
<td>Day 4</td>
<td>475±52*</td>
<td>414±85*</td>
<td>288±92*</td>
<td>70±62*</td>
</tr>
<tr>
<td>Δ</td>
<td>-100±51*</td>
<td>-152±82*</td>
<td>-257±83*</td>
<td>-423±76*</td>
</tr>
</tbody>
</table>

Values are means ± SE. N, nitrogen; Δ, day 4 group mean minus day 3 group mean (effect of LPS or saline treatment). Significant main effects (ANOVA, P < 0.05): 1tumor effect; 2LPS effect; 3interaction (tumor × LPS). *Significant difference between day 4 and day 3 mean for that treatment group (paired t-test, P < 0.05). a, b, c: within each line: groups with same letter are not significantly different (P < 0.05).

Fig. 1. Daily feed intake in tumor-bearing [Yoshida ascites hepatoma (YAH)] and nontumor-bearing (Control) rats over 5 days of treatment. Low-dose endotoxin (LPS) or saline was given at the start of study day 4 (n = 7 rats per group). *Significant YAH effect (ANOVA, P < 0.05). Groups with same letter are not significantly different (P < 0.05).

Fig. 2. Nitrogen absorbed (A) and %absorbed nitrogen retained (B) in tumor-bearing (YAH) and nontumor-bearing (Control) rats during 24 h before (day 3) and after (day 4) low-dose endotoxin (LPS) or saline administration (n = 7 rats per group). A: significant main effect (ANOVA, P < 0.05) of tumor days 3 and 4. B: significant main effect of LPS and interaction effect (tumor × LPS) day 4. For each day, groups with same letter are not significantly different (P < 0.05). *Significant difference between study day 4 and day 3 for that group (paired t-test, P < 0.05).
Table 4. In vitro skeletal muscle protein degradation 24 h after LPS (+LPS) or saline (−LPS) administration in YAH-bearing rats (+tumor) and non-tumor-bearing controls (−tumor)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>−Tumor</th>
<th>+Tumor</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>−LPS</td>
<td>2.112 ± 0.147a</td>
<td>2.906 ± 0.163b</td>
<td>0.794 ± 0.220 ±37.6%</td>
</tr>
<tr>
<td>+LPS</td>
<td>2.164 ± 0.180a</td>
<td>3.438 ± 0.182a</td>
<td>1.274 ± 0.255 ±58.9%</td>
</tr>
<tr>
<td>Δ</td>
<td>0.052 ± 0.232</td>
<td>0.532 ± 0.244</td>
<td>+2.4% NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7/group in nmol Phe·mg protein−1·h−1. NS, not significant; Δ, effect of LPS or tumor. Significant main effect of tumor (ANOVA, P < 0.05), a, b, c: groups with same letter are not significantly different (P < 0.05).

Discussed in the next paragraph.

Discussion

The primary catabolic effects of tumors on skeletal muscle protein have been well established in animal models (2), and here we hypothesized a possible sensitization of protein catabolism to secondary factors in the tumor-bearing state. Our study shows that a superimposed inflammatory stimulus in the tumor-bearing state results in a larger catabolic response than would be expected on the basis of the independent responses to tumor and endotoxin alone. Low-dose endotoxin reduced nitrogen retention and elevated skeletal muscle proteolysis when a tumor known to be associated with catabolism induced by TNF-α and eicosanoids was present. The tumor model used produces an inflammatory response. Thus activation of proteolytic systems by a primary inflammatory response might predispose skeletal muscle to catabolism by a secondary stimulus such as endotoxin.

Fig. 3. A: mRNA expression of components of ATP-ubiquitin proteolytic system in gastrocnemius muscle from tumor-bearing (YAH, n = 13) and non-tumor-bearing (Control, n = 14) rats 24 h after low-dose endotoxin (LPS) or saline administration. LPS had no effect in either Control or YAH rats; therefore, data were pooled. YAH was in the 5th day of tumor growth. Results are expressed as %control. E2, ubiquitin conjugating enzyme; MuRF-1, muscle-specific RING Finger 1; *Significant main effect of tumor (ANOVA, P < 0.05). B: representative Northern blots for the components of ATP-ubiquitin proteolytic system and 18S rRNA for Control and YAH rats given LPS or saline, with analyzed bands indicated. mRNA levels were standardized using 18S rRNA.
Endotoxin produces a classic response mediated by proinflammatory cytokines (15). A variety of studies have established evidence for a heightened sensitivity to endotoxin in various pathological states. Studies using a “two-hit” model in rats have shown that activation of inflammatory genes by hemorrhagic shock primes macrophages for an exaggerated cytokine (TNFα) response to low-dose endotoxin (11, 29). This theme is seen in the literature on tumor-bearing animals. Rats bearing the Ward colon tumor had a mortality rate of 83% compared with 8% in control rats in the 24 h after the administration of endotoxin (5 mg/kg) (17). A dose-response study in mice bearing the Lewis lung carcinoma is also associated with an increased lethality of endotoxin (26). The LD₅₀ of endotoxin decreased from 700 μg in healthy mice to 60 and 1 μg in mice with 2.7-cm³ and 6.0-cm³ tumors, respectively (26). Rats bearing a Yoshida sarcoma given 1 mg/kg endotoxin exhibited a plasma TNF-α response 45-fold higher than in nontumor-bearing controls (7), and it is easy to imagine reaching a threshold for endotoxic shock under these conditions. Collectively, these studies suggest a sharp left shift in the endotoxin dose-toxicity relationship in the tumor-bearing state, which may be related to an amplified production of TNF-α. TNF-α mediates endotoxin-induced shock as well as a catabolic response in skeletal muscle (24, 25). The high levels of TNF-α production in tumor-bearing animals may account not only for increased mortality but also a shift in the catabolic response to endotoxin.

Animals with YAH experienced a large increase in muscle protein catabolism associated with an induction of proteolytic gene expression. Although changes in muscle protein synthetic rates were not observed in the current study, the catabolic state of in vitro muscle incubations might have masked any alterations by tumor or endotoxin. In agreement with previous studies that have used this model (3, 21), gene expression for key enzymes in the ubiquitin-proteasome pathway were elevated. At least four E3s have been found to have increased expression in states with muscle atrophy (24, 25). These include E3α/Ubr1, a second Ubr1 homolog E3αII, MuRF-1, and atrogin-1/MAFbx (also known as SCF atrogin-1). Studies in gene knockout mice and differential gene analysis in rat models of atrophy indicate that atrogin-1/MAFbx and MuRF-1 are involved in the enhanced protein degradation associated with muscle wasting (4, 21). Mice with atrogin-1/MAFbx gene deletion demonstrate a 56% reduction in denervation-induced muscle wasting, whereas the reduction in MuRF-1 gene-deficient mice was 36% (4). Atrogin-1/MAFbx mRNA expression displayed the greatest degree of YAH-induced elevation, along with an increase in protein levels, in our study. Despite the higher rates of skeletal muscle protein breakdown, endotoxin in YAH-bearing rats did not increase the expression of genes involved in ubiquitin conjugation, suggesting that other mechanisms of increasing substrate flux through the pathway are involved. The relative contribution of different ligases to muscle wasting associated with tumors or endotoxin is not known. Two muscle specific ligases, MuRF-1 and atrogin-1/MAFbx, were examined in this study, and the protein level was determined only for atrogin-1/MAFbx. Therefore, it is possible that other ligases were more responsive to endotoxin in the tumor-bearing state, which may be related to an amplified production of TNF-α. TNF-α mediates endotoxin-induced shock as well as a catabolic response in skeletal muscle (24, 25). The high levels of TNF-α production in tumor-bearing animals may account not only for increased mortality but also a shift in the catabolic response to endotoxin.
results in muscle protein breakdown greater than expected from the sum of both. Endotoxin at relatively high doses (10 mg/kg) in rats has been shown to increase skeletal muscle breakdown through activation of the ATP-ubiquitin proteolytic pathway (6, 7). In the design of the present study, we selected a much lower dose of endotoxin, which would be expected to cause little or no perturbation in protein metabolism. Accordingly, the effects on N metabolism elicited by low-dose endotoxin in healthy control rats were minimal; however, like the study of muscle sensitivity to cortisol, muscles of tumor-bearing rats had an enhanced degradative response to endotoxin. Our related work with the same low dose of endotoxin that demonstrated an induction of TNF and IL-6 receptors in skeletal muscle (38) provides a possible basis for this sensitization. This pattern of enhanced sensitivity of muscle protein degradation to catabolic factors may be an important component of complex wasting disorders where tumor, inflammation or infection, inactivity, elevated levels of glucocorticoids, and other factors may frequently be simultaneously present. The present study highlights the complexity involved in defining the mechanism responsible for and treatment of muscle wasting in disease states. The etiology of wasting may not be attributable to a single factor but rather to a series of factors that disastrously potentiate each other’s catabolic effects, and the magnitude of the overall effect cannot be predicted on the basis of the separate effects of each.

The unfortunate outcomes of tumor and endotoxin/inflammation may be a feature of tumors that are themselves associated with some degree of inflammatory mediator production or speculation that episodes of infection or inflammation may be a feature of tumors that are themselves associated with indices of inflammation. Many human tumors that are associated with wasting of skeletal muscle are associated with indices of inflammation (12, 13, 33), and these in turn are known to have prognostic significance and to be related to shortened survival (10, 31). The present study relates clinically to the metabolic response to infection in cancer patients. Infections are very common in patients with cancer (27) and are often the cause of death in advanced-cancer patients (18, 19). Our results generate the speculation that episodes of infection or inflammation may be associated with exaggerated catabolic responses in the tumor-bearing state.

GRANTS

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