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

Michelle L. Mackenzie,1 Nathalie Bedard,3 Simon S. Wing,3 and Vickie E. Baracos1,2
1Department of Agricultural, Food, and Nutritional Science; 2Department of Oncology, University of Alberta, Edmonton, Alberta, Canada; and 3Polypeptide Laboratory, Department of Medicine, McGill University, Montreal, Quebec, Canada

Submitted 4 February 2005; accepted in final form 27 May 2005

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 muscle 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-activated enzyme (E1) activates and transfers ubiquitin to an ubiquitin-conjugating enzyme (E2), which cooperates 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 muscle wasting; inflammation; ubiquitin proteolytic pathway

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 metabolic 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...
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 were 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 \(\times\) 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, 1.24 mM NaH2PO4, 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 diakyl, 1.5 ml) 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.

**Statistical Analysis**

**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 1. Initial body weight and changes in body weight before (day 4) and after (day 5) LPS or saline administration in tumor-bearing and non-tumor-bearing controls**

<table>
<thead>
<tr>
<th>Weight</th>
<th>CON (n = 7)</th>
<th>CON + LPS (n = 7)</th>
<th>YAH (n = 7)</th>
<th>YAH + LPS (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0, g</td>
<td>238±3(^a)</td>
<td>243±3(^a)</td>
<td>243±3(^a)</td>
<td>245±4(^a)</td>
</tr>
<tr>
<td>Day 4, g</td>
<td>276±5(^a)</td>
<td>283±5(^b)</td>
<td>295±5(^b)</td>
<td>286±7(^b)</td>
</tr>
<tr>
<td>Day 5, g</td>
<td>283±6(^a)</td>
<td>286±4(^b)</td>
<td>307±5(^a)</td>
<td>283±7(^a)</td>
</tr>
</tbody>
</table>

\(\Delta^{2,3}\) 7±2\(^a\) 3±2\(^b\) 12±3\(^a\) 4±3\(^c\)

Values are means ± SE. YAH, Yoshida ascites hepatoma; CON, control; Δ, day 5 group mean minus day 4 group mean (effect of LPS or saline treatment). Significant main effects (ANOVA, \(P < 0.05\)): tumor effect; LPS effect; interaction (tumor × LPS). *Significant difference between day 5 and day 4 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 (\(P < 0.05\)).
of the ascites tumor resulted in a higher body weight at the start of study days 4 (main effect \( P = 0.049 \)) and 5 (\( P = 0.005 \) vs. control). However, muscle mass (Table 2) was reduced (gastrocnemius \( P = 0.002 \)) in the tumor-bearing group, suggesting non-tumor weight loss. Body weight increased during the final 24 h (\( P = 0.007 \)). Tumor-bearing rats had a reduction in feed intake (Fig. 1) on study days 3 (main effect \( P = 0.01 \)) and 4 (\( P = 0.002 \)).

N intake, loss, and balance data are shown in Table 3 and Fig. 2. On study day 3, YAH-bearing rats compensated for a lower total N absorbed (\( P = 0.005 \)) through a reduction in urinary N loss (\( P = 0.0008 \)) and thus maintained N balance at levels similar to that of controls. Despite a similar pattern on study day 4 (\( P = 0.009 \) for N absorbed and \( P < 0.0001 \) for urinary N), the tumor-bearing group had a lower N balance compared with day 3 (\( P = 0.038 \)).

YAH increased muscle protein degradation (\( P = 0.0028 \)) by 37% (Table 4). Expression of ubiquitin and E2\(_{1,4k}\) mRNA (Fig. 3) was elevated in the YAH group (main effect \( P = 0.003 \) and 0.0113, respectively). The two ubiquitin ligases (Figs. 3 and 4) measured also had higher expression in the tumor-bearing group: the atrogin-1/MAFbx ligase mRNA and protein were increased almost 5-fold (main effect \( P < 0.0001 \)) and 1.7-fold (main effect \( P = 0.0001 \)), respectively, and MuRF-1 mRNA was 1.5-fold higher (main effect \( P = 0.0116 \)).

Effects of LPS Injection in Non-Tumor-Bearing Animals

Mean body and muscle weight of rats that received LPS did not differ from those that received saline in the control groups.
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−1·2 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).

Synergy Between YAH and LPS

There was a significant interaction effect (P = 0.0228) between tumor and LPS treatment on weight (P = 0.0028) and weight gain (P = 0.0068). Tumor-bearing rats receiving LPS had a lower weight (P = 0.013) and weight gain (P = 0.0007) compared with the tumor-bearing rats that received saline despite similar feed intake (Table 1 and Fig. 1).

LPS stimulated N loss in the tumor-bearing rats, as indicated by a reduction in N balance (P = 0.0015) in the 24 h after LPS administration (Table 3). This lowering of N retention was attributed to both a decrease in N intake (P = 0.0013) and total N absorbed (P = 0.0014) without a change in N loss. N absorbed was similar to that in rats bearing YAH alone (Fig. 2); however, there was a drastic reduction in the percentage of absorbed N retained in the tumor-bearing rats given LPS (P = 0.0051 vs. YAH alone). Tumor-bearing rats that received LPS were the only group with N balance significantly lower than controls (P = < 0.0001 vs. control and P = 0.0034 vs. LPS).

There was a main effect of YAH on the weight (P = 0.0263) and protein content (P = 0.0125) of the epimysialis muscle (Table 2); however, only the tumor-bearing rats that received LPS had lower values compared with controls (P = 0.0265 for weight and P = 0.041 for protein content). Protein synthesis rates were not affected by either tumor implantation or LPS treatment (CON: 0.970 ± 0.070; CON + LPS: 1.084 ± 0.051; YAH: 1.111 ± 0.094; YAH + LPS: 1.008 ± 0.097 nmol Phe·mg protein−1·2 h−1). The major effect of the study treatments on protein turnover was on the process of protein degradation, which was strongly elevated by the tumor (+38%) and further elevated 18% by LPS treatment in the tumor-bearing group (P = 0.0336 vs. YAH alone) (Table 4). However, this activation of catabolism above that induced by the tumor alone was not associated with any further increase in the mRNA expression of the elements of the ATP-ubiquitin proteolytic system studied (ubiquitin, P = 0.4; E2, P = 0.7; MuRF-1, P = 0.8; atrogin-1/MAFbx, P = 0.9) or protein levels of atrogin-1/MAFbx (Fig. 4; P = 0.3). Because LPS did not have an effect on the mRNA expression of ubiquitin, E24k, MuRF-1, or atrogin-1/MAFbx, nor protein level of atrogin-1/MAFbx, the data were pooled into two groups: control or YAH bearing (Fig. 3).

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.
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 (TNF-α) response to low-dose endotoxin (11, 29). This theme is seen in the literature on tumor-bearing animals. Rats have shown that activation of inflammatory genes by tumor or endotoxin. In agreement with previous studies that have used this model (3, 21), gene expression for atrogin-1/MAFbx (40.9 kDa) with molecular mass markers on the left. Indicated band migrates at ~38 kDa, consistent with it being atrogin-1/MAFbx, given the error of molecular mass estimation based on electrophoretic migration of prestained standard markers. In support of this assignment, this band, like the mRNA for atrogin-1/MAFbx (see Ref. 4), is present only in skeletal muscle and heart, but not in brain, kidney, lung, liver, testis, and spleen (data not shown). Protein levels were standardized using GAPDH.

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 (20, 22). 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 rats. E3αII mRNA expression is elevated in response to YAH as well as TNF (20), and thus may have a role in sensitizing skeletal muscle of tumor-bearing rats to endotoxin. It is also possible that the increased expression of genes in the conjugation pathway is a part of an increase in the infrastructure within proteolytic pathways that allows for more catabolism to take place upon the arrival of a second stimulus, in this case endotoxin. The second stimulus may, for example, cause enhanced gene translation, posttranslational activation of enzymatic activity, or proteasome regulation, which are sites downstream of gene transcription that may affect proteolytic rates. As well, the availability of substrates for ubiquitin conjugation may be elevated by endotoxin.

There is some evidence of a synergistic effect of catabolic factors on skeletal muscle protein breakdown. A low dose of cortisol, which had a minimal effect on muscle protein catabolism in normally active healthy young men, elevated skeletal muscle protein breakdown rates threefold in the same subjects after bed rest for 2 wk (14). Although both inactivity and cortisol stimulate muscle protein breakdown, coadministration
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 host inflammatory response. The YAH used here and the other tumor models where enhanced sensitivity to endotoxin was demonstrated (7, 26) are associated with an inflammatory response. 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 infections 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

This work was supported by a Natural Sciences and Engineering Research Council of Canada Grant and Canadian Institutes of Health Research Grant MT-12121. AntiMAFbx antibody was a gift from Regeneron Pharmaceuticals.

REFERENCES


