

Activation of Protein Breakdown and Prostaglandin E₂ Production in Rat Skeletal Muscle in Fever Is Signaled by a Macrophage Product Distinct from Interleukin 1 or Other Known Monokines

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Abstract

During sepsis or after injection of endotoxin into rats, there is a large increase in muscle protein breakdown and prostaglandin E₂ (PGE₂) production. Prior studies showed that partially purified interleukin 1 (IL-1) from human monocytes can stimulate these processes when added to isolated rat muscles. The availability of pure recombinant IL-1 and other monokines has allowed us to investigate the identity of the active agent in this process. Incubation of muscles with recombinant human or murine IL-1 α or IL-1 β or with IL-1 plus a phorbol ester did not stimulate muscle proteolysis or PGE₂ production. Homogeneous natural porcine IL-1 ("catabolin") and mouse or human IL-1 β were also not effective in vitro. In addition, a variety of other human cytokines, including tumor necrosis factor ("cachectin"), epidermal thymocyte-activating factor, eosinophil cytotoxicity-enhancing factor, interferon- α , β , and γ , platelet-derived growth factor, and transforming growth factor (TGF) β , which are all released by activated macrophages, TGF- α , or mixtures of these polypeptides, also failed to activate proteolysis or PGE₂ production. By contrast, a large increase in net protein breakdown could be induced in the rat soleus by polypeptides released from porcine monocytes or by the serum from febrile cattle which had been injected with *Pasteurella haemolytica* or bovine rhinotracheitis virus. Therefore, a still-unidentified product of activated monocytes appears to be responsible for the negative nitrogen balance that accompanies infectious illness.

Introduction

In patients with fever or sepsis, traumatic injuries, and certain neoplastic diseases, there is a marked loss of weight and body protein and a generalized muscle wasting (1). These conditions lead to a severe negative nitrogen balance that can exceed that seen in fasting (2-9). Several observations indicate that the nitrogen loss during sepsis results primarily from accelerated degradation of cell proteins (2-7), which may also be accompanied by a reduction in tissue protein synthesis (4, 7-9). Skeletal muscle, which constitutes the major protein reserve in mammals, seems to be the primary site of this increased proteolysis (2-8). In experimental animals, the administration of

live *Streptococcus pneumoniae* (6) or *Escherichia coli* endotoxin (2, 7) causes a marked loss of muscle mass and more rapid breakdown of muscle proteins. Similarly, in humans with sepsis, there is clear evidence of increased muscle proteolysis (3) and net release of amino acids from this tissue (3).

Work from this laboratory (4, 5) and that of Clowes et al. (3) indicated that a circulating factor(s) released by activated macrophages (4) acts directly on skeletal muscle to signal the enhanced protein degradation. Our early studies (4, 5) utilized partially purified human interleukin 1 (IL-1) obtained from the medium of activated human monocytes. When added to rat muscles in vitro, this material stimulated protein breakdown within 2 h without affecting protein synthesis, and also caused a large increase in prostaglandin E₂ (PGE₂) formation by the muscle (4, 5). Furthermore, inhibition of PG production with indomethacin blocked the enhancement of muscle proteolysis (4, 5). Similarly, pretreatment of animals with indomethacin was found to prevent the rise in muscle proteolysis induced in vivo with endotoxin (2). In addition to its essential role in activating protein catabolism, the increase in PGE₂ production may account for the muscle pain that generally accompanies fever and infectious diseases (4).

These observations (4, 5) strongly suggest that the acceleration of protein breakdown in muscle is coordinately regulated with a number of other host responses to infection (1, 10, 11), including fever, activation of T and B cells, production of neutrophils and lymphocytes, and the induction of acute phase proteins in the liver (1, 10, 11). During infectious illness or after trauma, the mobilization of amino acids from muscle protein can be advantageous by providing essential precursors for gluconeogenesis, wound repair, or protein synthesis in liver and white blood cells (4, 5).

There is now very strong evidence that these host responses to infection (10) are initiated by circulating factors released by macrophages upon interaction with bacteria, viruses, or antigen-antibody complexes. The best studied of these monokines is IL-1 (10), which can elicit many of these defensive responses to infection. However, a number of other potent polypeptide factors, including tumor necrosis factor (TNF),¹ the interferons, and several growth factors are also released by activated macrophages. The specific roles of these different agents in host defenses are still unclear. Of particular interest in this regard are the effects of TNF (12), a macrophage product pro-

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1. Abbreviations used in this paper: ECEF, eosinophil cytotoxicity-enhancing factor; ETAF, epidermal cell-derived thymocyte-activating factor; IBR, infectious bovine rhinotracheitis (virus); PDGF, platelet-derived growth factor; r, recombinant; TGF, transforming growth factor; TNF, tumor necrosis factor.

posed to play a critical role in the pathogenesis of shock (13, 14) and of cachexia (15–17; also see accompanying article). In fact, this factor was called “cachectin” by Beutler et al. (16) based on its ability to inhibit lipoprotein lipase (16) and to block the differentiation of adipocytes in vitro (17).

Because of the availability of homogenous recombinant IL-1 (18–21) and the discovery of multiple forms of IL-1 (α and β) (18, 20), we set out to clarify its precise role in the regulation of muscle proteolysis. The pure polypeptides failed to elicit the effects seen with the partially purified human IL-1. The latter material had been obtained by gel chromatography of culture medium from macrophages stimulated with *Staphylococcus albus* (4). Such preparations contain 17-kD proteins that show the classic IL-1 activities (e.g., stimulation of lymphocyte proliferation and pyrogenicity), but they also contain many other proteins, including TNF. We therefore undertook a systematic investigation of the effects on muscle protein breakdown and PGE₂ production of various pure preparations of IL-1, other cytokines released by macrophages, the transforming growth factors, and serum from febrile cattle infected with *Pasteurella haemolytica* and infectious bovine rhinotracheitis (IBR) virus.

Methods

Young male rats of the CD (Charles River Breeding Laboratories, Inc., Wilmington, MA) or Sprague-Dawley (University of Alberta, Bio-Sciences Animal Unit) strains, and young male mice of the CD-1 strain (Charles River Breeding Laboratories) were maintained on Purina Lab Chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. Soleus or extensor digitorum longus muscles were dissected with tendons intact. Rat muscles weighed 20–25 mg; mouse muscles weighed 7–10 mg. In experiments where muscles were incubated under tension at their resting length, tissues were mounted on supports made of surgical stainless steel, as described previously (22).

Tissues were incubated in 2.0 ml of Krebs-Ringer bicarbonate buffer (KRB) equilibrated with 5% CO₂/95% O₂. KRB was supplemented with 5 mM glucose and 0.1 U/ml insulin and branched chain amino acids at concentrations similar to those found in plasma (22). Muscles were routinely preincubated at 37°C for 30 min to 1 h, then transferred to fresh media and incubated further for 2 or 3 h. In experiments where natural (monocyte) products were studied, these factors were added to the medium during the latter 3-h incubation only. Protein synthesis, net protein degradation, protein degradation, and production of PGE₂ were measured as previously described (4, 23). (The various polypeptides used and their sources are listed in Tables II and III.)

Bovine pneumonic pasteurellosis, a severe respiratory disease of cattle, results from impaired pulmonary antimicrobial defense mechanisms after stress and/or a viral infection, which increases the susceptibility of the lungs to infection with *Pasteurella haemolytica* (24). An experimental model of this infection involves exposure to IBR virus, followed 4 d later by intranasal inoculation of *P. haemolytica* (25). Samples of blood serum from 11 infected cattle and prechallenge controls from each were generously provided by Drs. S. D. Acres and L. A. Babiuk of the Veterinary Infectious Disease Organization (Saskatoon, Canada). The body temperature of each animal was measured at 9:00 a.m., using a portable digital thermometer with a Cu/Co thermocouple inserted 10 cm into the rectum. Blood samples were collected daily after the temperature measurements. Sera were prepared for assay by ultrafiltration to remove high molecular mass material (> 50 kD) as described by Clowes et al. (3).

Data are presented as the means±SEM, and all experiments used at least seven individual muscles for each condition.

Results and Discussion

Effects of IL-1. When rat soleus muscles were incubated with partially purified IL-1 derived from activated human monocytes (26), net protein degradation increased by ~ 100% (Table I) and PGE₂ production increased severalfold, in accord with our previous observations (4). However, incubation of the muscles with any of several preparations of recombinant(r) human or murine IL-1 consistently failed to influence net protein degradation or PGE₂ production (Tables I and II). A broad range of concentrations of IL-1 were studied, and both IL-1 α and IL-1 β , corresponding to the pI 5 and pI 8 species (18, 20), respectively, were tested individually and together. These polypeptides were all ineffective in promoting muscle protein degradation (Table II), even though the same preparations had been shown to stimulate lymphocyte proliferation or fever in the laboratories that provided them for our studies (Table II). Negative results were obtained both with muscles maintained under passive tension, where they approach neutral nitrogen balance (22), and muscles incubated without tension, which are in a very catabolic state (22). This failure to stimulate proteolysis cannot be ascribed to species specificity, since the human or mouse rIL-1 induce other physiological responses in rats or isolated rat cells (10, 11, 18–21).

Since the rIL-1 produced in *E. coli* may have lacked some essential posttranslational modifications, we investigated the effects of the homogeneous natural molecules (which had not been available at the time of our previous study). The two species of porcine IL-1, pI 5(α) and pI 8(β), and natural human IL-1 β had no effect on either net protein degradation or PGE₂ production (Table II). It has been suggested that in vivo or in vitro, the increased muscle catabolism is signaled by a fragment of IL-1 which is generated by proteolytic cleavage (3, 26). To test whether a serum protease may be necessary for the activation of IL-1, human rIL-1 was incubated in normal human serum for 24 h at 37°C. After this treatment, the material still did not stimulate net protein degradation in rat muscle. Similarly, when rIL-1 β was incubated with trypsin under

Table I. Effects of Crude Natural and rIL-1 on Net Protein Degradation and PGE₂ in Rat Soleus Muscle

Treatment	Net protein degradation	PGE ₂ production
	pmol tyrosine/ mg muscle per 3 h	pg/mg muscle per 3 h
No additions	199±17	32±3
Crude monocyte IL-1 human (1 µg/ml)	410±8*	103±9*
rIL-1 β human (1 µg/ml)	237±30	29±4

Rat soleus muscles were preincubated for 30 min in KRB medium containing 5 mM glucose, branched chain amino acids (0.85 mM isoleucine, 0.5 mM leucine, and 1.0 mM valine) and 0.1 U/ml insulin. Muscles were then transferred to fresh medium containing the additions indicated, and were incubated for a further 2 or 3 h. Crude monocyte IL-1 and rIL-1 β (26) were kindly provided by Dr. C. A. Dinarello (Tufts University School of Medicine, Boston, MA). The statistical analysis was done using Student's *t* test.

* *P* < 0.001 (n = 10).

Table II. Lack of Effect of Other Interleukins, by Themselves or in Combination with Other Monokines, on Net Protein Degradation in Rat Soleus Muscles

IL-1 added	Net protein degradation	Concentrations used
	<i>pmol tyr/mg muscle per 2 h</i>	
Natural		
Experiment 1		
None	115±0.008 (*)	
1. Porcine, pI 5 or 8	116±0.008 (*)	200 pg/ml; 20, 200 ng/ml
Experiment 2		
None	88±5	
2. Human β	83±6	100 U/ml
Recombinant		
Experiment 1		
None	79±3	
3. Murine α, β	84±4	100, 1,000, 5,000 U/ml
4. Human β	83±4	10 μ g/ml
5. Plus PDGF	95±6	+1 or 150 ng/ml
6. Plus murine rTNF	79±4	+6,000 U/ml
7. Plus human rTNF	82±5	+10 ⁵ U/ml
8. Plus PMA	79±5	+20 nM or 2 μ M
9. Murine IL-1 + ETAF + rTNF	83±4	5,000 U/ml + 80 U/ml + 6,000 U/ml
Experiment 2		
None	253±22 (*)	
10. Human β	217±17 (*)	2.5, 10 μ g/ml
11. Human α, β	231±18 (*)	5,000 U/ml (50 ng/ml)
Human α plus β	219±25 (*)	

Rat soleus muscles were incubated as in Table I. In the experiments indicated (*), when the muscles were incubated without their tendon attached to a support, they shortened and the rate of protein breakdown was higher than when they were incubated under tension. When more than one concentration was used, the results shown in the Table correspond to the highest one tested. No significant difference was observed with any of the concentrations used. The number of muscles used in each condition was always greater than seven. Sources: (1) Dr. J. Saklatvala (Strangeways Laboratory, Cambridge, MA); (2) Dr. J. Singh (Collaborative Research, Inc., Waltham, MA); (3) Dr. P. Lomedico (Hoffman-La Roche Inc., Nutley, NJ); (4, 7, 10) Dr. Alan Shaw (Biogen Research Corp., Geneva, Switzerland); (5) Dr. H. Antoniades (Harvard School of Public Health, Boston, MA); (6) Dr. P. Tavernier (University of Ghent); (8) Sigma Chemical Co., St. Louis, MO (PB139); (9) IL-1 and TNF from Biogen Research Corp. and ETAF from Dr. D. Sauder (McMaster University, Hamilton, Ontario, Canada); (11) Dr. D. Cosman (Immunex Corp., Seattle, WA).

conditions reported to generate an active fragment of natural IL-1 (26), it was still ineffective.

One other possibility is that IL-1 requires another factor from the macrophages to enhance proteolysis. Since IL-1 and phorbol-12-myristate-13-acetate (PMA) are synergistic in stimulating PGE₂ production in many cell types (27), we tested whether an effect of the rIL-1 could be demonstrated when this potent activator of protein kinase C was also present. However, in the rat muscles, the phorbol ester by itself or in combination with rIL-1 β did not influence either protein turnover or PGE₂ release (Table II).

Effects of other cytokines. We examined the possible effects on muscle protein turnover of other polypeptides known to mediate certain host responses to infection. Epidermal cell-derived thymocyte-activating factor (ETAF) is a protein released by keratinocytes with many IL-1-like activities (28, 29). It was also found not to affect net protein degradation in these muscles. We undertook similar studies with human and murine TNF ("cachectin"), which has been suggested to signal the marked weight loss during infectious illness (15, 17, 30). Although rTNF does stimulate PGE₂ release in many cells (30, 31), it did not affect PGE₂ production or protein balance in

skeletal muscles (Table III, 32). In the accompanying study (32), we found that injection of rTNF into rats also has no effect on muscle protein balance, even though this polypeptide did cause fevers (32, 33). Partially purified preparations of natural murine TNF also did not affect muscle protein balance, nor did the addition of rIL-1 and rTNF together (Table II). These negative results are noteworthy because the crude preparations of IL-1 that are effective (4, Table I) must contain both IL-1 and TNF.

These findings suggest that some other monocyte product by itself or with these monokines stimulates muscle protein degradation. Monocytes, like platelets, can release platelet-derived growth factor (PDGF) (34). Although PDGF can stimulate PGE₂ production in many cells (34), it did not enhance this process, nor did it affect protein turnover, in skeletal muscle (Table III). Recently, Lee and Pekala (35) found a polypeptide that stimulates glucose uptake in myotubes and that is released by a macrophage cell line (RAW 264.7 cells) exposed to endotoxin. This monokine differs from TNF in its chemical properties and biological effects. When the soleus muscles were incubated with this activity, no increase in protein breakdown was found. Since the unfractionated medium from

these cells exposed to endotoxin also was ineffective, this cell line may fail to generate the critical component(s). (Alternatively, this factor may be unstable under these conditions or ineffective at the concentrations used.)

Another polypeptide produced by endotoxin-stimulated human monocytes is eosinophil cytotoxicity-enhancing factor (M-ECEF). This polypeptide, which can increase the ability of human eosinophils to kill larvae of *Schistosoma mansoni* (36), has also been purified from the U937 cells treated with phorbol ester and endotoxin (36). When rat skeletal muscles were incubated with this factor (U937-ECEF), or with partially purified preparations of ECEF, which also contain activities resembling those of TNF, no effect on protein degradation was observed (Table III).

Analogous experiments were carried out with α , β , and γ -interferons. Like IL-1 and TNF, these polypeptides are pyrogenic and can induce PGE₂ production in the hypothalamus (37). Nevertheless, none of the interferons tested altered muscle protein breakdown or PGE₂ production in vitro (Table III). Similarly, when human recombinant interferon- α_2 was injected intraperitoneally into mice (10⁴, 10⁶, and 10⁷ U/kg), and their muscles were incubated in vitro 2.5 and 12 h later, protein degradation and PGE₂ production were not significantly different than in controls.

Finally, we studied the effects of transforming growth factors α and β (TGF α and β), which are produced by many tumor cells (38–40), but may also be produced by certain non-neoplastic cells (38). Since increased amounts of TGF activity are found in the urine of patients with cancer (39, 40), we examined the possibility that TGF α and β may initiate the cachexia accompanying cancer. Although these polypeptides induce proliferation of fibroblasts and have effects resembling those of EGF (37, 40), TGF α and β are catabolic in a number of biological systems. Like TNF, TGF- β can block the differentiation of fibroblasts into adipocytes (41), and when administered to newborn mice, TGF retards their growth (42). Both TGF α and β are also potent stimulators of bone resorption by a mechanism involving PGE₂ (43, 44), and TGFs may be humoral factors activating bone catabolism in neoplastic disease (43, 44). However, no effect of TGFs on protein degradation or synthesis was found in skeletal muscle (Table III).

The failure of PGE₂ levels to rise dramatically with various treatments contrasts with our prior results with partially purified IL-1 (4), and with in vivo studies of muscles of rats injected with endotoxin or live *E. coli* (2). Experiments by Ruff and Secrist (6) also suggest that muscle wasting during sepsis requires PG production. Thus, the failure of these polypeptides to promote muscle PGE₂ production correlated with, and can account for, their inability to stimulate proteolysis in this tissue.

Proteolysis-inducing activity exists in serum. Because of these consistently negative results, we reinvestigated in different experimental systems our earlier conclusions that factors that are catabolic to skeletal muscles are released by macrophages and are present in the serum of infected animals. These efforts confirmed that partially purified fractions from monocyte supernatants can stimulate muscle protein degradation in vitro (4, 5, 8, Table I) and can alter protein and amino acid metabolism when injected in vivo (9, 45, 46). To test whether factors that stimulate muscle proteolysis can actually be demonstrated in serum of infected individuals, as reported in

Table III. Other Cytokines that Failed to Affect Net Protein Breakdown in Rat Soleus

Polypeptides	Concentration tested	Additional assays
TNF (α)		
1. Murine cachectin (partially purified)	1 nM	*, ‡
2. Human rTNF α	10 μ g/ml	
3. Human rTNF α	6,000, 10 ⁵ U/ml	‡
Interferons		
4. Human α_2	10, 100, 1,000 U/ml	*, §,
5. Murine α 1	10, 100, 1,000, 5,000 U/ml	*, §,
6. Murine α 7	10, 100, 1,000 U/ml	‡
7. Murine α	1,000 U/ml	‡
8. Murine	1,000 U/ml	‡
9. Murine α_2, β (natural)	25,000 U/ml	*, ‡, §,
Other		
10. Human PDGF	1, 150 ng/ml	*
11. Murine TGF β	10 ng/ml	§,
12. Human rTGF α	0.5, 5.0, 10.0 μ g/ml	§,
13. Human ETAF (partially purified)	20, 80 U/ml	
14. ETAF + murine TNF	80 U/ml + 6,000 U/ml	
15. ETAF + human TNF	80 U/ml + 6,000 U/ml	
16. ECEF	0.33, 33 ng/ml	

Rat soleus muscles were incubated as in Table I, except in the case of murine interferons, which were assayed on mouse muscles. In all experiments net protein degradation was determined. No difference was observed with any of the concentrations used. In some experiments, the absolute rate of protein degradation ([†]), protein synthesis ([‡]), PGE₂ production (^{*}) were also measured in the soleus, as indicated. None of these additions had any effect on these parameters (n \geq 7). In some experiments ([‡]), net protein degradation in the extensor digitorum longus muscle was also studied, as indicated.

Sources: (1) Dr. B. Beutler (Rockefeller University, New York); (2, 7) Dr. S. Sheppard (Genentech Inc., South San Francisco, CA); (3, 6) Biogen Research Corp; (4) Schering-Plough Corp, Kenilworth, NJ; (5) Dr. Charles Weismann (University of Zurich); (8) Dr. W. Fiers (University of Ghent); (9) Dr. P. Lengyel (Yale University, New Haven, CT); (10) Dr. H. Antoniades (Harvard School of Public Health); (11) Dr. A. Roberts (National Cancer Institute, Bethesda, MD); (12) Dr. M. Winkler (Genentech Inc.); (13) Dr. D. Sauder (McMaster University); (14, 15) TNF and IL-1 (Biogen Research Corp.); (16) Dr. David Silberstein (Harvard Medical School, Boston, MA).

humans by Clowes et al. (3), we studied sera from cattle injected with experimental bovine pneumonic pasteurellosis, a severe respiratory disease associated with high fever (24). This experimental model of sepsis is induced by sequential injection with infectious bovine rhinotracheitis virus and *P. haemolytica* (25). The infected animals showed high fever for 8 d (Table IV). Serum collected during this period stimulated net protein breakdown in rat muscles by up to 150% (Table IV) compared to the serum taken prior to the bacterial challenge.

Presumably, this response is due to factors released from the activated macrophages. In fact, in related experiments (Baracos, V. E., and J. Saklatvala, manuscript in preparation), the medium from activated porcine monocytes was fraction-

Table IV. Effect of Serum from Infected Cattle on Net Protein Degradation in Rat Skeletal Muscle

Day	Body temperature	Net protein degradation
	°C	nmol tyr/mg per 3 h
-6	38.6±0.13	0.117±0.008
0	38.9±0.10	0.117±0.011
After injection of IBR virus		
+2	40.8±0.15*	0.235±0.019*
+4	40.8±0.08‡	0.208±0.017*
After injection of <i>P. haemolytica</i>		
+6	40.7±0.17*	0.294±0.023‡
+8	39.3±0.15*	0.294±0.021‡
+10	ND	0.251±0.023*

Eleven cattle were infected sequentially with IBR virus and *P. haemolytica*, an experimental model of bovine pneumonia (25). Two prechallenge samples were taken from each animal. Sera were treated by ultrafiltration to remove high molecular mass material (> 50 kD) (3), and added to incubations at a dilution of 1:12. All samples were assayed in quadruplicate. Incubation blanks contained serum but no muscles and were used to correct for the initial tyrosine content of the serum samples. Rat soleus muscles were preincubated for 30 min in KRB medium containing 5 mM glucose and 0.1 U/ml insulin. Muscles were then transferred to fresh medium containing the sera, and were incubated for an additional 3 h. Values for net protein degradation and body temperature on different days after infection were compared to the pooled prechallenge values, by Student's *t* test. Difference from prechallenge controls: * $P < 0.01$; ‡ $P < 10^{-4}$ ($n = 11$); ND, not determined.

ated according to size by gel filtration, and each fraction incubated with rat muscles in vitro. Three distinct fractions were found that were capable of inducing net protein breakdown in muscle. Of particular interest is a peak that coeluted with the IL-1 activity that stimulated proteolysis. Two further peaks which promoted net protein breakdown were eluted subsequently. The first of these activated protein degradation and the other, of low molecular mass (< 8 kD), had a catabolic effect by inhibiting protein synthesis. However, the active polypeptides in these fractions of culture medium or in serum that stimulate muscle protein breakdown remain to be identified, and work to clarify the nature of these polypeptide(s) is in progress.

Blood monocytes and fixed tissue macrophages are known to release a large variety of defined molecules, including prostanooids, enzymes, monokines, etc. which can act in a coordinated fashion to mediate the antimicrobial, antitumor, and inflammatory activity of these cells. One or more factor(s) appear responsible for systemic alterations in the turnover of cellular proteins during fever. These experiments and the accompanying study (32) indicate that the critical component(s) leading to negative nitrogen balance is not IL-1, TNF, or any of the presently known monokines, although we can not eliminate for certain their possible involvement in concert with some still unknown agent.

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