Clinical Trials for the Treatment of Secondary Wasting and Cachexia

Muscle Protein Breakdown and the Critical Role of the Ubiquitin-Proteasome Pathway in Normal and Disease States¹,²

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All intracellular proteins and many extracellular proteins are continually "turning over"; i.e., they are being hydrolyzed to their constituent amino acids and replaced by new synthesis. This continual degradation of cell proteins was first discovered through the classic experiments of Schoenheimer and coworkers in the 1940s using ¹⁵N-labeled amino acids in adult animals (Schoenheimer 1942). However, after this surprising discovery, knowledge about protein breakdown developed quite slowly. Since the late 1960s, there has been growing evidence that protein degradation plays many essential roles in the functioning of organisms from bacteria to mammals, but only in the past decade have the proteolytic systems been elucidated and the fundamental importance of protein degradation been generally recognized.

Individual proteins in the nucleus and cytosol, as well as in the endoplasmic reticulum and mitochondria, are degraded at widely differing rates. For example, while most proteins in a rat liver might turn over once every one to two days, some regulatory enzymes have half-lives as short as 15 minutes. On the other hand, actin and myosin in skeletal muscle are much more stable, perhaps turning over only once every one to two weeks, and hemoglobin can last the lifetime of the red blood cell (three months in humans). Mammalian cells contain multiple proteolytic systems to carry out this degradation process and complex regulatory mechanisms to ensure that this continual proteolysis is highly selective and to prevent excessive breakdown of cell constituents. Also, the overall rates of protein synthesis and degradation in each cell must be precisely balanced, since even a small decrease in synthesis or a small acceleration of degradation, if sustained, can result in a marked loss of mass in the organism as a whole.

PHYSIOLOGICAL IMPORTANCE OF PROTEIN BREAKDOWN

Although the continual destruction of cell proteins might appear wasteful, this process serves several important homeostatic functions:

The rapid removal of critical regulatory proteins (e.g., transcription factors or enzymes and inhibitory factors) is essential for control of cell growth and metabolism. Unlike most regulatory mechanisms, protein degradation is inherently irreversible. Destruction of a protein component can lead to a complete, rapid and sustained termination of a process and a change in cell composition. Examples of important regulatory proteins rapidly inactivated by proteolysis include transcription factors (e.g., c-jun [Treier et al. 1994] and c-fos [Stancovski et al. 1995]) and the tumor suppressor p53 (Scheffner et al. 1993). In many cases, phosphorylation of a protein dramatically alters its half-life by either enhancing or inhibiting its susceptibility to degradation.

This type of regulation is responsible for rapid elimination of the inhibitory factor, IkB during the inflammatory response (Alkalay et al. 1995; Scherer et al. 1995) and of the various cyclins that control progression through the cell cycle (King et al. 1994).

The rapid degradation of specific proteins permits adaptation to new physiological conditions and changes in cell composition. For example, in the fasting state, hepatic gluconeogenic enzymes for glucose storage disappear and the synthesis of gluconeogenic enzymes increases, but within hours after food has been ingested, this pattern is reversed.

In all cells, protein breakdown provides an essential quality control mechanism that selectively eliminates abnormally folded or damaged proteins that have arisen by missense or nonsense mutations, biosynthetic errors, damage by oxygen radicals, or by denaturation (especially at high temperatures). For example, the abnormal globins produced in some “unstable hemoglobinopathies” cannot bind heme, fail to fold correctly, and are degraded within minutes after synthesis. Similarly, in cystic fibrosis, the mutant form of the transmembrane conductance regulator


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protein, (CFTR) is selectively degraded and therefore fails to reach the cell surface (Jensen et al. 1995, Ward et al. 1995).

In states of inadequate caloric intake or catabolic disease, the overall breakdown of cell proteins, especially in skeletal muscle, increases to provide the organism with amino acids essential for gluconeogenesis, new protein synthesis and energy production (Mitch and Goldberg 1996).

Protein degradation is critical for the functioning of the immune system in its continual monitoring for abnormal (e.g., viral) proteins within cells and in the extracellular space. A fraction of the small peptides generated during breakdown of intracellular proteins in proteasomes is taken up into the endoplasmic reticulum and transported to the cell surface bound to major histocompatibility complex (MHC) class I molecules (Rock et al. 1994). Circulating lymphocytes continually monitor cell surfaces for these small fragments presented on MHC molecules, and if non-native peptides are encountered, the cell is destroyed. Similarly, the development of antibodies against foreign proteins requires their uptake by cells and digestion in the lysosomal-endosomal compartment to peptides that are presented to the immune system on MHC Class II molecules.

**METHODS FOR THE STUDY OF PROTEIN BREAKDOWN**

The continuous turnover of cell protein is typically measured in cells using isotopic amino acid tracers in "pulse-chase" protocols (Goldberg and St. John 1976). After a brief labeling of proteins, the decay of radioactive polypeptides is followed with time. To prevent the reincorporation of radioactive amino acids released by proteolysis, cells are administered large amounts of nonradioactive amino acids, or protein synthesis is blocked with antibiotic inhibitors. The disappearance of the radioactive protein or class of proteins follows an exponential decay curve. These "pulse-chase" approaches can be used most readily in bacteria, yeast and cells in culture, but have also been used with incubated tissues and organs. Besides enabling measurement of degradation of cell protein generally, pulse-chase analysis can be used to analyze the kinetics of degradation of any specific protein of interest, provided a specific antibody or affinity tag is available to separate it from the rest of the radiolabeled polypeptides. By the use of specific inhibitors of the various cellular proteases (see below) the responsible degradative pathway can also be identified.

In isolated tissues, the overall rate of protein degradation can be measured most readily by following the accumulation of an amino acid that is neither synthesized nor metabolized in tissue, for example tyrosine in skeletal muscle (Fulks et al. 1975). Tyrosine is particularly useful in these types of experiments, since its accumulation in the medium directly reflects loss of cell protein and since it can be easily measured fluorometrically. The major contractile proteins in muscle, actin and myosin, contain N-methylhistidine, a special amino acid generated by postsynthetic methylation of certain histidine residues. Because it cannot be reincorporated into protein or metabolized when generated by muscle proteolysis (Huszar 1972), production of N-methylhistidine reflects the breakdown of these myofibrillar proteins in isolated muscle. Also their degradation in animals can be assessed by measuring urinary N-methylhistidine excretion (Young and Munro 1978). Similarly, the breakdown of extracellular collagen can be followed by the appearance of the specialized amino acid hydroxyproline, which is also generated by postsynthetic modification.

In addition, rates of degradation in patients and intact organisms have been assessed by more indirect approaches involving extrapolation from rates of protein synthesis during continual infusion of labeled amino acids (e.g., $^{15}$N-leucine). In general, these methods are less precise and are only really valid in steady-state conditions. In clinical settings, $^{15}$N-labeled precursors have often been used in patients in such studies, utilizing complex assumptions (e.g., about tissue distribution of precursors and rates of amino acid metabolism), whose validity remains unclear. It is still uncertain if these approaches give similar results as well-validated studies with isolated organs.

**CELLULAR DEGRADATIVE PATHWAYS**

All cells contain multiple pathways for protein breakdown (Fig. 1). One main site for proteolysis is within the lysosome, but protein breakdown also occurs in the cytosol and nucleus.

**A. Lysosomal System**

Extracellular proteins, such as plasma proteins or hormones and phagocytosed bacteria, are taken up by endocytosis and are completely degraded within lysosomes. These organelles contain several acid-optimal proteases, including cathepsins B, H, and D, and many other acid hydrolases. Plants and yeast also contain multiple proteases in a membrane-enclosed space, the vacuole. Proteolysis within the lysosome also generates peptides that are presented to the immune system in association with MHC class II molecules. Endocytosis of cell surface receptors is followed by their degradation in lysosomes, and this process is often accelerated after ligand binding. Some cytosolic proteins are degraded in lysosomes after being engulfed in autophagic vacuoles that fuse with lysosomes (Dice 1990, Lardeux and Mortimore 1987). This process is accelerated in most cells by the lack of insulin or essential amino
acids, and in liver by glucagon (Gronostajski et al. 1984). Also, in such cells, there exists a specific mechanism involving hsp70 for transport of certain cytosolic proteins directly into the lysosome (Chiang et al. 1989). It is possible to quantitate the contribution of the lysosomes to a proteolytic process by using agents that block lysosomal acidification (e.g., chloroquine and methylamine) or by using inhibitors of the lysosomal cysteine proteases, cathepsins B, H, and L (e.g., leupeptin or E64) (Furuno and Goldberg 1986). Use of these inhibitors has demonstrated that the lysosomal pathway is mainly involved in degrading surface membrane proteins and endocytosed, extracellular proteins, rather than having a major role in the normal turnover of cytosolic proteins under normal conditions (Furuno and Goldberg 1986, Lowell et al. 1986).

**B. Miscellaneous cytosolic proteases**

The bulk of protein metabolism in the cytosol occurs by the ubiquitin (Ub)-proteasome pathway (discussed in depth below); however, other cytosolic proteolytic systems also exist in mammalian cells. One Ca\(^{2+}\)-activated (ATP-independent) proteolytic process involves the cysteine proteases termed calpains (Mellgren 1987, Murachi et al. 1980, Waxman 1981). Like many lysosomal proteases, these enzymes are inhibitable by E64 and leupeptin. Two forms of calpains have been described which differ in their affinity for Ca\(^{2+}\). A specific protein inhibitor of the calpains, calpistatin, is also present in mammalian cytosol and is able to bind and inactivate four calpain molecules (Murachi et al. 1980). These proteases appear to be activated when cells are injured and cytosolic Ca\(^{2+}\) rises, and so they may play an important role in tissue injury, necrosis and autolysis (Goll et al. 1992). However, it is still unclear whether they function in the normal turnover of any cell proteins (Furuno and Goldberg 1986).

Another important family of cytosolic proteases is the caspasases or ICE (interleukin-\(\beta\)-converting enzyme)-related proteases. These enzymes, which are cysteine proteases, are involved in the apoptotic pathway in eukaryotes (Salvesen and Dixit 1997). They cleave all proteins after aspartic acid and are synthesized as inactive precursors. In response to a variety of toxic stimuli (e.g., DNA damage or circulating signals), the various caspases are activated, leading to programmed cell death.

**C. Mitochondrial proteases**

These organelles contain a complete system for protein turnover within the mitochondrial matrix, where there exists an ATP-dependent pathway for breakdown of organellar proteins. This system does not involve Ub but involves high molecular weight protease complexes similar to ones found in bacteria, and can digest polypeptides or free protein subunits to amino acids.

**THE UB-PROTEASOME PATHWAY**

It is now clear that the bulk of all intracellular protein is degraded by the Ub-proteasome pathway (Fig. 2) (Rock et al. 1994). Protein substrates of this major pathway are first marked for degradation by the covalent attachment of chains of Ub molecules. Proteolysis is then catalyzed by the 26S proteasome complex that degrades proteins to small peptides (Kisselev et al. 1998). This ATP-dependent pathway was first demonstrated in the late 1970s and shown to catalyze the breakdown of abnormal proteins and short-lived regulatory cellular components. However, this system is also responsible for the slow turnover of the long-lived proteins that comprise the bulk of cells (Mitch and Goldberg 1996, Rock et al. 1994) and generates the peptides presented on MHC class I molecules during the immune response (Rock et al. 1994). In addition, this pathway plays a special role in the accelerated breakdown of myofibrillar proteins in fasting and disease states (see below).

Ub is a 76 amino acid heat-stable polypeptide, and was first shown primarily by Hershko, Ciechanover and coworkers, to be an essential cofactor in the ATP-dependent degradation of proteins in reticulocyte extracts (Ciechanover et al. 1978, Hershko et al. 1980). Their experiments showed that Ub must first be activated at its C-terminal glycine residue prior to conjugation to a substrate (Hershko et al. 1981). The activated Ub molecule is covalently linked by an isopeptide bond to an \(\epsilon\)-NH\(_2\) group of a lysine in the substrate protein (Busch 1984, Chau et al. 1989). Then the C-terminal glycine of another Ub is linked to a specific lysine of the first Ub to form long multi-Ub chains. Rapid degradation by the 26S proteasome requires association with more than five Ub moieties linked to the substrate (Pickart 1997). The 26S proteasome also contains enzymes to disassemble the Ub chain and release Ub for reuse in subsequent proteolytic cycles (Hadari et al. 1992, Kam et al. 1997, Papa and Hochstrasser 1993, Wilkinson et al. 1995). At least three enzymes function to link Ub to protein substrates:

**A. E1: Ub-Activating Protein**

E1 utilizes ATP to generate a highly reactive form of Ub. Hydrolysis of ATP drives the formation of a covalently bound C-terminal Ub adenylate (Haas and Rose 1982). The activated Ub is then transferred to an active site sulffhydryl group on the enzyme, liberating AMP, in a reaction similar to the charging of tRNAs by amino acyl-tRNA synthetase. This high-energy thiolester form of Ub, generated by E1, is essential for the eventual linkage to substrate in a series of reactions requiring two other Ub conjugating enzymes, E2 and E3. E1 is an abundant 110 kD protein that is essential for cell viability. In mammalian organisms, unlike the large number of E2s and E3s, only a single functional E1 protein has been found (Handler et al. 1991). Temperature-sensitive mutants of E1 have been isolated, which have been very useful in implicating the Ub-proteasome pathway in the degradation of specific proteins (Finley et al. 1984, Michalek et al. 1993).

**B. E2s: Ub-Carrier Proteins**

Once activated, the Ub bound to E1 is transferred to a sulffhydryl group of one of at least a dozen Ub carrier proteins or E2s (Jentsch 1992). They have a limited ability to transfer the activated Ub to certain proteins, but in vivo, E2s generally serve as Ub-carriers in a process catalyzed by the E3s. The E2s are generally small proteins that share a conserved 16kD core containing the cysteine that forms a thiolester linkage with the activated Ub (Jentsch 1992). A large family of E2 enzymes has been isolated due to their propensity to bind to a Ub affinity matrix in the presence of E1 and ATP (Haas and Bright 1988, Pickart and Rose 1985). The large number of E2s help to generate the specificity of the ubiquitination system, since particular E2s function in the degradation of different types of substrates.

**C. E3s: Ub-Protein Ligases**

Finally, the activated Ub is transferred from E2 to the protein substrate by E3, the Ub-protein ligase. Most E3s car-
alyze the formation of long Ub chains, which means that the E3 active site(s) are able to transfer Ub directly to either a lysine of the substrate or, processively, to the preceeding Ub moieties to form a chain. The E3 proteins contain specific binding sites for the substrate, for E2, and at least one (or possibly two) Ub molecules. It is believed that the E3 alone, or in a complex with an E2, generates the specificity of the ubiquitination process. A variety of structural determinants in the substrate are recognized by different E3s, such as its amino-terminal residue (Bachmair et al. 1986, Varshavsky 1992), specific domains (i.e., the “destruction box” found within various proteins degraded during mitosis) (Glotzer et al. 1991), and phosphorylated domains (Hoyt 1997). Only a small number of E3s (about 10) have been described to date. These fall into several disparate families, with much less structural similarity than among E2s.

**D. The Proteasome**

Proteins marked for degradation by Ub are digested to small peptides within the 20S proteasome particle. This 600 kDa particle is a major cell constituent, comprising up to 1% of cellular proteins. The 20S particle is a barrel-shaped structure of four stacked rings, each composed of seven subunits surrounding a central cavity (Coux et al. 1996, Goldberg et al. 1995b, Lowe et al. 1995). The two inner β rings enclose a large central chamber containing the sites where protein is degraded, while the two outer α rings surround a small opening through which protein substrates must enter. Because of its small diameter, polypeptides must first be unfolded to enter this opening in the α rings (Coux et al. 1996, Goldberg et al. 1995b). The presence of a powerful proteolytic enzyme system within the cell requires the evolution of mechanisms to safeguard against the nonspecific digestion of essential proteins. The first safeguard is the requirement for Ub-conjugation prior to degradation of the protein. Second, the active sites of the proteasome are geographically isolated within the central chamber and away from cytosolic milieu. Third, only unfolded proteins can enter the narrow opening in the α rings on either end of the 20S proteasome. On either end of the 20S proteasome, there is a large 19S (700 kDa) regulatory particle. This structure appears to provide specificity to proteolysis by binding ubiquitinated substrates and catalyzing the entry of the polypeptide into the 20S particle (Chu-Ping et al. 1994, Coux et al. 1996, Rechsteiner et al. 1993). Interestingly, the 19S regulatory complex also contains at least 6 different ATPases (Glickman et al. 1998) and it appears likely that the consumption of ATP by the 19S complex enables it to unfold protein substrates, to inject them into the 20S proteasome and to activate this particle (Larsen and Finley 1997). Thus, the marking of substrates by Ub-conjugation, the organization of the 26S complex and the energy requirement for proteolysis, all appear to have evolved to provide a remarkable degree of selectivity and regulation to the degradative process.

The mechanism by which peptide bonds are cleaved in the proteasome is also unique. Proteasomes do not fit into the standard classification of proteolytic enzymes according to their active sites (e.g., serine, cysteine, acidic or metalloproteases). The sequences of their β subunits are not homologous.
to those of known enzymes and the pattern of sensitivity to various inhibitors differs from that of any known protease family. The recent X-ray diffraction studies and mutagenesis of different amino acids in the proteasome have uncovered a new type of proteolytic mechanism (Groll et al. 1997, Lowe et al. 1995, Seemuller et al. 1995). The active site nucophile of the proteasome is the hydroxyl group of a threonine at the amino terminus of the β subunit. This novel mechanism has permitted selective inhibitors of the proteasome to be synthesized (Bogyo et al. 1997, Rock et al. 1994). Also, a natural product of streptomycetes, lactacystin, was recently discovered to inhibit intracellular protein degradation by reacting selectively with this terminal threonine (Bogyo et al. 1997, Dick et al. 1996, Fenteany et al. 1995). It is unclear why this unique proteolytic mechanism has evolved in the active sites of all proteasomes, from bacteria to man.

The mammalian proteasome contains at least three active sites that differ in their specificity for different types of bonds (Vinitski et al. 1994). One activity cleaves preferentially after basic amino acids, others after large hydrophobic amino acids, after small, neutral amino acids or after acidic residues. These activities which function together to catalyze the complete digestion of proteins seem to be associated with different β subunits. There are intriguing adaptations in proteasome subunit composition that can aid in the generation of diverse antigenic peptides. For example, in disease states, the cytokine, γ-interferon, which enhances antigen presentation in most cells, induces the expression of three novel β subunits that are incorporated into the 20S proteasome in place of β subunits present normally (Gaczynska et al. 1994, Goldberg et al. 1995a). These components alter the way peptide bonds are cleaved in order to favor the production of peptides appropriate for antigen presentation (Coux et al. 1996, Goldberg et al. 1995a).

### ACTIVATION OF THE Ub-PROTEASOME PATHWAY IN CATABOLIC STATES

Early studies of the rapid loss of muscle mass in rats during denervation atrophy, fasting or treatment with glucocorticoids suggested that the marked loss of cell protein was primarily due to enhanced proteolysis, especially of myofibrillar proteins (Tables 1 and 2)(Goldberg 1996, Goldberg and Goodman 1969). However, definitive conclusions and rigorous quantitation of rates of protein breakdown were only achieved by studies using isolated rat muscles incubated under defined conditions (Tawa and Goldberg 1994). The increase in proteolysis seen in such atrophying muscles was not inhibited when the atrophying muscles were incubated in vitro with agents that blocked the activity of lysosomes or Ca2+-activated proteases. However, when inhibitors of ATP production were added, muscle protein degradation decreased to the levels measured in control muscles (Mitch et al. 1994, Wing and Goldberg 1993). By this simple approach, the ATP-dependent, nonlysosomal degradative process could be measured in muscles under defined conditions in vitro. Fasting and denervation were found to stimulate the ATP-dependent process two- to three-fold in muscles, and this response accounted for the increase in overall proteolysis. After food removal, this ATP-dependent process increased progressively, especially in the pale fiber muscles, but fell to control levels within one day of refeeding.

More definitive evidence that the Ub-proteasome pathway was stimulated by denervation and fasting was the finding that levels of Ub-protein conjugates were increased in muscles at the same time that protein degradation was maximally accelerated. When the fasted rats were refed, proteolysis decreased, as did the levels of ubiquitinated proteins (Wing et al. 1995). The accumulation of Ub-conjugates is clear evidence that ubiquitination of cell proteins and the flux of substrates through this pathway are accelerated in the atrophying muscles. Additional evidence indicating an activation of this pathway was the observation that the levels of mRNAs encoding Ub and several different subunits of the 20S proteasome increased during fasting and denervation atrophy (Medina et al. 1991 and 1995). These mRNAs increased at the same time as the total RNA content in muscle decreased, and upon refeeding the fasted rats, Ub and proteasome RNA decreased when total RNA rose. Fasting also increases the mRNA encoding at least one Ub-conjugating enzyme, E214k (Wing and Goldberg 1993). In fact, there is evidence that this series of adaptations involves changes in gene expression. Results from a nuclear run-on experiment using nuclei isolated from muscle of rats with acute diabetes or chronic uremia showed that higher levels of mRNA could be attributed in part to increased transcription of genes encoding ubiquitin and the C3 subunit of the proteasome (Bailey et al. 1996, Price et al. 1996). Together, these findings strongly suggest a coordinated series of biochemical adaptations in the atrophying muscle that enhance the capacity of the Ub-proteasome pathway and lead to muscle wasting. More recently, direct evidence for the role of this pathway in muscle atrophy has come from experiments utilizing the proteasome inhibitors in isolated muscles (Bailey et al. 1996, Price et al. 1996, Tawa et al. 1997). In these studies, the increased proteolysis

### TABLE 1

**Experimental observations in models of muscle wasting**

<table>
<thead>
<tr>
<th>Observation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>ATP-dependent proteolysis in isolated muscles</td>
</tr>
<tr>
<td>2</td>
<td>Susceptibility of proteolysis to proteasome inhibitors</td>
</tr>
<tr>
<td>3</td>
<td>Ubiquitin content of muscles</td>
</tr>
<tr>
<td>4</td>
<td>Content of ubiquitin-protein conjugates</td>
</tr>
<tr>
<td>5</td>
<td>Ubiquitin mRNA (total mRNA falls)</td>
</tr>
<tr>
<td>6</td>
<td>mRNA for ubiquitin-carrier proteins (E2s)</td>
</tr>
<tr>
<td>7</td>
<td>mRNA of proteasome subunits</td>
</tr>
<tr>
<td>8</td>
<td>Ub-conjugation to muscle proteins</td>
</tr>
</tbody>
</table>

### TABLE 2

**Conditions that stimulate protein degradation by the ubiquitin-proteasome pathway**

<table>
<thead>
<tr>
<th>Human Disease</th>
<th>Experimental Model in Rats</th>
</tr>
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<tbody>
<tr>
<td>Eating Disorders</td>
<td>Fasting</td>
</tr>
<tr>
<td>Renal Tubular Defects, Diabetes</td>
<td>Metabolic Acidosis</td>
</tr>
<tr>
<td>Uremia</td>
<td>Partial Nephrectomy</td>
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<tr>
<td>Neuromuscular Disease</td>
<td>Muscle Denervation</td>
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<tr>
<td>Immobilization</td>
<td>Hind Limb Suspension</td>
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<td>Diabetes</td>
<td>Streptozotocin</td>
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<td>Burn Injuries</td>
<td>Administration</td>
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<tr>
<td>Sepsis</td>
<td>Thermal Injury</td>
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<tr>
<td>Sepsis</td>
<td>Cecal Puncture, Endotoxin Injection</td>
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<tr>
<td>Cancer Cachexia</td>
<td>Tumor Implantation</td>
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<tr>
<td>Hyperadrenocortisolism</td>
<td>Glucocorticoid</td>
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<tr>
<td>Hyperthyroidism</td>
<td>Administration</td>
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</tbody>
</table>
measured in muscles from diabetic or uremic rats (Bailey et al. 1996, Price et al. 1996) rats after denervation, or after induction of sepsis or hyperthyroidism (Tawa et al. 1997) was reduced to control levels upon the addition of MG132 to the bathing media.

These experimental approaches opened a new area of research and provided insights into the mechanisms of muscle protein loss seen in a number of other catabolic illnesses. In fact, in every experimental model studied, where there is rapid muscle wasting, a similar pattern of changes was found in the muscles. For example, metabolic acidosis induced in rats by feeding NH₄Cl was shown to suppress normal growth and stimulate protein degradation in muscle (May et al. 1986, Mitch et al. 1994). The same experimental strategy of sequentially blocking different proteolytic pathways in incubated muscles demonstrated that metabolic acidosis, like starvation and denervation, stimulated an ATP-dependent proteolytic pathway. Metabolic acidosis also caused an increase in the levels of mRNAs for Ub and two subunits of the 20S proteasome specifically in muscle (Mitch et al. 1994, Price et al. 1994). Similar findings were also made in muscles of acidotic rats with acute or chronic renal failure, both of which also stimulate muscle protein degradation (Bailey et al. 1996, Clark and Mitch 1983, May et al. 1987, Reaich et al. 1994). The accumulation of acid is critical in initiating these responses, since correction of the acidosis (at least in CRF) not only blocks the stimulation of muscle proteolysis, but also prevents the increase in mRNAs for Ub and proteasome subunits (Bailey et al. 1996).

Other conditions that cause a marked loss of lean body mass and muscle wasting in patients include sepsis, cancer, and burn injury. In experimental models that mimic these disease states in rats, strong evidence has been obtained for activation of the ATP-dependent proteolytic pathway. For example, muscle protein breakdown rises in rats within 6 hours after injection of endotoxin, live bacteria, or after puncture of the cecum to produce peritonitis and fulminant sepsis (Tiao et al. 1994). Presumably, this response has evolved to provide the infected organism with a source of amino acids for energy metabolism and synthesis of new proteins (e.g., the acute phase reactant proteins). Prolonged activation of muscle protein degradation, however, would contribute to the negative nitrogen balance, commonly associated with sepsis and AIDS (Clowes et al. 1976).

Patients with burns or other traumatic injuries experience a similar rapid loss of body protein primarily due to enhanced proteolysis in muscle (Mansoor et al. 1996). When a thermal injury is applied to the back of rats, the ATP-dependent degradative process is stimulated in leg muscles and there is as much as a 7-fold increase in the degradation of myofibrillar proteins and an increase in the level of Ub mRNA (Clark et al. 1984, Fang et al. 1995).

Inactivity can also promote muscle atrophy. This phenomenon is often seen in immobilized patients and those after prolonged bedrest, and most dramatically in astronauts after spaceflight. This atrophy has been modeled in hind-limb-suspended rats (Thomason and Booth 1990, Thomason et al. 1987), as well as in rats with denervated hind limbs (Furuno and Goldberg 1986). Muscle atrophy also has also been measured in rats exposed to microgravity (spaceflight) (Caiozzo et al. 1994 and 1996). As in the other types of atrophy discussed, the Ub-proteasome pathway appears responsible for the increased protein breakdown (Tawa et al. 1997, Wing et al. 1995 and S.L., V.S., K. Baldwin and A.G., unpublished observations).

A prominent feature of several types of cancer is cachexia. This profound loss of tissue mass in cancer cannot be explained simply by anorexia (Lawson et al. 1982). In fact, tumor-bearing rats show a greater loss of muscle protein and a higher rate of muscle proteolysis than rats fed an equal amount of calories (Baracos et al. 1995). Several studies indicate that transplantation of tumors into rats activates the Ub-proteasome pathway in muscle apparently via factors that act locally or systematically. For example, implantation of even a small amount of the rapidly growing Yoshida sarcoma into the leg muscle of rats produces atrophy of adjacent muscles, presumably because the tumor releases a locally active catabolic factor (Tempari et al. 1994). The muscle atrophy was due to an increase in the rates of protein degradation compared to rates measured in muscles of the contralateral leg. Studies with inhibitors of proteolytic pathways indicated there was increased activity of the ATP-dependent process, and there was an increase in the content of Ub mRNAs.

Similar responses in muscles were observed in studies of the effects of injecting Yoshida ascites hepatoma cells into the peritoneal cavity. Within several days, there was a rapid loss of body weight and muscle mass, even though the tumor accounted for less than 3% of body weight (Baracos et al. 1995). It is interesting that the weights of the kidney and liver did not decrease (in contrast to muscle weight). Food intake fell in these cachectic rats, but the increase in muscle protein breakdown exceeded that in control rats fed the same amount of calories and protein. The marked loss of muscle mass was due to activation of the ATP-dependent proteolytic system, although the capacity for lysosomal proteolysis also increased slightly and protein synthesis fell, contributing to the decrease in muscle mass. In the atrophying muscle, there were also coordinated increases in the mRNAs for Ub, and at least four subunits of the 20S proteasome, even though total RNA fell by 20%. Aside from muscle, skin was the only other tissue exhibiting an increase in these mRNAs. Finally, the content of Ub-conjugated proteins and certain proteasome subunits was increased in the muscles of these cachectic rats providing strong evidence for an acceleration of the Ub-proteasome pathway.

CONSERVATION OF MUSCLE CELL PROTEIN BY SUPPRESSION OF THE Ub-PROTEASOME PATHWAY

While many physiological and disease processes can stimulate the breakdown of proteins and amino acids in muscle, mammals also have adaptive mechanisms that conserve cell protein and essential amino acids. For example, when there is an inadequate intake of amino acids in the diet but adequate caloric intake, there are very different series of biochemical adaptations than are seen in catabolic states. Rats fed protein-deficient diets fail to grow normally, but they exhibit little or no loss of muscle mass for extended periods, in sharp contrast to fasted, acidic or tumor-bearing rats (Baracos et al. 1995, May et al. 1986 and 1991, Medina et al. 1995, Tawa and Goldberg 1992). In such rats or humans, protein turnover in muscle (and presumably in other tissues) is suppressed, as is the degradation of amino acids (May et al. 1991, Tawa and Goldberg 1992, Young 1986). Muscles of protein-deficient rats have a reduced capacity to degrade proteins, to synthesize new proteins and to oxidize branched-chain amino acids (May et al. 1991, Tawa and Goldberg 1992, 1994). This reduced proteolysis is associated with a suppression in muscle of the ATP-dependent proteolytic pathway, a decrease in the pro-
proteasomes) indicates that Ub conjugation to muscle atrophy (in the face of increased breakdown of such proteins Ub-protein conjugates observed in several types of muscle atrophy). Accordingly, the increase in the levels of their degradation is Ub conjugation (i.e., the step catalyzed by the E2-E3 pair). These findings indicate a special role in the regulation of protein turnover in skeletal muscle, and in muscle wasting.

FIGURE 3 125I-Ub conjugation to muscle proteins rises in extracts from tumor-bearing rat muscles. Shown are SDS-PAGE (left panel) and graphic representation (right panel) of 125I-Ub conjugation to endogenous proteins in soluble extracts from pair-fed control rats (C) and rats bearing Yoshida Ascites Hepatoma (T) for three days. Tumor implantation was performed as described previously (Baracos et al., 1995). Free Ub runs at the dye front and is not visible in this gel. Amount of radioactivity incorporated into high molecular weight conjugates was measured and plotted as a function of time. Ub = ubiquitin.

BIOCHEMICAL MECHANISMS OF MUSCLE ATRPHY

A full understanding of the biochemical basis for the acceleration of muscle protein catabolism in these catabolic states will require identification of the critical adaptations that accelerate flux of proteins through this pathway. Various studies of the breakdown of specific regulatory proteins, as described above, have indicated that the rate-limiting step in their degradation is Ub conjugation (i.e., the step catalyzed by an E2-E3 pair). Accordingly, the increase in the levels of Ub-protein conjugates observed in several types of muscle atrophy (in the face of increased breakdown of such proteins by proteasomes) indicates that Ub conjugation to muscle proteins is accelerated in these catabolic conditions. Because a general increase of protein ubiquitination might trigger the increased protein breakdown, it appeared important to measure directly the rates of Ub-conjugation in these different disease states.

Recently, we have developed cell-free preparations from rodent muscles in which the overall rate of Ub conjugation to endogenous muscle proteins changes in a manner correlating with overall proteolysis (Solomon et al. submitted). These studies have shown that the stimulation of muscle protein degradation in a wide variety of catabolic states, including cancer cachexia, sepsis, diabetes, chronic renal failure, hypothyroidism, and disuse induced by hind-limb suspension, is associated with an increase in Ub conjugation in the atrophying muscle (Solomon et al. submitted for publication). For example, in soluble extracts of atrophying muscles from cachectic rats bearing Yoshida hepatoma, rates of 125I-Ub conjugation to endogenous proteins were two- to three-fold faster than in control extracts (Fig. 3). These changes coincide with and account for the enhancement in overall proteolysis. In principle, increased ubiquitination could be due to a modification in cell proteins that enhances their susceptibility to certain E2s or E3s, or it may result from an activation of the ubiquitination system. In these extracts of atrophying muscle, the rates of ubiquitination of exogenously added 125I-lysosome also increased in a similar way. Therefore, in these muscles, accelerated ubiquitination seems to involve an activation or induction of enzymes for Ub-conjugation.

As discussed above, in certain nutritional and endocrine states, such as in hypothyroidism, overall protein breakdown in muscle decreases below normal. In extracts of muscles from hypothyroid rats induced by thyroidectomy or hypophysectomy, Ub-conjugation to soluble proteins as well as to exogenously added 125I-lysosome decreased by 50%, and these differences were reversed upon treatment of the hypothyroid animals for several days with T3 (Solomon et al. submitted). These observations are the first evidence that overall rates of Ub-conjugation in tissues are precisely controlled by hormones, as well as by cytokines and contractile activity, and that the activation of the protein breakdown in disease states is due primarily to changes in the enzymes for Ub conjugation, although additional adaptations are probably also occurring. Thus, the increase (or decrease) in these ubiquitination enzymes in muscle seems to represent a very general mechanism for enhancing (or suppressing) muscle proteolysis. These preparations have also enabled us to begin to define which ubiquitination enzymes are responsible for the more rapid conjugation in atrophying muscles. A number of our recent observations (Solomon et al. submitted) have indicated that E24k and E3α are responsible for ubiquitination of large fraction of muscle proteins normally. Moreover, this Ub-conjugation system becomes of even greater importance in these various catabolic states and is suppressed in muscles of hypothyroid animals. These ubiquitination enzymes had been in the selective elimination of abnormal proteins with unusual N-termini (the "N-end" pathway). However, these exciting findings indicate a special role in the regulation of protein turnover in skeletal muscle, and in muscle wasting.

ENDOCRINE SIGNALS REGULATING MUSCLE PROTEOLYSIS

In skeletal muscle, overall rates of protein breakdown vary with changes in nutrient supply, endocrine factors and the degree of contractile activity (Tawa and Goldberg 1994). For example, since the conversion of muscle protein to amino acids is as a reduction in content of lysosomal hydrolases (Tawa and Goldberg submitted). These metabolic responses appear to involve a suppression of thyroid function, and are similar to changes seen in experimentally induced hypothyroidism (Tawa and Goldberg submitted). These adaptations to protein deficiency resemble ones seen in humans in prolonged starvation or malnutrition. Humans have evolved physiological mechanisms that enable us to withstand little or no calorie intake for prolonged periods, primarily because there are large reserves of triglycerides in adipose tissue, which are an excellent source of energy. However, since continued loss of cell proteins is lethal, the capacity to mobilize protein from cells as a source of energy is quite limited and would be quickly reached in fasting if proteolysis were continued at an accelerated level. In the initial days of a fast or catabolic illness, protein reserves are mobilized and amino acids are metabolized for energy. After several days of food deprivation, this catabolic response ceases, and other mechanisms are activated to suppress muscle protein breakdown and amino acid oxidation, just as occurs with amino acid-deficient diets. In both prolonged fasting and protein deficiency, muscle protein conservation occurs concomitantly with a reduction in oxygen consumption and metabolic rate (Tawa and Goldberg 1994). Thus, the biochemical and physiological adaptations that suppress muscle protein degradation contrast sharply with those leading to protein loss and mobilization of amino acids in an acute fast or catabolic disease. 

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acids can be a major determinant of the rate of gluconeogenesis, muscle proteolysis is controlled by several glucoregulatory hormones, especially insulin and glucocorticoids. In denervated or immobilized muscles, events within the inactive cells must stimulate protein breakdown in the muscle, whereas in fasting, acidosis, sepsis, etc., muscles must respond to circulating hormones and cytokines.

A. Glucocorticoids and Insulin

One important factor activating these catabolic responses are glucocorticoids (Wing and Goldberg 1993, Wing et al. 1995). For example, the rise in muscle protein degradation in fasting rats fed NH$_4$Cl to induce metabolic acidosis requires glucocorticoids. If rats are adrenalectomized and then fed NH$_4$Cl (and reach the same degree of acidemia) or deprived of food, muscle proteolysis does not increase unless they are given replacement doses of dexamethasone (May et al. 1986). Administration of glucocorticoids to such animals is also necessary for the increase in levels of mRNAs encoding Ub and subunits of the proteasome in muscle (Price et al. 1994). In response to fasting, muscles of adrenalectomized rats (unlike those of normal rats) are not able to increase protein breakdown nor to increase the levels of Ub mRNA or of Ub-protein conjugates, and all of these coordinated responses are restored with glucocorticoids (Wing and Goldberg 1993). The glucocorticoids, through changes in muscle gene expression, therefore, allow proteolysis to rise and combat the challenge to energy homeostasis. A failure to produce glucocorticoids impairs the organism’s ability to maintain blood glucose in fasting or acute renal failure. An inability to enhance muscle proteolysis would reduce the supply of amino acids to other organs; in acidosis, increased supply of glutamine is necessary for the renal response to acidosis, and in fasting, increased release of amino acids from muscle is essential for hepatic gluconeogenesis.

This catabolic response to glucocorticoids is not seen in the fed state because of the high levels of insulin which inhibit the activation of proteolysis. Although addition of dexamethasone to muscles from adrenalectomized, fasted rats stimulates protein breakdown and the rise in Ub mRNA, the presence of insulin in incubation media blocks this response (Wing and Goldberg 1993). In the fed state, both suppress protein breakdown and enhance protein synthesis in muscle. Thus, the activation of muscle proteolysis in fasting and probably in diabetes (Price et al. 1996) seems to require two signals, glucocorticoids and a fall in levels of insulin. Furthermore, when glucocorticoids are administered in pharmacologic doses (as in the iatrogenic Cushing’s syndrome), the steroids can overcome the inhibitory effect of insulin and activate muscle protein breakdown and muscle wasting even in the fed state.

These effects of glucocorticoids are through a direct action on the muscle cells (Isozaki et al. 1996, May et al. 1986, Wing and Goldberg 1993). In myocytes, protein degradation was shown to increase when the pH of the culture media was reduced but only if dexamethasone was also added (Isozaki et al. 1996), and blockers of the proteolytic response were accompanied by increased levels of mRNAs encoding Ub and subunits of the proteasome, as occurs in muscles of rats during acidosis. Additional evidence for a key role of glucocorticoids was that a receptor antagonist that prevents activation of gene transcription by glucocorticoids (RU 486) blocked this response.

In both fasting and acidosis, glucocorticoids at physiological concentrations appear to play a “permissive role” in the catabolic response; they are necessary, but not sufficient to account for activation of proteolysis. Besides promoting proteolysis in muscle, glucocorticoids also inhibit protein synthesis by decreasing translation of mRNAs encoding muscle proteins and suppressing amino acid entry into muscle. Simultaneously, in the liver, glucocorticoids induce gluconeogenic enzymes that convert amino acids into glucose. Thus, the homeostatic function of glucocorticoids in fasting involves coordinated actions in muscle to mobilize amino acids and in the liver to produce glucose from these precursors.

B. Cytokines and other factors

In sepsis and certain cancers, activation of the Ub-proteasome pathway in muscle appears to be signaled by cytokines released from activated macrophages. When macrophages, phagocytose bacteria, endotoxin or antigen-antibody complexes, etc., they release circulating mediators, such as TNF and IL-1, which elicit host defense responses, including fever, increased production of acute phase proteins, leukocytosis, and muscle protein catabolism. Sepsis, certain types of cancer, and burn injury are all associated with the release of large amounts of TNF and other monokines (and glucocorticoids), and these mediators appear to function together to signal the associated muscle wasting. Experimentally, injection of large amounts of TNF can activate muscle proteolysis (Goodman 1991). However, TNF is known to cause anorexia, shock, and to release other monokines, including IL-1 and IL-6, making it difficult to identify the signal acting directly on the muscle. Direct action of any of these cytokines in isolated muscles to stimulate proteolysis has not been demonstrated (Flakoll et al. 1995). On the other hand, administration of pentoxifylline (an inhibitor of TNF release from macrophages) was shown to suppress the muscle proteolytic response to sepsis in rats (Breuille et al. 1993) and injections of an antibody against TNF suppressed the proteolytic response occurring in muscles of rats injected with tumor cells, treated to produce sepsis, or injected with endotoxin (Costelli et al. 1993, Zamir et al. 1992). Blocking IL-1 actions by injecting soluble IL-1 receptors, which serve as a decoy to bind cytokines, prevents activation of muscle protein breakdown in response to endotoxin (Attaix and Goldberg unpublished observations). It would appear, therefore, that both cytokines are essential for muscle protein catabolism during sepsis, and in fact, injection of TNF and IL-1 together under conditions where neither is effective alone, stimulates ATP-dependent muscle protein degradation and a rise in Ub mRNA. There also is evidence that overproduction of IL-6, as occurs in sepsis, can induce muscle proteolysis (Goodman 1994). The precise signal activating this process in sepsis is uncertain, but seems to involve TNF as well as other inflammatory mediators.

There may exist other factors in disease states that stimulate excessive proteolysis. For example, a proteoglycan has been isolated from a murine adenocarcinoma that appears to cause weight loss and stimulate proteolysis in skeletal muscle (Todorov et al. 1996). An immunologically similar material has been reported in the urine from humans with a variety of different neoplasms (Cariuk et al. 1997), but these reports await independent confirmation.

C. Thyroid Hormone

As discussed above, another class of hormones that appears to be important in the normal regulation of muscle protein breakdown is the thyroid hormones. Following thyroideectomy or hypophysectomy, rats exhibit a decrease in muscle protein degradation but administration of T3 or thyroxine (T4) stim-
ulates overall protein catabolisim in muscle to normal or excessive levels (N. Tawa, unpublished data). When given experimentally in high doses, or in patients with hyperthyroidism, thyroid hormones cause excessive proteolysis and a loss of muscle mass, and administration of T3 has been found to raise the content of proteasomes in muscle (as well as lysosomal proteases) and to increase the ATP-dependent proteolytic process (as well as lysosomal proteolysis) in muscle (Tawa et al. 1997).

On the other hand, when T3 production falls, there is reduced activity of the Ub-proteasome pathway in muscle, as well as other adaptations that help to conserve muscle protein mass (e.g., reduced proteasome content). Similar changes are seen in muscles of rats fed a low-protein diet and such animals have reduced thyroid function. Clinically, individuals fed a very-low-protein diet (but adequate calories) also exhibit a reduction in T3 levels, and there is a decrease in overall protein degradation (Young 1986). As noted above, a reduced thyroid status also undermines the adaptations in muscle that occur in prolonged fasting, and these appear opposite to those occurring in highly cachetic states. Since alternative sources of calories are available in such conditions, suppression of muscle proteolysis would help preserve body protein and essential amino acids.

APPLICATIONS IN DRUG DEVELOPMENT

The recent advances in knowledge about the Ub-proteasome pathway have led to real progress in our understanding of the biochemical mechanisms of muscle wasting. It is now clear that the loss of body weight and muscle protein in a wide variety of clinical diseases involves a very similar series of adaptations in the muscle leading to accelerated protein breakdown. These developments, however, are not only of importance for pathophysiology but also should have practical applications. Clearly, the methods described here for measuring proteolysis in isolated muscles and the series of biochemical changes characteristic of atrophying tissue (e.g., the increases in Ub or proteasome subunit mRNA and especially in changes in Ub conjugation) should also prove useful in drug development (e.g., in allowing the precise monitoring of experimental models of human disease). In addition, many of these methods which we developed for studies of rat or mouse tissues are applicable to human muscle biopsies.

More importantly, these various discoveries about muscle wasting also make it likely that inhibition of the Ub-proteasome pathway or its activation represents an attractive new approach for the rational treatment of cachexia and negative nitrogen balance. In recent years, our laboratory and others have identified small molecules that can inhibit selectively the active sites of the proteasome (Coux et al. 1996, Goldberg et al. 1995b, Rock et al. 1994). Because a number of these inhibitors can readily enter cells and inhibit the proteasome-mediated degradative pathway, these agents have proven highly useful for basic investigations of the importance of the Ub-proteasome pathway in diverse cellular processes. In vitro, peptide aldehyde inhibitors of the proteasome have also been shown to inhibit selectively the increased protein breakdown seen in muscle atrophy due to denervation, sepsis or acidosis. In addition to providing further evidence for the critical role of this pathway in muscle atrophy, these findings raise the possibility that pharmacological inhibition of this process might be useful in combatting the progressive wasting and debilitation seen in these cachetic diseases. Already, certain proteasome inhibitors are eliciting appreciable interest as potential human therapeutic agents, because they have potent anti-inflammatory effects (e.g., by blocking iκB) and anti-cancer effects (e.g., by affecting cell cycle regulation). Because of the pleiotropic effects of inhibiting the proteasome, they may not be appropriate for treatment of muscle wasting. A more interesting pharmacological target would be the development of inhibitors of protein ubiquitination in muscle. Our recent discovery that certain E2s and E3s are especially important in muscle atrophy suggests that their inhibition may have particular therapeutic potential. Clearly, greater knowledge about the acceleration of proteolysis in these states may well prove of major medical benefit in the future.

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