Inhibition of Proteasome Activity by Selected Amino Acids

Frederick G. Hamel, Jennifer L. Upward, Gerri L. Siford, and William C. Duckworth

Cellular protein homeostasis is a balance between synthesis and degradation. Protein degradation is regulated by hormones (eg, insulin) and nutrients (eg, amino acids). Certain amino acids are capable of decreasing cellular protein degradation, with evidence that this is mediated through altered lysosomal function. However, proteasomes, the major cytosolic protein degrading machinery, are being shown to play a central role in the control of protein turnover in the cell. In this study we show that the amino acids, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, lysine, and arginine are capable of inhibiting the chymotrypsin-like activity of the proteasome in a dose-dependent manner. Leucine, tyrosine, and phenylalanine have a substantial effect at normal serum concentrations. The effect was greater in a proteasome preparation derived from muscle compared to a similar preparation from liver. On the assumption that amino acid–induced alterations in cellular protein degradation reflect the inhibitory changes in proteasomal activity shown here, we may conclude that amino acid control of cellular protein degradation is mediated, at least in part, through proteasomes.

THE CONTROL and the mechanisms of cellular protein degradation are complex. Mortimore and colleagues conducted and published an extensive series of studies of cellular protein turnover.[1-6] Among many other findings they demonstrated inhibition of protein degradation by insulin and amino acids with interactions among these regulators. Of interest was that not all amino acids have equal effects, but the overall effect of a mixture of amino acids can be duplicated by a small selected group of amino acids.[2,3,5-8] Leucine has the greatest inhibitory effect, producing 20% to 50% of the total activity of a combination of amino acids. In studies from several groups insulin has approximately the same overall effect as a mixture of all amino acids at 5 times the normal serum concentrations.[1,8,9] The effects are not additive but at normal serum amino acid concentrations an insulin effect is evident.[4] Insulin has less effect on cellular protein degradation in the total absence of amino acids.[10] These data suggest a common site of action for insulin and amino acids on protein degradation.

Traditionally, lysosomes have been considered the primary cellular site of protein degradation. These organelles are clearly involved in many types of degradation ranging from removal of endocytosed material to autophagy of cytoplasm in starvation.[11,12] Recent work, however, has suggested that for selective cellular protein degradation proteasomes may be more important (eg, the ubiquitin system).[13-15] The role of insulin in control of proteasome activity has been controversial since Mortimore and Poso showed an effect of insulin on lysosomal autophagy,[2,3] but we recently showed a direct effect of this hormone on proteasome activity in intact cells.[16,17] Previously we had shown that insulin inhibited proteasome activity directly in an in vitro partially purified proteasome preparation.[18,19]

In the present study, we report the effect of individual amino acids and combinations of amino acids on in vitro proteasome activity. As in intact cells, selected amino acids inhibit proteasome activity with differences between proteasomes derived from liver and proteasomes derived from muscle.

MATERIALS AND METHODS

Crystalline human insulin was provided by Dr Ronald Chance (Lilly Research Laboratories, Indianapolis, IN). Enzyme-grade ammonium sulfate was purchased from ICN Biomedicals (Irvine, CA). The fluorogenic peptides succinyl-leu-leu-val-tyr-7-amido-4-methyl coumarin (LLVY) and boc-leu-ser-thr-arg-7-amido-4-methyl coumarin (LSTR), the proteasome inhibitor acetyl-leu-leu-norleucinal (ALLN, calpain inhibitor I), and the amino acids were obtained from Sigma (St Louis MO). The proteasome inhibitor N-boc-ile-glu(*O-t*-butyl)-ala-leucinal (PSI) was from Peptide International (Louisville, KY). All other chemicals were reagent grade or better.

Partially purified insulin-degrading enzyme/multicatalytic protease was prepared from male rat (Sprague-Dawley) muscle or liver by ultracentrifugation and ammonium sulfate precipitation similar to that described previously.[20] Hind limb skeletal muscle or liver was homogenized in 0.35 mol/L sucrose (5 mL/g of tissue) in a blender for 90 seconds. The homogenate was centrifuged at 13,000 × *g* for 15 minutes. The supernatant fraction was then centrifuged at 100,000 × *g* for 1 hour and the pellet discarded. The supernatant was fractionated with 30% ammonium sulfate (0.21 g NH_4SO_4 per mL of supernatant) with the pH maintained at 6.2. The suspension was centrifuged at 15,000 × *g* for 20 minutes, and the precipitate discarded. The soluble fraction was then treated with 60% ammonium sulfate, centrifuged as before. The precipitate was dissolved in 20 mmol/L sodium acetate, pH 6.2 at 1/15 the original homogenate volume. The solution was dialyzed against at least 20 vol of 20 mmol/L sodium acetate, pH 6.2, overnight with 3 changes of the dialysis fluid. The preparation was then frozen and stored until use.

The degradation of the fluorogenic peptides was performed as described previously.[19] The enzyme sample was incubated with 13μmol/L LLVY or LSTR in 1 mL of 100-mmol/L Tris, pH 7.5, with additions as noted, for 1 hour (except for the time course experiment). For the kinetics experiments, the LLVY concentration was varied from 7.0 to 67.0 μmol/L. The reaction was stopped with 0.2 mL of ice-cold ethanol and activity expressed as by the change in fluorescence over time (excitation. and emission wavelengths of 390 nm and 440 nm, respectively).

From the Research Service, Department of Veterans Affairs Medical Center, Omaha, NE; Departments of Internal Medicine and Pharmacology, University of Nebraska Medical Center, Omaha, NE; and the Carl T. Hayden Medical Center, Department of Veterans Affairs, Phoenix, AZ.

Submitted March 18, 2002; accepted February 11, 2003.

Supported by the Medical Research Service of the Department of Veterans Affairs, and the Bly Memorial Research Fund.

Address reprint requests to Frederick G. Hamel, PhD, Department of Veterans Affairs Medical Center, 4101 Woolworth Ave,Omaha, NE68105

0026-0495/03/5207-0006$30.00/0

doi:10.1016/S0026-0495(03)00094-5

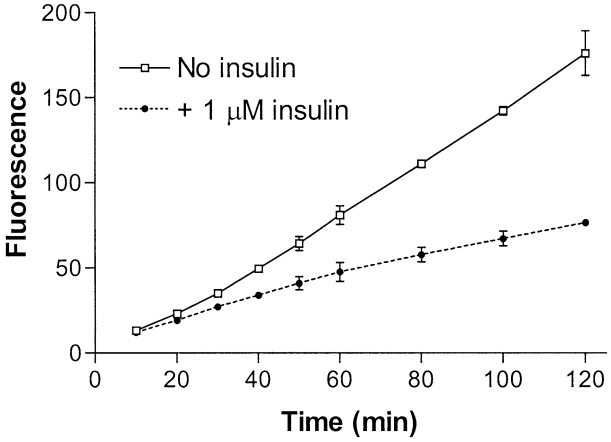

Fig 1. Time course of LLVY degradation by skeletal muscle proteasome preparation. Data are means ± SEM (n = 2). The lines are linear out to 2 hours (no insulin: $R^2 = 0.997$; with insulin: $R^2 = 0.989$).

All data are shown as the mean ± SEM. Statistical significance was determined by analysis of variance (ANOVA) with a Dunnett's multiple comparison test using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). The kinetic data were analyzed by fitting the data with a nonlinear regression to the Michaelis-Menton equation (per GraphPad Prism) and then plotting the lines based on the derived V_{max} and K_m parameters.

RESULTS

Figure 1 demonstrates that under our assay conditions degradation of proteasome peptide substrates is linear with respect to time for at least 2 hours. It also shows, as we have previously demonstrated,[19] that insulin inhibits this activity without any lag period. This indicates that the insulin inhibition is not the result of the production of competing hormone fragments or amino acids generated by insulin degradation. Subsequent assays were done using 1-hour incubations.

Proteasome activity is altered by selected amino acids. Figure 2 shows the effect of various amino acids on LLVY (Fig 2A) and LSTR (Fig 2B) degradation by proteasome preparations from muscle. Amino acids were used at concentrations selected to be 5 times those found in normal plasma, to screen for inhibitory activity. Inhibition of LLVY degradation was seen with leucine, phenylalanine, tryptophan, lysine, and arginine. Isoleucine and tyrosine showed a similar magnitude of inhibition, but did not achieve statistical significance. Histidine appeared to stimulate the proteasome, while the remaining amino acids had no effect. Inhibition of LSTR degradation was apparent for all the amino acids, but only tryptophan and lysine showed statistically significant inhibition.

Figure 3 shows similar studies with liver proteasome preparations. Hepatic organelles had significantly different responses compared to muscle proteasomes. A similar pattern is seen on the graph, but none were statistically significant. Lysine was the only amino acid to show inhibition of LSTR degradation. The maximal response of liver to an individual amino acid (lysine) was a 21% inhibition, whereas in muscle phenylalanine produced a 40% reduction. Thus, the muscle proteasome preparations are more sensitive to amino acids than are the liver proteasome preparations.

The above studies were all done at 5 times normal plasma concentration of the individual amino acids. Figure 4 shows dose-response curves of the active amino acids on proteolysis by isolated muscle proteasomes. Phenylalanine, leucine, tryptophan, and tyrosine suppressed proteolysis in a dose-dependent fashion with effective suppression even at normal serum concentrations, which are comparable to intramuscular concentrations.[21,22] Isoleucine, lysine, and arginine also were effective inhibitors, but generally at higher concentrations.

Figure 5 shows a Lineweaver-Burk plot of LLVY degrading activity inhibited with tryptophan. Increased tryptophan concentrations cause an increase in the K_m with the V_{max} staying essentially constant. Similar results were seen with leucine as the inhibiting amino acid (data not shown). These data indicate that the amino acids inhibit in a competitive manner.

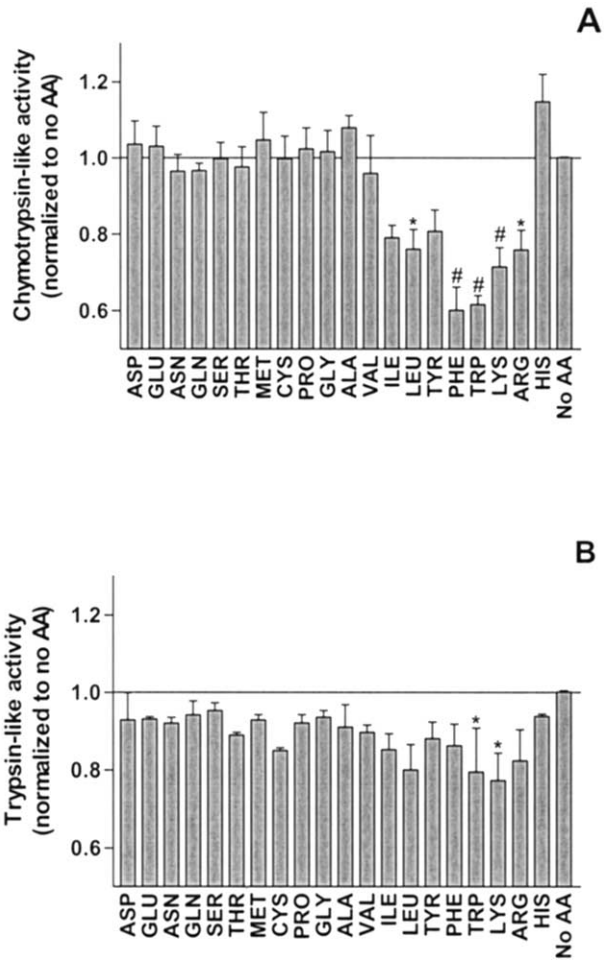

Fig 2. The effect of amino acids on skeletal muscle proteasome activity. The chymotrypsin-like (A) and trypsin-like (B) activities of the proteasome are shown in the absence and presence of 5 times the normal serum concentration of each of the 20 amino acids. Data are means ± SEM (LLVY degradation, n = 4; LSTR degradation, n = 3; *$P < .05$, #$P < .001$). The line indicates the activity in the absence of amino acids and is shown for clarity.

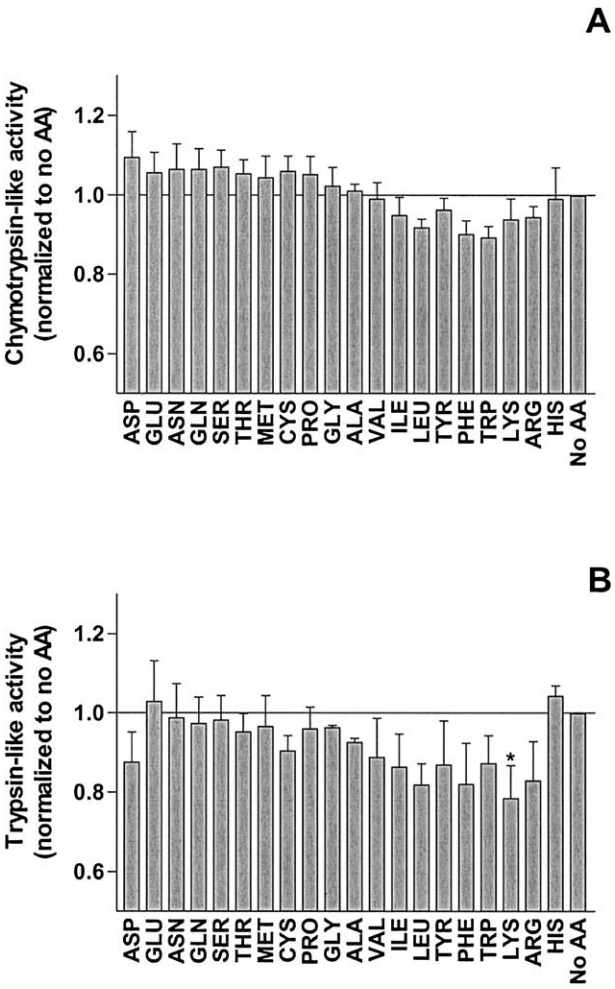

Fig 3. The effect of amino acids on liver proteasome activity. The chymotrypsin-like (A) and trypsin-like (B) activities of the proteasome are shown in the absence and presence of 5 times the normal serum concentration of each of the 20 amino acids. Data are means ± SEM (LLVY degradation, n = 4; LSTR degradation, n = 3; *$P < .05$). The line indicates the activity in the absence of amino acids and is shown for clarity.

DISCUSSION

Protein homeostasis is a balance between protein synthesis and protein degradation, which are independent processes with different regulatory systems. A great deal of information has been obtained about the mechanism of protein synthesis and its regulation for both individual and total cell protein. Relatively less is known about protein degradation. Overall cellular protein degradation is controlled by several different mechanisms. Hormones, especially insulin and glucocorticoids, and nutrients (ie, amino acids), are the 2 major regulators. Lysosomes and proteasomes are the 2 major organelles involved. Lysosomes are involved in many types of degradation ranging from removal of endocytosed material to autophagy of cytoplasm in starvation.[11,12] However, the proteasome is assuming a central role in cellular degradation as more information is being obtained.[13,15]

The proteasome is a cytosolic organelle with multiple proteolytic activities, including chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activities.[23-27] The fundamental unit is a barrel-shaped 20S (700 kd) structure that can associate with an adenosine triphosphate (ATP0-independent activator called PA28 (or 11S regulator). The activity of the core particle can be regulated by controlling substrate entry or exit from the catalytic center.[28,29] The 20S proteasome can also associate with the 19S regulator (or PA700) resulting in the 26S proteasome (~2.5 million kd), which is ubiquitin= and ATP-dependent. Altered or abnormal proteins, as well as some individual proteins, are targeted for degradation by the ubiquitin-dependent pathway.[30,31] The assays used in our study cannot distinguish between the various forms.

Consistent with proteasomes as the central proteolytic mechanism, hormones and nutrients have direct regulatory effects on the proteolytic organelles. We have shown here (Fig 1) and previously that insulin has direct inhibitory action on isolated proteasomes and on proteasome activity in intact cells.[16-19]

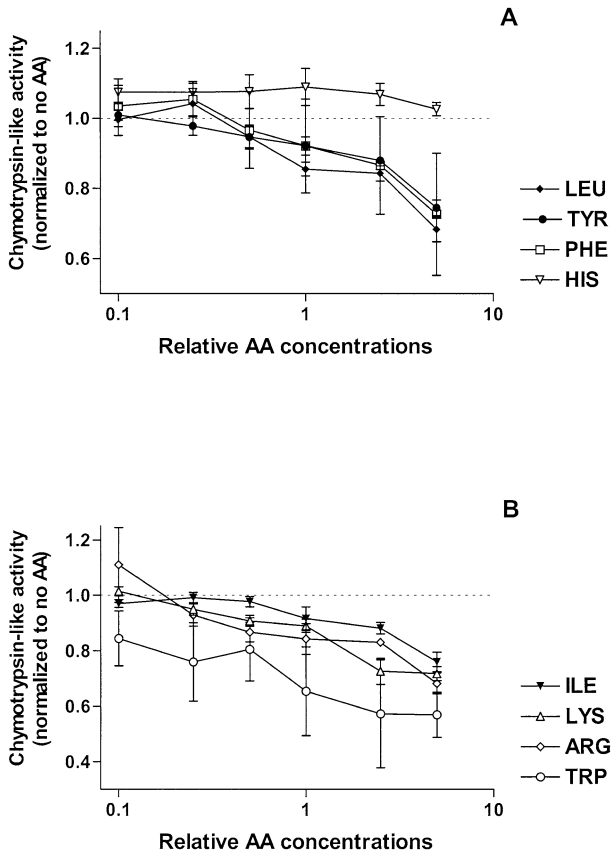

Fig 4. Dose-response of selected amino acids to inhibit proteasome activity. The graphs show the dose-dependent inhibition of the chymotrypsin-like activity of the proteasome from muscle. The x-axis shows the relative amino acid concentration with the normal serum concentration of each amino acid set at 1. Data are normalized to proteasome activity in the absence of amino acids, which is indicated by the dotted line. (A) Leu, tyr, and phe, as well as histidine, which had a slight stimulatory effect. (B) Ile, lys, arg, and trp. Data are means ± SEM (n = 3).

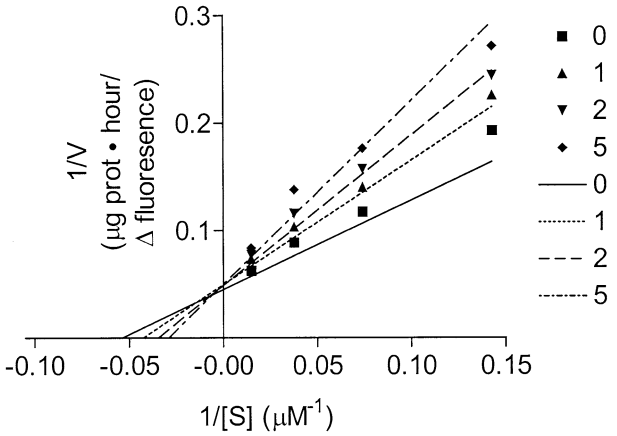

Fig 5. Lineweaver-Burk plot of tryptophan inhibition of the chymotrypsin-like activity of the proteasome from muscle. Data shown represent the means of 5 experiments without error bars shown for clarity. The lines show the results of nonlinear regression of the data from a substrate-velocity plot redrawn on a double reciprocal plot.

Conversely, glucocorticoids have been shown to activate the proteasome.[32] Thus, hormonal control of protein degradation can be mediated through proteasome effects. Nutrient effects on proteasomes have not previously been examined.

In this report we show (Figs 2 through 4) that selected amino acids have a direct, dose-dependent inhibitory effect on proteasome activity, at concentrations equivalent to intramuscular concentrations.[21,22] The effective amino acids are consistent with the amino acids reported by Mortimore and others to have inhibitory effects on overall cellular protein degradation,[2,3,5-8] which was attributed to lysosomal effects. While the correspondence is not complete, leucine, phenylalanine, tyrosine, and certain other amino acids are effective in the whole animal and in isolated proteasomes. Thus, under physiological conditions, amino acids may work by modifying proteasome activity as much or more than any effect they have on lysosomes. The competitive nature of the inhibition (Fig 5) would suggest the inhibition to be important when the cells have a relative abundance of amino acids. Competitive inhibition also suggest that the amino acids may be working by interfering with the active site, or entry into the catalytic core. Inhibitor studies using PSI or ALLN (data not shown) indicated that the amino acids are proteasome-specific since, in general, they did not have additional effects on degradation. However, because ALLN is also an inhibitor of calpains, we cannot exclude the possibility that there was some effect on those proteases as well.

The difference between muscle and liver proteasomes (Figs 2 and 3) is of considerable interest. Both insulin and the effective amino acids have greater effects on muscle than on liver, with some additional qualitative differences. The different responses may be a reflection of different distribution of the 20S proteasome subtypes present, the relative association of PA28 or 19S regulatory modules, the phoshorylation state of the subunits, or the subcellular distribution (and thus presence in our preparation) in the 2 tissue preparations.[27,33-36] The various forms of the proteasome may have different sensitivities to hormonal and nutritional regulation. Since protein breakdown in muscle and in liver serve different physiological roles, some differences would be expected.

Insulin and amino acids have similar inhibitory effects on cellular protein breakdown. Both have been implicated in lysosomal activity in intact cells, but recent studies have suggested that prelysosomal (cytosolic) actions are more important in insulin action.[37] While the physiological mechanisms remain uncertain, in general, these studies of proteasome inhibition by amino acids and insulin support similar processes. Extrapolation from previous studies in combination with the present one suggests that both insulin and selected amino acids may alter proteasome activity through a common mechanism, such as substrate availability to the active site, and be dependent on proteasome subtype. This could explain differences in the sensitivity of protein degradation of various tissues and cell types.

REFERENCES

1. Lardeux BR, Mortimore GE: Amino acid and hormonal control of macromolecular turnover in perfused rat liver. Evidence for selective autophagy. J Biol Chem 262:14514-14519, 1987

2. Mortimore GE, Poso AR: The lysosomal pathway of intracellular proteolysis in liver: regulation by amino acids. Adv Enzyme Regul 25:257-276, 1986

3. Mortimore GE, Poso AR: Intracellular protein catabolism and its control during nutrient deprivation and supply. Annu Rev Nutr 7:539-564, 1987

4. Mortimore GE, Poso AR, Kadowaki M, et al: Multiphasic control of hepatic protein degradation by regulatory amino acids. J Biol Chem 262:16322-16327, 1987

5. Poso AR, Mortimore GE: Requirement for alanine in the amino acid control of deprivation- induced protein degradation in liver. Proc Natl Acad Sci USA 81:4270-4274, 1984

6. Poso AR, Wert JJ Jr, Mortimore GE: Multifunctional control of amino acids of deprivation-induced proteolysis in liver. Role of leucine. J Biol Chem 257:12114-12120, 1982

7. Seglen PO, Gordon PB: Amino acid control of autophagic sequestration and protein degradation in isolated rat hepatocytes. J Cell Biol 99:435-444, 1984

8. Seglen PO, Gordon PB, Poli A: Amino acid inhibition of the autophagic/lysosomal pathway of protein degradation in isolated rat hepatocytes. Biochim Biophys Acta 630:103-118, 1980

9. Peavy DE, Edmondson JW, Duckworth WC: Selective effects of inhibitors of hormone processing on insulin action in isolated hepatocytes. Endocrinology 114:753-760, 1984

10. Flakoll PJ, Kulaylat M, Frexes-Steed M, et al: Amino acids augment insulin's suppression of whole body proteolysis. Am. J. Physiol 257:E839-847, 1989

11. Blommaart EF, Luiken JJ, Meijer AJ: Autophagic proteolysis: Control and specificity. Histochem J 29:365-385, 1997

12. Seglen PO, Bohley P: Autophagy and other vacuolar protein degradation mechanisms. Experientia 48:158-172, 1992

13. Goldberg AL, Akopian TN, Kisselev AF, et al: New insights into the mechanisms and importance of the proteasome in intracellular protein degradation. Biol Chem 378:131-140, 1997

14. Tanaka K: Proteasomes: Structure and biology. J Biochem (Tokyo) 123:195-204, 1998

15. Wilkinson KD: Roles of ubiquitinylation in proteolysis and cellular regulation. Annu Rev Nutr 15:161-189, 1995

16. Duckworth WC, Bennett RG, Hamel FG: Insulin acts intracel-

lularly on proteasomes through insulin-degrading enzyme. Biochem Biophys Res Commun 244:390-394, 1998

17. Hamel FG, Bennett RG, Harmon KS, et al: Insulin inhibition of proteasome activity in intact cells. Biochem Biophys Res Commun 234:671-674, 1997

18. Bennett RG, Hamel FG, Duckworth WC: Characterization of the insulin inhibition of the peptidolytic activities of the insulin-degrading enzyme-proteasome complex. Diabetes 46:197-203, 1997

19. Duckworth WC, Bennett RG, Hamel FG: A direct inhibitory effect of insulin on a cytosolic proteolytic complex containing insulin degrading enzyme and multicatalytic proteinase. J Biol Chem 269: 24575-24580, 1994

20. Duckworth WC, Heinemann M, Kitabchi AE: Purification of insulin specific protease by affinity chromatography. Proc Natl Acad Sci USA 69:3698-3702, 1972

21. Engelen MP, Wouters EF, Deutz NE, et al: Factors contributing to alterations in skeletal muscle and plasma amino acid profiles in patients with chronic obstructive pulmonary disease. Am J Clin Nutr 72:1480-1487, 2000

22. Ebeling P, Tuominen JA, Laipio ML, et al: Carbohydrate depletion has profound effects on the muscle amino acid and glucose metabolism during hyperinsulinaemia. Diabetes Obes Metab 3:113-120, 2001

23. Rechsteiner M, Hoffman L, Dubiel W: The multicatalytic and 26S proteases. J Biol Chem 268:6065-6068, 1993

24. Coux O, Tanaka K, Goldberg AL: Structure and functions of the 20S and 26S proteasomes. Annu Rev Biochem 65:801-847, 1996

25. Voges D, Zwickl P, Baumeister W: The 26S proteasome: A molecular machine designed for controlled proteolysis. Annu Rev Biochem 68:1015-1068, 1999

26. Orlowski M, Wilk S: Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex. Arch Biochem Biophys 383:1-16, 2000

27. Cardozo C, Eleuteri AM, Orlowski M: Differences in catalytic activities and subunit pattern of multicatalytic proteinase complexes (proteasomes) isolated from bovine pituitary, lung, and liver. Changes in LMP7 and the component necessary for expression of the chymotrypsin-like activity. J Biol Chem 270:22645-22651, 1995

28. Kohler A, Bajorek M, Groll M, et al: The substrate translocation channel of the proteasome. Biochimie 83:325-332, 2001

29. Hill CP, Masters EI, Whitby FG: The 11S regulators of 20S proteasome activity. Curr Top Microbiol Immunol 268:73-89, 2002

30. Hochstrasser M: Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr Opin Cell Biol 7:215-223, 1995

31. Kornitzer D, Ciechanover A: Modes of regulation of ubiquitin-mediated protein degradation. J Cell Physiol 182:1-11, 2000

32. Wing SS, Goldberg AL: Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. Am J Physiol 264:E668-676, 1993

33. Noda C, Tanahashi N, Shimbara N, et al: Tissue distribution of constitutive proteasomes, immunoproteasomes, and PA28 in rats. Biochem Biophys Res Commun 277:348-354, 2000

34. Dahlmann B, Ruppert T, Kuehn L, et al: Different proteasome subtypes in a single tissue exhibit different enzymatic properties. J Mol Biol 303:643-653, 2000

35. Rivett AJ, Bose S, Brooks P, et al: Regulation of proteasome complexes by gamma-interferon and phosphorylation. Biochimie 83: 363-366, 2001

36. Ferrell K, Wilkinson CR, Dubiel W, et al: Regulatory subunit interactions of the 26S proteasome, a complex problem. Trends Biochem Sci 25:83-88, 2000

37. Barrett EJ, Jahn LA, Oliveras DM, et al: Chloroquine dose not exert insulin-like actions on human forearm muscle metabolism. Am J Physiol 268:E820-E824, 1995