Insulin inhibition of the proteasome is dependent on degradation of insulin by insulin-degrading enzyme

R G Bennett[1,2], J Fawcett[3,4], M C Kruer[3], W C Duckworth[3,4,5] and F G Hamel[1,2]

[1]Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska, USA
[2]Department of Medical Research, Veterans Affairs Medical Center, Omaha, Nebraska, USA
[3]Endocrinology Section, Carl T Hayden VA Medical Center, Phoenix, Arizona, USA
[4]Molecular and Cellular Biology Program, Arizona State University, Tempe, Arizona, USA
[5]Department of Medicine, University of Arizona, Tucson, Arizona, USA
(Requests for offprints should be addressed to R G Bennett; Email: rgbennet@unmc.edu)

Abstract

A consequence of insulin-dependent diabetes mellitus is the loss of lean muscle mass as a result of accelerated proteolysis by the proteasome. Insulin inhibition of proteasomal activity requires interaction with insulin-degrading enzyme (IDE), but it is unclear if proteasome inhibition is dependent merely on insulin–IDE binding or if degradation of insulin by IDE is required. To test the hypothesis that degradation by IDE is required for proteasome inhibition, a panel of insulin analogues with variable susceptibility to degradation by IDE binding was used to assess effects on the proteasome. The analogues used were [Lys^{B28}, Pro^{B29}]-insulin (lispro), [Asp^{B10}]-insulin (Asp^{B10}) and [Glu^{B4}, Gln^{B16}, Phe^{B17}]-insulin (EQF). Lispro was as effective as insulin at inhibition of degradation of iodine-125 (^{125}I)-labeled insulin, but Asp^{B10} and EQF were somewhat more effective. All agents inhibited cross-linking of ^{125}I-insulin to IDE, suggesting that all were capable of IDE binding. In contrast, although insulin and lispro were readily degraded by IDE, Asp^{B10} was degraded more slowly, and EQF degradation was undetectable. Both insulin and lispro inhibited the proteasome, but Asp^{B10} was less effective, and EQF had little effect. In summary, despite effective IDE binding, EQF was poorly degraded by IDE, and was ineffective at proteasome inhibition. These data suggest that insulin inhibition of proteasome activity is dependent on degradation by IDE. The mechanism of proteasome inhibition may be the generation of inhibitory fragments of insulin, or by displacement of IDE from the proteasome.

Journal of Endocrinology (2003) **177,** 399–405

Introduction

Insulin-dependent diabetes mellitus (IDDM) is characterized by loss of lean muscle mass caused by increased proteolysis (Smith *et al.* 1989). The protein loss occurring in IDDM is reversible with insulin treatment (Rooyackers & Nair 1997, Grizard *et al.* 1999). Although insulin induces synthesis of some proteins, the increase in cellular protein induced by the hormone is attributable mostly to a decrease in the rate of overall cellular proteolysis (Gelfand & Barrett 1987). The major contributor to protein loss in IDDM is an increase in the activity of the proteasome (Price *et al.* 1996, Merforth *et al.* 1999, Galban *et al.* 2001). The proteasome is a macromolecular complex that is responsible for degradation through the energy-dependent ubiquitin pathway (Coux *et al.* 1996). The increased proteasome activity is suppressible with insulin treatment, suggesting that the proteasome pathway is the primary target of the anti-catabolic effect of insulin (Rooyackers & Nair 1997, Grizard *et al.* 1999, Galban *et al.* 2001). One contributing mechanism may be altered expression of mRNA for proteins involved in the ubiquitin pathway of protein degradation, such as proteasome subunits, ubiquitin and ubiquitin-conjugating enzymes (Wing & Banville 1994, Price *et al.* 1996, Lecker *et al.* 1999). However, these changes in the various mRNA species have not been consistently observed or always correlated with changes in proteasome activity (Larbaud *et al.* 1996, 2001, Wing & Bedard 1996). In addition, there is evidence that insulin can decrease the catalytic activity of the proteasome: it has been found to inhibit the peptide-degrading activity of the proteasome in cell-free extracts and in intact cells (Duckworth *et al.* 1994, 1998*a*, Hamel *et al.* 1997, Li *et al.* 2000). Furthermore, this same inhibition by insulin was seen when the ATP- and ubiquitin-dependent activity of the 26S form of the proteasome was measured both in a cell-free system and in cell culture (Bennett *et al.* 2000*b*).

The ability of insulin to inhibit proteasome activity requires the presence of insulin-degrading enzyme (IDE).

0022–0795/03/0177–399

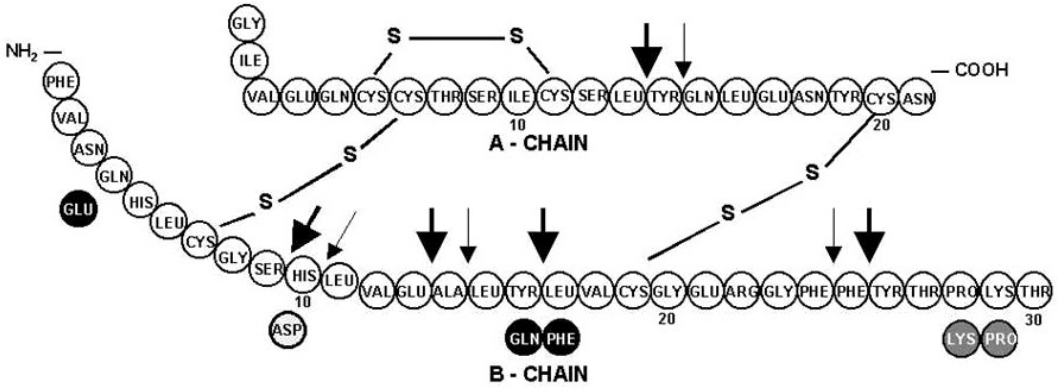

Figure 1 Sequence of human insulin and the substituted amino acids in the analogues used in the study. Amino acid substitutions are shown for lispro (gray), Asp^{B10} (white), and EQF (black). The primary and secondary IDE cleavage sites are indicated by the heavy and light arrows respectively.

IDE and the proteasome co-purify through a number of chromatographic steps (Duckworth *et al.* 1994, Bennett *et al.* 2000*b*). When IDE is either removed from the proteasome or inhibited, insulin has no influence on proteasome activity (Duckworth *et al.* 1994, 1998*a*, Hamel *et al.* 1998). In addition to insulin, other IDE substrates, such as atrial natriuretic peptide and relaxin, also inhibit the proteasome (Bennett *et al.* 1997). A key observation in this study was that proinsulin also inhibited the proteasome, but less effectively than insulin. It was shown previously that, although proinsulin binds to IDE with an affinity similar to that of insulin, it is itself a poor substrate for degradation by IDE, being degraded much more slowly than insulin (Duckworth *et al.* 1972). This suggests that binding to IDE is insufficient to induce proteasome inhibition, and that IDE must degrade the substrate to result in proteasome inhibition.

To explore the relationship between IDE binding and degradation and the ability of insulin to inhibit proteasome activity, we used a panel of insulin analogues, all based on biosynthetic human insulin (Fig. 1). The [Asp^{B10}]-insulin (Asp^{B10}) and [Glu^{B4}, Gln^{B16}, Phe^{B17}]-insulin (EQF) analogues were developed as potential 'long-acting' insulin replacements (Slieker *et al.* 1992). Both Asp^{B10} and EQF contain amino acid substitutions at key sites of cleavage by IDE, and thus were selected for probable resistance to degradation by IDE. The analogue [Lys^{B28}, Pro^{B29}]-insulin (lispro) has increased solubility compared with native insulin (DiMarchi *et al.* 1994). Lispro has no substitutions at IDE cleavage sites, and was included for comparison with Asp^{B10} and EQF. In this study, it was found that these analogues bind to IDE, but differ in their ability to be degraded by IDE. This approach allowed distinction between the ability of insulin or its analogues to inhibit the proteasome, and to relate this effect to their relative degradation by IDE.

Materials and Methods

Preparation of IDE–proteasome

Partially purified preparations of rat skeletal muscle extract containing both IDE and proteasome were prepared as described previously (Bennett *et al.* 2000*a*). Briefly, male Sprague–Dawley rats (140–160 g) were killed by decapitation under pentobarbital anesthesia, the hind leg muscle was excised and homogenized, and the cytosolic fraction was purified by 30–60% ammonium sulfate fractionation followed by weak anion exchange chromatography. In this preparation, IDE is the only insulin-degrading enzyme activity. For highly purified IDE preparations, the material was further purified by hydrophobic interaction, chromatofocusing and strong anion-exchange chromatography steps, as described previously (Bennett *et al.* 2000*a*). All determinations were made using independently prepared enzyme samples. All animals were used in accordance with the US National Institutes of Health Guide for the Use and Care of Laboratory Animals, and all procedures were approved by the Omaha VA Medical Center Institutional Animal Care and Use Committee.

Insulin degradation assay

The degradation of ^{125}I-insulin was measured by the trichloroacetic acid solubility assay (Duckworth & Kitabchi 1974), using appropriate concentrations of partially purified enzyme preparation to limit insulin degradation to the linear boundaries of the assay ($\leq$25%). Competitive inhibition of insulin degradation was carried out by including unlabeled biosynthetic human insulin or an insulin analogue. Insulin and insulin analogues, and [^{125}I-A14]-labeled insulin were provided by Drs Ronald Chance and Bruce Frank of Lilly Research Laboratories.

Covalent cross-linking of ^{125}I-insulin to IDE

Preparations of partially purified IDE were covalently cross-linked to ^{125}I-insulin using the homobifunctional amine-reactive cross-linker, disuccinimidyl suberate (Pierce, Rockford, IL, USA), by a procedure described previously (Shii *et al.* 1985). To determine the specificity of binding, excess (0·1 mg/ml) unlabeled insulin or an insulin analogue was included in appropriate samples. The samples were diluted in reducing SDS-PAGE sample buffer, resolved on 7·5% SDS-PAGE, and dried. Bands of radioactivity were visualized by PhosphorImager analysis.

Insulin and analogue degradation analysis using Tris/Tricine gels

The degradation of insulin and insulin analogues was determined by disappearance of the intact substrate on Tris/Tricine gels. In a total volume of 10 µl, insulin or insulin analogue (1 µg) was incubated with highly purified IDE or vehicle (50 mM HEPES, pH 7·4, 0·15 M NaCl), for 4 h at 37 °C. The samples were diluted into non-reducing SDS-PAGE buffer, boiled for 4 min, then immediately resolved on 16·5% Tris/Tricine gels (Schagger & von Jagow 1987). After fixation, proteins were detected by silver staining. To confirm that IDE was the enzyme responsible for degradation of insulin and the analogues, some samples also received the IDE inhibitor 1,10-phenanthroline (1 mM).

Proteasome activity assays

The chymotrypsin-like, trypsin-like and peptidyl-glutamyl hydrolyzing activities of the proteasome were measured using the fluorogenic peptide substrates *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY), boc-Leu-Ser-Thr-Arg-7-amino-4-methylcoumarin (LSTR) and CBZ-Leu-Leu-Glu-2-naphthylamide respectively, as described previously (Bennett *et al.* 1997). Partially purified IDE–proteasome preparations were incubated with the peptide substrates in the presence or absence of insulin or insulin analogues, and proteasome activity was determined by increased fluorescence resulting from hydrolysis of the substrates.

Results

The relative affinities of insulin and the analogues for IDE were analyzed by competitive inhibition of ^{125}I-insulin degradation (Fig. 2). Unlabeled insulin inhibited ^{125}I-insulin degradation with the expected effective concentration. The lispro analogue was similarly effective. The Asp^{B10} analogue was somewhat more effective than insulin, but EQF was more effective by approximately an order of magnitude. Therefore, each analogue is capable of

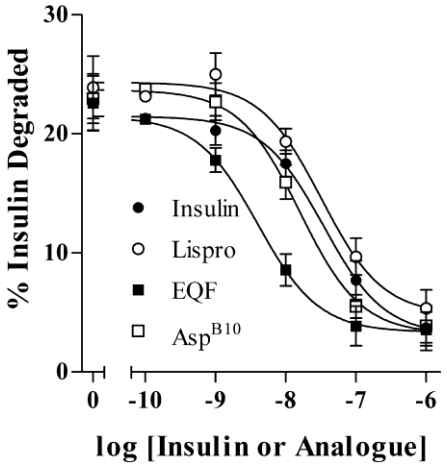

Figure 2 Competitive inhibition of ^{125}I-insulin degradation by insulin and analogues. Partially purified IDE was incubated with ^{125}I-insulin in the presence of the indicated concentrations of unlabeled insulin or analogue. Data are expressed as the percentage of insulin degraded as measured by trichloroacetic acid solubility assay (mean ± S.E.M., $n=4$).

interaction with IDE, but with different affinities. To confirm that the analogues bind to IDE, their ability to compete with ^{125}I-insulin for cross-linking to IDE was examined (Fig. 3). In the absence of unlabeled analogue, a band of radioactivity was readily detected at 110 kDa, consistent with cross-linking of insulin to IDE. When insulin or any of the analogues was included, the cross-linking was completely abolished, suggesting that all three analogues interact with the insulin-binding site of IDE.

Because all the analogues appear to bind IDE, they can potentially be degraded by IDE. Previous studies have indicated that radiolabeled lispro is degraded by IDE at a rate similar to that of native insulin, whereas radiolabeled Asp^{B10} is degraded at a slower rate (Hamel *et al.* 1999). The ability of EQF to be degraded by IDE was unknown. To study degradation of insulin and its analogues, the disappearance of the native molecules in the presence of IDE was monitored on non-reducing Tris/Tricine SDS-PAGE gels (Fig. 4). In the absence of IDE, insulin and analogues were readily detectable as single bands (left column). In the presence of IDE (middle column), both

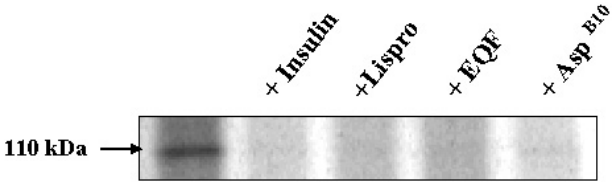

Figure 3 Inhibition of covalent cross-linking of ^{125}I-insulin to IDE. Purified IDE was incubated with ^{125}I-insulin in the presence of excess (0·1 mg/ml) unlabeled insulin or analogue. Bound proteins were covalently cross-linked with disuccinimidyl suberate and visualized by SDS-PAGE and PhosphorImager analysis.

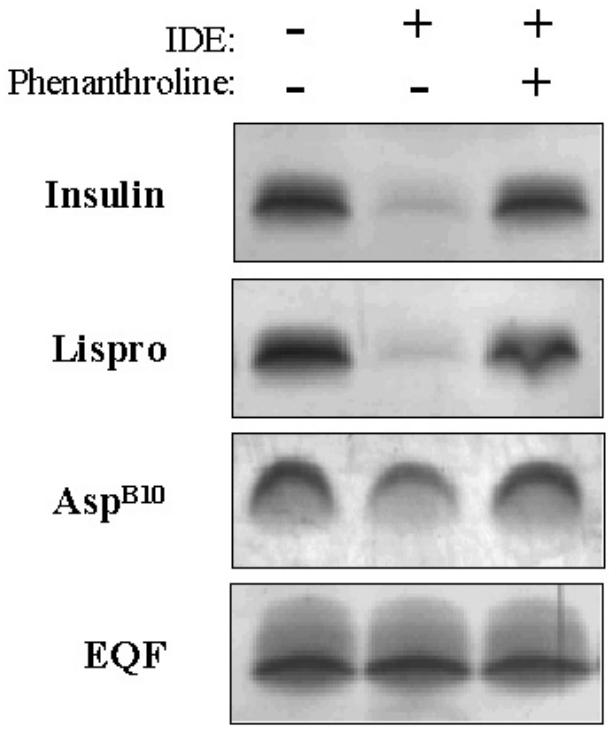

Figure 4 Degradation of insulin and analogues by IDE. Purified IDE was incubated with insulin or analogue, in the presence or absence of the IDE inhibitor 1,10-phenanthroline (1 mM). The samples were resolved on 16·5% Tris/Tricine SDS-PAGE gels and visualized by silver staining. Degradation was monitored by disappearance of the substrate. The results are representative of three independent observations.

insulin and lispro bands disappeared almost completely. The Asp^{B10} analogue was diminished, but to a lesser degree than native insulin. Degradation of the EQF analogue was undetectable. To confirm that IDE was responsible for the degradation of the analogues, the IDE inhibitor 1,10-phenanthroline was included (right column). In all cases, any disappearance of the substrates as a result of degradation was completely inhibited. Thus, consistent with the studies using radiolabeled substrates, insulin and lispro were readily degraded by IDE, whereas Asp^{B10} was degraded more slowly. Furthermore, this experiment showed that the EQF analogue, despite the ability to bind to IDE and the increased inhibition of the degradation of insulin by IDE, is not itself degraded by IDE to a measurable degree.

Insulin inhibits the chymotrypsin-like and trypsin-like activities of the 20S proteasome, and this inhibition involves interaction of insulin with IDE. When the chymotrypsin-like activity of the proteasome was measured (Fig. 5), insulin and lispro had the characteristic inhibitory effect seen previously (Duckworth *et al.* 1994, Bennett *et al.* 1997). In contrast, Asp^{B10} was much less effective, and EQF was essentially without effect.

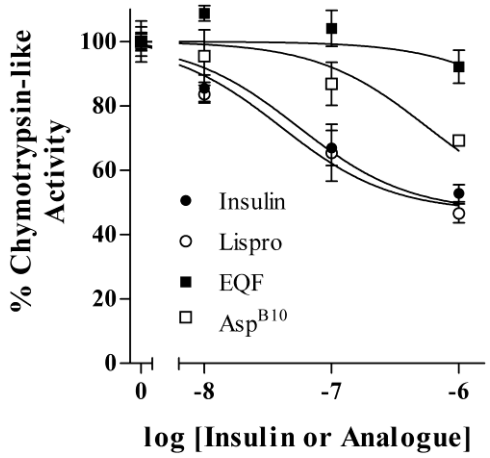

Figure 5 Inhibition of chymotrypsin-like proteasome activity. Partially purified IDE–proteasome was incubated with insulin or analogues, and proteasome activity measured with the fluorogenic substrate, LLVY. The data are expressed as the percentage of LLVY degradation in the absence of insulin (mean ± S.E.M., $n=3$).

Similar results were seen with the trypsin-like proteasome activity (Fig. 6). Previous studies showed that, unlike the chymotrypsin-like and trypsin-like activities, the peptidyl-glutamyl hydrolyzing activity of the proteasome was unaffected by insulin. When this activity was monitored, neither insulin nor any of the analogues had any effect on the peptidyl-glutamyl hydrolyzing proteasome activity (data not shown). The effective doses of insulin and analogues were greater than those seen with competitive inhibition of insulin degradation (Fig. 2). This was, at least in part, attributable to the nature of the proteasome assays, which require much greater (5–10-fold) enzyme concentrations than the insulin degradation assay.

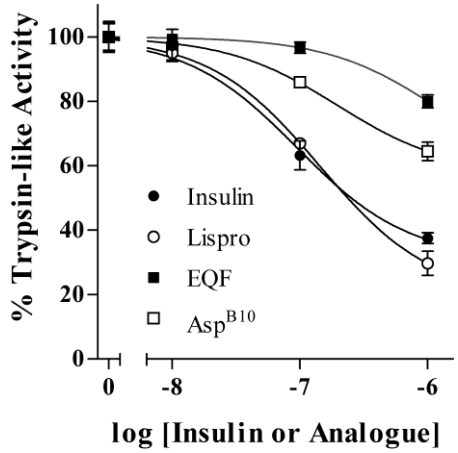

Figure 6 Inhibition of trypsin-like proteasome activity. Partially purified IDE–proteasome was incubated with insulin or analogues, and proteasome activity measured with the fluorogenic substrate, LSTR. The data are expressed as the percentage of LSTR degradation in the absence of insulin (mean ± S.E.M., $n=3$).

Table 1 Summary of the effects of insulin and its analogues. The EC_{50} for inhibition of the degradation of ^{125}I-insulin by IDE, or inhibition of the proteasomal chymotrypsin-like (LLVY) and trypsin-like (LSTR) activities was calculated from the curves produced in Figs 1, 4 and 5

	Inhibition of degradation EC_{50} (nM)			**Degradation by IDE**
	^{125}I-Insulin	LLVY	LSTR	
Insulin	34	54	99	+
Lispro	32	40	101	+
Asp^{B10}	16	562	833	+/−
EQF	4	6459	2479	−

+, readily degraded by IDE; +/−, somewhat degraded by IDE; −, not degraded by IDE.

The overall findings are summarized in Table 1. The ability to bind IDE, as measured by competitive inhibition of ^{125}I-insulin degradation, was greatest for the EQF and Asp^{B10} analogues. Conversely, these analogues were the least effective inhibitors of proteasome activities. Finally, the Asp^{B10} analogue displayed impaired degradation by IDE compared with native insulin, whereas no evidence of EQF degradation was seen.

Discussion

The major target of the antiproteolytic action of insulin is the proteasome (Price *et al.* 1996, Merforth *et al.* 1999, Galban *et al.* 2001). In intact cells from both rodent and human origin, insulin decreases both the peptide-degrading and ubiquitin-dependent proteolytic activities of the proteasome (Hamel *et al.* 1997, Duckworth *et al.* 1998*a*, Bennett *et al.* 2000*b*, Li *et al.* 2000). This may be, at least in part, the result of regulation of the expression of genes involved in the ubiquitin pathway (Wing & Banville 1994, Price *et al.* 1996, Lecker *et al.* 1999); studies using cell-free IDE–proteasome preparations have shown that insulin can inhibit both the 20S proteasome and 26S proteasome involved in the ubiquitin pathway (Duckworth *et al.* 1994, Bennett *et al.* 2000*b*). After removal or inhibition of IDE from the preparation, the ability of insulin to inhibit the proteasome is lost (Duckworth *et al.* 1994, Bennett *et al.* 1997, 2000*b*, Hamel *et al.* 1998). These data suggest that association with IDE maintains the proteasome in a more active state, and that insulin treatment causes dissociation of IDE, and a resultant decrease in proteasome activity to a basal level.

Consistent with the notion that IDE degrading activity is required for proteasome inhibition, a number of IDE substrates, including relaxin and atrial natriuretic peptide, also readily inhibited the proteasome (Bennett *et al.* 1997). However, proinsulin had an intermediate effect on proteasome activity, and was able to attenuate the ability of insulin to inhibit the proteasome (Duckworth *et al.* 1994, Bennett *et al.* 1997). Proinsulin has been shown previously to bind IDE with an affinity approaching that of insulin, but is degraded much more slowly than insulin (Kitabchi *et al.* 1971). This finding raised the possibility that inhibition of the proteasome through IDE required enzymatic degradation of the substrate, and that mere binding to IDE was insufficient to produce inhibition.

In this study, analogues of insulin with variable susceptibilities to degradation by IDE were used. The Asp^{B10} analogue, like proinsulin, displayed an impaired rate of degradation by IDE compared with native insulin or lispro, confirming the findings of an earlier study (Hamel *et al.* 1999). At the same time, binding of Asp^{B10} to IDE was readily detectable by competitive inhibition and cross-linking studies. However, the ability of Asp^{B10} to inhibit proteasome activity was considerably less than that of insulin. The EQF analogue was more effective than insulin at competitive inhibition of IDE, yet was not detectably degraded by IDE. There was very little inhibition of the proteasome with EQF, strongly suggesting that degradation of substrates by IDE is required for proteasome inhibition. These findings are consistent with the effects seen in cultured L6 myotubes, in which EQF displayed impaired inhibition of cellular proteolysis compared with insulin (Fawcett *et al.* 2001).

The impaired IDE degradation of Asp^{B10} and EQF is probably attributable to the positions of the amino substitutions (Fig. 1): the Asp substitution at B10 and the two substitutions in EQF at the B16 and B17 positions correspond to IDE cleavage sites (Duckworth *et al.* 1988*b*). Furthermore, these same three substitutions lie in regions that have been shown to be involved in fibril formation (Nielsen *et al.* 2001). The substrate specificity of IDE appears to be for amyloidogenic peptides, as indicated by its affinity for insulin, glucagon, atrial natriuretic peptide, Alzheimer's beta-amyloid polypeptide and amylin (Duckworth *et al.* 1998, Kurochkin 2001). However, amyloid formation itself is not a requirement, because insulin at neutral pH and rodent amylin do not form amyloid fibrils, yet are degraded by IDE (Bennett *et al.* 2000*a*). Therefore, the amino acid substitutions in EQF and Asp^{B10} may have resulted in structural alteration of the molecule to the point that IDE was able to bind these molecules, but was unable to complete the degradation process.

The data presented strongly suggest that degradation of insulin by IDE is necessary for inhibition of the proteasome. One possible explanation is that IDE produces insulin degradation fragments that then compete with the proteasome substrate for degradation. However, several lines of evidence argue against this mechanism. Firstly, the kinetics of inhibition of the proteasome by insulin were consistent with a non-competitive mechanism (Bennett *et al.* 1997). Secondly, a study was performed in which insulin was predegraded to varying degrees and then added back to the proteasome (Bennett *et al.* 1997). The

more completely the insulin was degraded, the less effect it had on the proteasome. Finally, two of the initial fragments of insulin produced by IDE (B10–B16 and B11–B16) were synthesized, and tested for inhibition of the proteasome. No effect on the proteasome was detected in either case (R G Bennett, unpublished observations). Therefore, although proteasome inhibition by insulin fragments cannot be ruled out, evidence for this mechanism is lacking. In contrast, as discussed above, insulin can induce dissociation of IDE from the proteasome. It has been proposed that IDE can bind to potentially amyloidogenic peptides, resulting in a conversion to a β-sheet conformation at the IDE binding site, which acts as a template for a corresponding β-sheet conversion in the substrate, and allowing degradation of the substrate (Kurochkin 2001). We speculate that the amino acid substitutions in the β-sheet-forming regions of Asp^{B10} and EQF do not allow for the correct structural change necessary for complete degradation of the substrate and dissociation of IDE from the proteasome. Therefore, it is possible that the EQF analogue and, to a lesser degree, the Asp^{B10} are unable to cause dissociation of IDE from the proteasome as a result of the inability of IDE to degrade them efficiently.

In conclusion, these studies suggest that the antiproteolytic effect of insulin is dependent on degradation by IDE, and may provide a mechanism for the reversal of the muscle wasting effect seen in uncontrolled IDDM.

Acknowledgements

This work was supported by the Department of Veterans Affairs Research Service and the Bly Memorial Research Fund, University of Nebraska Medical Center.

References

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

Bennett RG, Duckworth WC & Hamel FG 2000*a* Degradation of amylin by insulin-degrading enzyme. *Journal of Biological Chemistry* **275** 36621–36625.

Bennett RG, Hamel FG & Duckworth WC 2000*b* Insulin inhibits the ubiquitin-dependent degrading activity of the 26S proteasome. *Endocrinology* **141** 2508–2517.

Coux O, Tanaka K & Goldberg AL 1996 Structure and functions of the 20S and 26S proteasomes. *Annual Review of Biochemistry* **65** 801–847.

DiMarchi RD, Chance RE, Long HB, Shields JE & Slieker LJ 1994 Preparation of an insulin with improved pharmacokinetics relative to human insulin through consideration of structural homology with insulin-like growth factor I. *Hormone Research* **41** (Suppl 2) 93–96.

Duckworth WC & Kitabchi AE 1974 Insulin and glucagon degradation by the same enzyme. *Diabetes* **23** 536–543.

Duckworth WC, Heinemann M & Kitabchi AE 1972 Purification of insulin specific protease by affinity chromatography. *Proceedings of the National Academy of Sciences USA* **69** 3698–3702.

Duckworth WC, Hamel FG, Peavy DE, Liepnieks JJ, Ryan MP, Hermodson MA & Frank BH 1988 Degradation products of insulin generated by hepatocytes and insulin protease. *Journal of Biological Chemistry* **263** 1826–1833.

Duckworth WC, Bennett RG & Hamel FG 1994 A direct inhibitory effect of insulin on a cytosolic proteolytic complex containing insulin degrading enzyme and multicatalytic proteinase. *Journal of Biological Chemistry* **269** 24575–24580.

Duckworth WC, Bennett RG & Hamel FG 1998*a* Insulin acts intracellularly on proteasomes through insulin-degrading enzyme. *Biochemical and Biophysical Research Communications* **244** 390–394.

Duckworth WC, Bennett RG & Hamel FG 1998*b* Insulin degradation: progress and potential. *Endocrine Reviews* **19** 608–624.

Fawcett J, Hamel FG, Bennett RG, Vajo Z & Duckworth WC 2001 Insulin and analogue effects on protein degradation in different cell types: dissociation between binding and activity. *Journal of Biological Chemistry* **276** 11552–11558.

Galban VD, Evangelista EA, Migliorini RH & do Carmo Kettelhut I 2001 Role of ubiquitin–proteasome-dependent proteolytic process in degradation of muscle protein from diabetic rabbits. *Molecular and Cellular Biochemistry* **225** 35–41.

Gelfand RA & Barrett EJ 1987 Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in man. *Journal of Clinical Investigation* **80** 1–6.

Grizard J, Dardevet D, Balage M, Larbaud D, Sinaud S, Savary I, Grzelkowska K, Rochon C, Tauveron I & Obled C 1999 Insulin action on skeletal muscle protein metabolism during catabolic states. *Reproduction, Nutrition, Development* **39** 61–74.

Hamel FG, Bennett RG, Harmon KS & Duckworth WC 1997 Insulin inhibition of proteasome activity in intact cells. *Biochemical and Biophysical Research Communications* **234** 671–674.

Hamel FG, Bennett RG & Duckworth WC 1998 Regulation of the multicatalytic enzyme activity by insulin and the insulin degrading enzyme. *Endocrinology* **139** 4061–4066.

Hamel FG, Siford GL, Fawcett J, Chance RE, Frank BH & Duckworth WC 1999 Differences in the cellular processing of AspB10 human insulin compared with human insulin and LysB28 ProB29 human insulin. *Metabolism* **48** 611–617.

Kitabchi AE, Duckworth WC, Brush JS & Heinemann M 1971 Direct measurement of proinsulin in human plasma by the use of an insulin degrading enzyme. *Journal of Clinical Investigation* **50** 1792–1799.

Kurochkin IV 2001 Insulin-degrading enzyme: embarking on amyloid destruction. *Trends in Biochemical Sciences* **26** 421–425.

Larbaud D, Debras E, Taillandier D, Samuels SE, Temparis S, Champredon C, Grizard J & Attaix D 1996 Euglycemic hyperinsulinemia and hyperaminoacidemia decrease skeletal muscle ubiquitin mRNA in goats. *American Journal of Physiology* **271** E505–E512.

Larbaud D, Balage M, Taillandier D, Combaret L, Grizard J & Attaix D 2001 Differential regulation of the lysosomal, Ca2+-dependent and ubiquitin/proteasome-dependent proteolytic pathways in fast-twitch and slow-twitch rat muscle following hyperinsulinaemia. *Clinical Science* (*London*) **101** 551–558.

Lecker SH, Solomon V, Price SR, Kwon YT, Mitch WE & Goldberg AL 1999 Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats. *Journal of Clinical Investigation* **104** 1411–1420.

Li BG, Fang CH & Hasselgren P 2000 Degradation of methoxysuccinyl-phe-leu-phe-7-amido-4-trifluoromethyl coumarin (FLF) in cultured myotubes and HepG2 cells is proteasome- and calpain/calcium-dependent. *International Journal of Biochemistry and Cell Biology* **32** 677–686.

Merforth S, Osmers A & Dahlmann B 1999 Alterations of proteasome activities in skeletal muscle tissue of diabetic rats. *Molecular Biology of Reproduction* **26** 83–87.

Nielsen L, Frokjaer S, Brange J, Uversky VN & Fink AL 2001 Probing the mechanism of insulin fibril formation with insulin mutants. *Biochemistry* **40** 8397–8409.

Price SR, Bailey JL, Wang X, Jurkovitz C, England BK, Ding X, Phillips LS & Mitch WE 1996 Muscle wasting in insulinopenic rats results from activation of the ATP-dependent, ubiquitin–proteasome proteolytic pathway by a mechanism including gene transcription. *Journal of Clinical Investigation* **98** 1703–1708.

Rooyackers OE & Nair KS 1997 Hormonal regulation of human muscle protein metabolism. *Annual Review of Nutrition* **17** 457–485.

Schagger H & von Jagow G 1987 Tricine–sodium dodecyl-sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166** 368–379.

Shii K, Baba S, Yokono K & Roth RA 1985 Covalent linkage of 125I insulin to a cytosolic insulin degrading enzyme. *Journal of Biological Chemistry* **260** 6503–6506.

Slieker LJ, Brooke GS, Chance RE, DiMarchi RD, Fan L, Hoffman JA, Howey DC, Long H, Mayer J, Shaw WN *et al.* 1992 Insulin and IGF-I analogs: novel approaches to improved pharmacokinetics. In *Peptides: Biology and Chemistry*, pp 7–10. Eds Y Du, JP Tam & Y Zhang. Leiden: Escom.

Smith OL, Wong CY & Gelfand RA 1989 Skeletal muscle proteolysis in rats with acute streptozocin-induced diabetes. *Diabetes* **38** 1117–1122.

Wing SS & Banville D 1994 14-kDa biquitin-conjugating enzyme: structure of the rat gene and regulation upon fasting and by insulin. *American Journal of Physiology* **267** E39–E48.

Wing SS & Bedard N 1996 Insulin-like growth factor I stimulates degradation of an mRNA transcript encoding the 14 kDa ubiquitin-conjugating enzyme. *Biochemical Journal* **319** 455–461.

Received 11 February 2003
Accepted 14 February 2003