Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats

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Insulin deficiency (e.g., in acute diabetes or fasting) is associated with enhanced protein breakdown in skeletal muscle leading to muscle wasting. Because recent studies have suggested that this increased proteolysis is due to activation of the ubiquitin-proteasome (Ub-proteasome) pathway, we investigated whether diabetes is associated with an increased rate of Ub conjugation to muscle protein. Muscle extracts from streptozotocin-induced insulin-deficient rats contained greater amounts of Ub-conjugated proteins than extracts from control animals and also 40–50% greater rates of conjugation of ^{125}I-Ub to endogenous muscle proteins. This enhanced Ub-conjugation occurred mainly through the N-end rule pathway that involves $E2_{14k}$ and E3α. A specific substrate of this pathway, α-lactalbumin, was ubiquitinated faster in the diabetic extracts, and a dominant negative form of $E2_{14k}$ inhibited this increase in ubiquitination rates. Both $E2_{14k}$ and E3α were shown to be rate-limiting for Ub conjugation because adding small amounts of either to extracts stimulated Ub conjugation. Furthermore, mRNA for $E2_{14k}$ and E3α (but not E1) were elevated 2-fold in muscles from diabetic rats, although no significant increase in $E2_{14k}$ and E3α content could be detected by immunoblot or activity assays. The simplest interpretation of these results is that small increases in both $E2_{14k}$ and E3α in muscles of insulin-deficient animals together accelerate Ub conjugation and protein degradation by the N-end rule pathway, the same pathway activated in cancer cachexia, sepsis, and hyperthyroidism.

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Introduction

A major feature of low-insulin states, such as diabetes mellitus and fasting, is a marked loss of muscle protein resulting in muscle atrophy (1, 2). In these insulin-deficient states, the amino acids released from muscle protein are used primarily for hepatic glucose production (2). Whereas some of the loss of muscle mass in acute diabetes is due to decreased protein synthesis, the rapid fall in tissue weight results primarily from increased protein degradation (2–5). Streptozotocin administration in rats leads to destruction of pancreatic beta cells and produces a useful model of acute insulin deficiency characterized by high blood glucose levels and ketoacidosis. Protein degradation in muscles isolated from such rats is also elevated (4, 5). Understanding the enzymatic adaptations that account for the enhanced muscle protein breakdown in uncontrolled diabetes and other catabolic diseases may help in the development of therapeutic regimens or pharmacological inhibitors of this excessive protein loss.

The bulk of proteolysis in skeletal muscle and most cultured cells occurs by the ubiquitin-proteasome (Ub-proteasome) pathway (6, 7), although degradation of membrane and organellar proteins within lysosomes also account for 10–20% of the overall protein breakdown (8). In the Ub-proteasome pathway, proteins to be degraded are first marked by covalent attachment of a chain of Ub molecules that targets them for destruction by the 26S proteasome (9). Ub molecules are linked to the protein substrate by a multienzyme pathway (10). The enzyme, E1, first activates Ub by forming a high-energy thiolester linkage between an internal Cys residue of E1 and the COOH-terminal Gly residue of Ub. Ub is transferred to a Ub-carrier protein (E2), which then transfers Ub to a lysine residue of the substrate in a reaction catalyzed by an E3 (Ub-protein ligase). Subsequently, E2 and E3 link additional Ub molecules onto the first Ub through isopeptide linkages to form a polyubiquitin chain. Whereas only a single E1 is present in cells (11), at least a dozen E2s and probably an even larger number of E3s exist (12, 13) to provide substrate specificity, because particular E2/E3 pairs appear to conjugate Ub to specific cell proteins or classes of proteins. Surprisingly, in rat, rabbit, and presumably in other mammals, the E2/E3 pair, $E2_{14k}$ and E3α, accounts for most of the Ub conjugation activity to endogenous proteins in soluble extracts of skeletal muscle (6). Ub conjugation by this pair of enzymes has been termed the "N-end rule pathway" (14), because the Ub-protein ligase E3α recognizes protein substrates that contain basic and large hydrophobic NH_2-terminal amino acids (14, 15), cat-

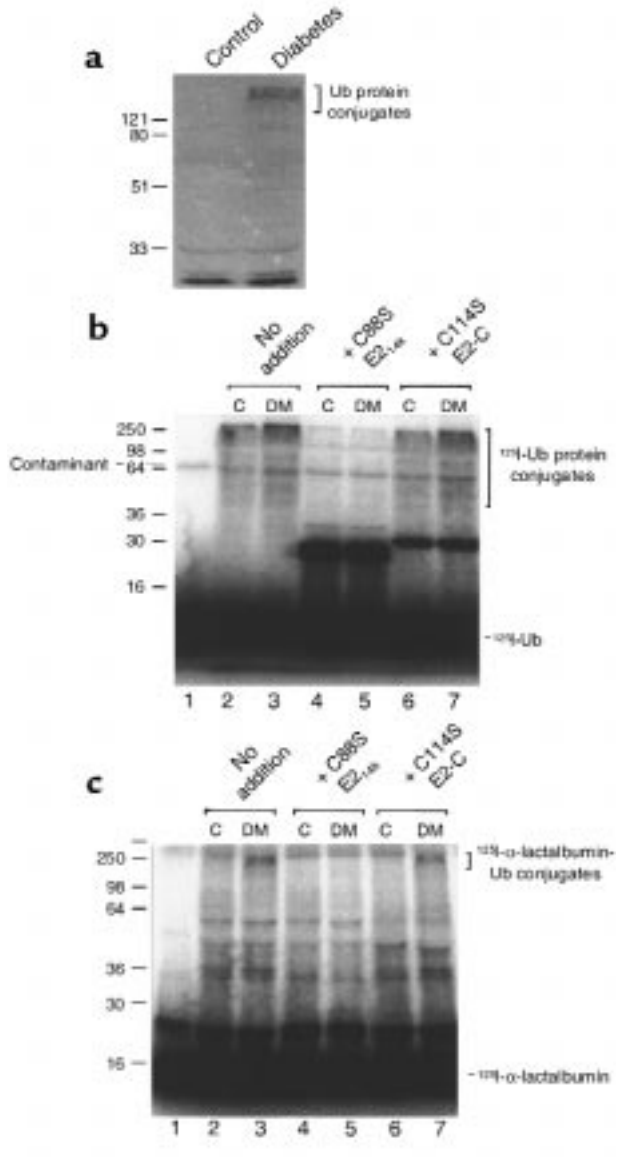

Figure 1

Ub protein conjugates and rates of Ub conjugation are increased in muscles from diabetic rats. (**a**) Anti-Ub immunoblot. Muscle extract protein (35 μg crude extract) from control or diabetic rats was subjected to 15% SDS-PAGE and probed with a polyclonal anti-Ub antibody at a 1/500 dilution. An increased amount of high-molecular-weight Ub-immunoreactive material (Ub conjugates) was present in the muscles from diabetic animals. (**b**) ^{125}I-Ub conjugation to soluble muscle proteins. Muscle protein fraction II from either control (C) or diabetic (DM) rats was used as the source of both Ub-conjugating enzymes and ubiquitination substrates. Upon addition of AMPPNP and ^{125}I-Ub, ^{125}I-Ub protein conjugates were formed (lanes 2 and 3). Lanes 4 and 5 are the same as lanes 2 and 3, except for the addition of 2 μM C88S $E2_{14k}$. Lanes 6 and 7 are the same as lanes 2 and 3, except for the addition of 2 μM C114S E2-C. Lane 1 has ^{125}I-Ub alone. Note that higher amounts of Ub-protein conjugates were formed in the extracts from diabetic muscles and that the dominant negative $E2_{14k}$, but not the dominant negative E2-C, inhibited this increase in Ub conjugation. The intensely radioactive bands at ~25 kD in lanes 4–7 are nonreducible ^{125}I-Ub-C88S $E2_{14k}$ and ^{125}I-Ub-C114S E2-C complexes. (**c**) Ub conjugation to α-lactalbumin. Same as in **a**, except that ^{125}I-Ub was replaced by Ub and ^{125}I-α-lactalbumin. Lane 1: ^{125}I-α-lactalbumin alone. Increased ^{125}I-α-lactalbumin-Ub conjugates were formed in the extracts from diabetic muscles. The dominant negative $E2_{14k}$, but not the dominant negative E2-C, again inhibited the enhanced Ub conjugation in the diabetic extracts.

alyzing their rapid ubiquitination and degradation.

The enhanced muscle protein breakdown in catabolic conditions seems to occur through activation of the Ub-proteasome pathway (16, 17). For example, in muscles removed from acidotic or septic animals and incubated in vitro, the proteasome inhibitor MG132 lowered protein degradation to control levels (5, 18–20). Many studies have shown increases in mRNA for Ub (5, 8, 21–25), $E2_{14k}$ (21, 26–29), and certain proteasomal subunits (5, 21, 23, 24, 27–29) in atrophying muscles. However, the increases in the amounts of these proteins in the muscles have not been demonstrated or even been measured in most of these cases. No studies have explored whether the increases in mRNA for Ub, $E2_{14k}$, or proteasomal subunits can, in fact, account for the enhanced protein breakdown in atrophying muscles, or if they are just associated adaptations. Perhaps the strongest indication that rates of ubiquitination are generally enhanced in atrophying muscles has been the finding of increased amounts of Ub conjugates in muscle from denervated, septic, and tumor-bearing rats (30–33). Recently, we demonstrated higher rates of Ub conjugation to muscle proteins in extracts from muscles of tumor-bearing, septic, and hyperthyroid animals (34). Surprisingly, enhanced Ub conjugation in these muscles again appeared to occur, in large part, by activation of the N-end rule system (34). However, the biochemical adaptations that activate this pathway have not been investigated.

In rats with acute insulin deficiency, overall protein degradation in muscle also appears to increase primarily through activation of the Ub-proteasome pathway, as suggested by greater sensitivity to proteasome inhibitors and increased mRNA for Ub and the proteasome (5). However, rates of Ub conjugation in diabetic muscles have not been investigated. We demonstrate here that the activation of proteolysis in insulin-deficient animals is associated with accelerated Ub conjugation and that activity of the N-end rule ubiquitination system is enhanced. To clarify how this system may influence proteolysis, we have examined whether its components, $E2_{14k}$ or E3α, are rate limiting for Ub conjugation and have tested whether the expression of these proteins increases and accounts for the enhanced Ub conjugation.

Methods

Reagents. Ub, anti-Ub antiserum, and human α-lactalbumin were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA), and rabbit reticulocytes were purchased from Pel-Freez Biologicals (Rogers, Arizona, USA). Chymostatin was obtained from Roche Molecular Biochemicals (Indianapolis, Indiana, USA), and diethylpyrocarbonate (DEPC) was from Fluka Chemie AG (Buchs, Switzerland). Radioiodination of protein substrates was performed by the chloramine T method, as described (35). The dominant negative inhibitor mutants of the E2s were expressed, purified, and kindly provided by Jackie Pierce, Margaret Read, and Vincent Chau (Proscript, Inc., Cambridge, Massachusetts, USA). *Escherichia coli* strains engineered to express rat $E2_{14k}$ and human E1 were kindly provided by S. Wing (McGill University, Montreal, Quebec, Canada) and Chikara Miyamoto (Nippon Roche K.K., Tokyo, Japan), respectively. Polyclonal rabbit antiserum against E1 was provided by A. Ciechanover (Technion,

Haifa, Israel) and antiserum against $E2_{14k}$ was provided by A. Haas (Medical College of Wisconsin, Milwaukee, Wisconsin, USA). Muscle extracts were prepared as described previously (6), with the following modifications. Fragments from multiple (> 4) frozen gastrocnemius muscles from control or diabetic rats were pooled, pulverized under liquid N_2, and weighed. Four to five volumes of ice-cold extract buffer (6) was added, and the suspension was homogenized using 3 pulses (5 seconds) of a Polytron tissue homogenizer (Brinkmann Intruments Inc., Westbury, New York, USA). Recombinant human E1 and $E2_{14k}$ were purified from overproducing strains of *E. coli* as described (6). An enriched preparation of E3α was prepared from a 100,000 *g* supernatant of rat muscle. The precipitate from a 40% wt/vol ammonium sulfate [$(NH_4)_2SO_4$] fractionation was centrifuged and resuspended in one-tenth the original extract volume of 20 mM Tris-hydrochloride (HCl), pH 7.6, 20 mM potassium chloride (KCl), 5 mM magnesium chloride ($MgCl_2$), 1 mM dithiothreitol (DTT), and 10% glycerol, and dialyzed overnight against more than 500 volumes of the same buffer. This fractionation removes approximately 70% of cell proteins and all $E2_{14k}$.

Diabetic animals. Diabetes was induced in male rats (~175 g) by a single injection of streptozotocin (125 mg/kg, freshly prepared in 0.1 M citrate buffer, pH 4.0) in the tail vein. Control rats were injected with buffer only. Treated and control rats were housed individually and fed a 23% protein diet; control rats were pair-fed with streptozotocin-injected rats as described (5, 36). Rats were studied on the third day after streptozotocin treatment, after fasting overnight. After anesthesia, arterial blood was collected to measure blood glucose, and gastrocnemius muscles were excised and immediately frozen in liquid nitrogen. Muscles were stored at –80°C until preparation of extracts.

Measurement of Ub conjugation to endogenous soluble muscle proteins. Fraction II (35 μg protein) was incubated with ^{125}I-Ub (~150,000 cpm, 5–10 μM) in buffer A (20 mM Tris-HCl, pH 7.6, 20 mM KCl, 5mM $MgCl_2$, 2 mM AMPPNP, 1 mM DTT, 30 μM MG132, and 10% glycerol). AMPPNP was used as the energy source for these experiments because it supports activation of Ub by E1 but not the degradation of ubiquitinated proteins by the proteasome (37). The reactions (20 μL total volume) were incubated at 37°C for 60 minutes and terminated by addition of 6 μl 5× Laemmli sample buffer. SDS-PAGE was then performed as described by Laemmli on 13% polyacrylamide gels (38). The gels were dried and analyzed using a Fuji Phosphorimager.

Measurement of Ub conjugation to human α-lactalbumin. Fraction II (35 μg protein), ^{125}I-α-lactalbumin (~150,000 cpm, ~1 μM), and Ub (50 μM) in buffer A (20 μL total volume) were incubated at 37°C for 60 minutes, terminated by addition of 6 μL 5× Laemmli sample buffer, electrophoresed, and analyzed as for the endogenous ubiquitination reaction above.

E3α assay. Crude muscle cell extracts (the 100,000 *g* supernatant) from control and diabetic rats were depleted of E2s by precipitation with $(NH_4)_2SO_4$ (40% wt/vol final concentration). After centrifugation, the $(NH_4)_2SO_4$ pellet was resuspended in one-tenth the original extract volume of 20 mM Tris-HCl, pH 7.6, 1 mM DTT, and 10% glycerol, and dialyzed overnight against more than 500 volumes of the same buffer. This material was completely dependent on $E2_{14k}$ for Ub conjugation activity (see Figure 6b). To measure E3α activity, 20 μg of the resuspended, dialyzed $(NH_4)_2SO_4$ precipitate was supplemented with E1 (90 nM), ^{125}I-α-lactalbumin (~100,000 cpm/reaction, 1–5 μM), and $E2_{14k}$ (5 μM) in buffer A (20-μL reaction volume). The reactions were incubated at

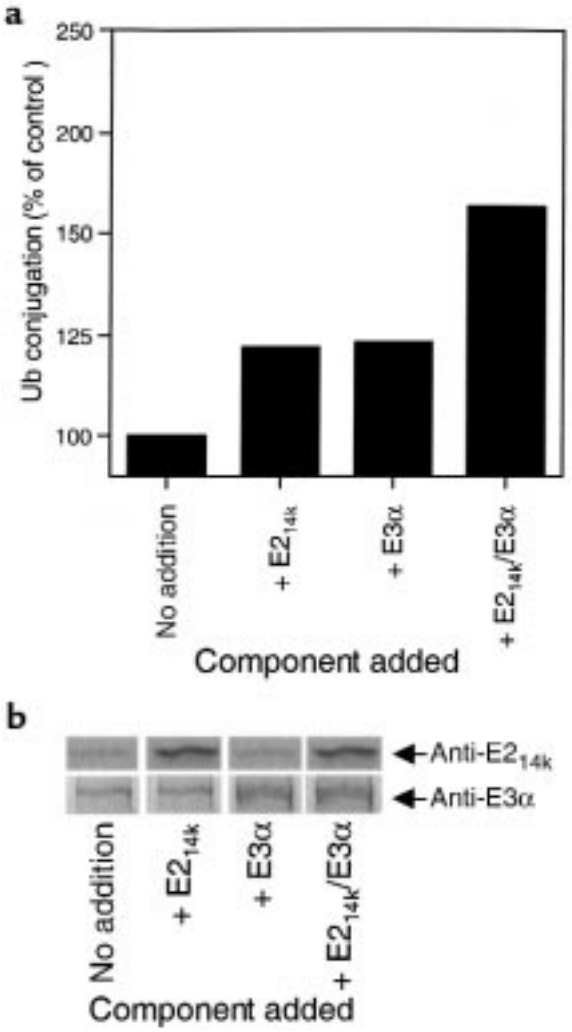

Figure 2
The combination of $E2_{14k}$ and E3α, when added to muscle extracts, increases the rate of Ub conjugation to an N-end rule substrate. Muscle extract fraction II (50 μg) was supplemented with recombinant $E2_{14k}$, E3α [as a 40% wt/vol $(NH_4)_2SO_4$ precipitate of crude muscle extract], or a combination of both proteins, and analyzed for Ub conjugation to ^{125}I-α-lactalbumin after addition of Ub (25 μM) and AMPPNP (2 mM). (**a**) Results are graphed as the amount of Ub conjugation measured in the extract supplemented with added $E2_{14k}$ or E3α divided by the amount of Ub conjugation measured in the extract without added $E2_{14k}$ or E3α × 100%. (**b**) The equivalent amount of fraction II with or without added $E2_{14k}$ and E3α as was used in **a** was analyzed by SDS-PAGE and immunoblot to directly view the quantities of $E2_{14k}$ and E3α added to the reactions. Densitometry analysis of the photographed immunoblots demonstrated that the reactions supplemented with $E2_{14k}$ contained approximately 3.5 times the amount of $E2_{14k}$ present in 50 μg of fraction II, and the reactions supplemented with E3α contained approximately 2.3 times the amount of enzyme present in 50 μg of fraction II.

37°C for 60 minutes and terminated by addition of 6 μl 5× Laemmli sample buffer. SDS-PAGE was then performed as described above.

Production of anti-E3α antibody — overexpression and purification of an NH_2-terminal E3α fragment. PCR probes were synthesized to introduce an *Nde*I site, just 5′ of the *Afl*II site at –6, and a stop codon and *Eco*RI site, just 3′ of the internal *Stu*I site at +1009 of the mouse UBR1 cDNA (39) (5′-AGCTGACCATATGCTTAAGATGGCGGACGAAGAG and 5′-TAGGAATTCTCAAGGCCTGGCAAAATATCTGTC). The probes were then used to generate the 1.0-kb PCR fragment, which was cut with *Nde*I and *Eco*RI and inserted into a pET28b vector (Novagen, Madison, Wisconsin, USA). The resulting plasmid (pSL1) contained a 35-kD NH_2-terminal fragment of the *UBR1/E3α* gene behind a His_6 tag and an IPTG-inducible promoter. This *E3α* fragment was purified from *E. coli* BL21(DE3) under denaturing conditions using the His_6 affinity matrix Ni-NTA Agarose (Qiagen, Chatsworth, California, USA) according to the manufacturer's instructions. Upon dialysis against PBS, approximately 50% of the eluted protein remained soluble.

Antibody production. Purified *E3α* fragment (150 μg) in complete Freund's adjuvant was injected into the popliteal fossas of 2 rabbits. This initial immunization was followed by 3 boosts with 100 μg denatured (i.e., not dialyzed), purified *E3α* fragment in incomplete Freund's adjuvant. George Deegan (Harvard University, Cambridge, Massachusetts, USA) performed antigen injection and serum collection. IgG was prepared from the crude rabbit serum using established protocols (40). Anti-E3α IgG was affinity purified from the pooled IgG from both animals according to the procedures of Harlow (40), using an Affigel-10 matrix (Bio-Rad Laboratories Inc., Hercules, California, USA) onto which the purified *E3α* fragment had been bound.

Northern blot analysis. Total RNA was extracted from muscle, subjected to electrophoresis on agarose-formaldehyde gels, and transferred to nylon membranes as described previously (36). Hybridization was performed according to the procedure of Church and Gilbert (41, 42). Hybridized membranes were analyzed using a Fuji Phosphorimager. Quantities of specific mRNA species were normalized for gel-loading differences by stripping the blots and rehybridizing with an 18S-rRNA probe. The following gel-purified DNA fragments were used as Northern probes: $E2_{14k}$, 0.5-kb *Xba*I-*Bam*HI fragment; E3α, 1.0-kb *Afl*II-*Eco*RI fragment; E1, 0.6-kb *Nde*I-*Hind*III fragment; 18S rRNA, 0.75-kb *Bam*HI-*Sph*I fragment.

Immunoblot analysis. After SDS-PAGE, proteins were transferred to PVDF membranes (Immobilon; Millipore Corp., Bedford, Massachusetts, USA) using a semi-dry transfer apparatus. Membranes were blocked with 5% nonfat dry milk in PBS and incubated with primary antibody (see individual figures) overnight at 4°C. Membranes were then washed twice with PBS plus 0.1% Tween-100 and once with PBS, then incubated with goat anti-rabbit IgG (Fc) alkaline phosphatase conjugate (Promega Corp., Madison, Wisconsin, USA), at a 1:4000 dilution in 5% milk/PBS for at least 3 hours. Membranes were washed again as above and viewed after treatment with NBT/BCIP color reagent (Promega) according to the manufacturer's instructions. The relative intensities of bands (i.e., $E2_{14k}$ and E3α) did not change, even when immunoblots were intentionally underdeveloped.

Table 1
Comparison of normal and diabetic rats

	Control	Diabetic	% of control
Initial body weight (g)	227 ± 3	226 ± 3	99
Final body weight (g)	209 ± 2	174 ± 3[A]	83
Blood glucose (mg/dL)	95 ± 9	288 ± 12[A]	303
Epitrochlearis weight (mg)	39.4 ± 1.0	31.8 ± 1.0[A]	81
Protein degradation in incubated epitrochlearis (nmol tyr/g per hour)	148 ± 5	208 ± 7[A]	141

Rats were injected with streptozotocin and sacrificed 3 days later. Control animals were injected with the buffer, pair-fed, and fasted for the 12 hours preceding muscle harvest. Results are expressed as mean ± SE. Protein degradation was measured as described previously (5). Protein degradation results are expressed as means ± SE. Both right and left muscles from each animal were measured independently. Each group contains 20 measurements. [A]$P < 0.001$ for control vs. diabetic groups by Student's t test; $n = 10$ for each group.

Results

As reported previously (5), 3 days after streptozotocin treatment, serum glucose in rats averaged 288 ± 12 mg/dL compared with 95 ± 9 mg/dL in the control group (Table 1). The treated animals lost 23% of their initial body weight during these 3 days, and individual epitrochlearis muscles underwent marked atrophy, finally weighing, on average, 19% less than epitrochlearis muscles from control animals (Table 1). To ensure that changes occurring in the muscles were not due to differences in food intake between the 2 groups, the control and streptozotocin-treated animals were pair-fed and fasted for 12 hours before muscle harvest. When the epitrochlearis muscles were isolated and incubated in vitro, those from diabetic rats showed protein degradation rates 40% greater than the controls (Table 1). This increase in protein degradation was largely through a proteasome-dependent pathway because it was markedly inhibited by the proteasome inhibitor, MG132, as well as ATP depletion (5).

Ub conjugation is increased in muscle extracts from diabetic rats. To test whether the increased protein degradation in the muscles of streptozotocin-treated rats was associated with an increase in Ub conjugation generally, we measured the content of Ub conjugates in muscles from control and diabetic animals by subjecting soluble muscle extracts to SDS-PAGE and immunoblot analysis using an anti-Ub antibody. The diabetic muscle extract showed greater amounts of high-molecular-weight Ub-conjugated proteins than the control muscles (Figure 1a). Larger amounts of Ub-protein conjugates were also found when whole muscles from diabetic rats were directly solubilized in SDS (data not shown). Thus, the increased amount of Ub conjugates was not the result of extract preparation. Because the increased protein degradation in the diabetic muscles was sensitive to proteasome inhibitors (5), the flux of substrates through the proteasome in these atrophying muscles must be increased, and this accumulation of Ub conjugates cannot be due simply to a failure of the conjugates to be degraded. Therefore, the conjugation of Ub to muscle protein must also be more rapid.

This build-up of conjugates in the face of increased protein breakdown suggests that in the streptozotocin-treated animals, proteasome function has become the rate-limiting step in the pathway.

To compare directly rates of Ub conjugation in the muscles of normal and diabetic rats, soluble extracts were prepared and ubiquitination rates measured in vitro as the formation of conjugates between ^{125}I-Ub and endogenous extract proteins (6). For these studies we used preparations (fraction II) that contain E1 and many E2s and E3s, including those required for the N-end rule pathway, but this fraction lacks Ub (which does not bind to the column) (43–45). Extracts from the diabetic and control muscles supported Ub-conjugation to muscle proteins, as measured by the ATP-dependent incorporation of ^{125}I-Ub into high-molecular-weight protein conjugates (Figure 1b, lane 2). Ub conjugates were formed at a faster rate in the extracts from diabetic animals than in those from controls (Figure 1b, lanes 3 vs. 2). These findings suggest that the acceleration of Ub conjugation is a critical adaptation leading to the enhanced proteolysis in muscles from rats with uncontrolled diabetes.

To test further whether the increase in Ub conjugation to muscle proteins was due to greater activity of the Ub-conjugating enzymes and not to some alteration in the protein substrates in the extracts, we studied Ub conjugation to an exogenously added substrate, ^{125}I-human α-lactalbumin (46). This protein has a basic amino terminus (Lys) and therefore is conjugated to Ub by the N-end rule pathway. This ubiquitination system, which involves the enzymes $E2_{14k}$ and E3α (46), selectively conjugates Ub to proteins with charged or large hydrophobic amino terminal residues (15), and our recent studies have indicated that this system plays a major role in Ub conjugation to proteins in skeletal muscle (6). Approximately twice as much of the ^{125}I-α-lactalbumin was conjugated to Ub in the extracts from diabetic rats than in the controls (Figure 1c, lanes 3 vs. 2). More rapid ubiquitination of this exogenous substrate suggests that the N-end rule pathway is activated in these atrophying muscles.

To test further whether the increase in Ub conjugation in these muscles from insulin-deficient rats was due to greater activity of the N-end rule pathway, the effects of a specific inhibitor of this pathway was studied. A mutant form of $E2_{14k}$ (Cys 88→Ser), which forms a stable ester bond with Ub instead of a thiolester linkage, can bind Ub but cannot transfer it to E3 or to substrates (47). This form of $E2_{14k}$ acts as a dominant negative inhibitor of E3α (47). When added to the Ub-conjugation assay, it inhibited most of the Ub conjugation to muscle proteins from both groups (Fig. 1, b and c, lanes 4 and 5) and also decreased or eliminated the difference between the diabetic and control extracts in the rates of Ub conjugation to endogenous substrates and to α-lactalbumin. By contrast, another dominant negative E2, E2-C (Cys 114→Ser), which is not involved in the N-end rule pathway but inhibits ubiquitination of some cell cycle proteins (48, 49), had no effect on ubiquitination rates in extracts from either control or diabetic animals (Figure 1, b and c, lanes 6 and 7).

Is $E2_{14k}$ or E3α rate limiting for Ub conjugation? Because Ub conjugation by the N-end rule pathway is enhanced in muscle extracts from the insulin-deficient animals, we investigated whether either of the enzymes of this pathway is rate limiting for ubiquitination in these extracts. Pure $E2_{14k}$, partially purified E3α, or a combination of both was added to rat muscle fraction II, and rates of Ub conjugation to ^{125}I-α-lactalbumin were measured (Figure 2). Addition of either $E2_{14k}$ or E3α in amounts sufficient to at least double cellular levels consistently increased ubiquitination of ^{125}I-α-lactalbumin, but only by approximately 20% (Figure 2a). However, increasing the content of $E2_{14k}$ and E3α together stimulated Ub conjugation of ^{125}I-α-lactalbumin by a larger amount than either alone (about 50%) (Figure 2a), as would be expected if the 2 proteins function together. Although increases in either $E2_{14k}$ or E3α appear to enhance Ub conjugation, the quantitative effects of increasing these factors are difficult to estimate accurately, because it is unclear to what extent the recombinant $E2_{14k}$ or the partially purified E3α have the same specific

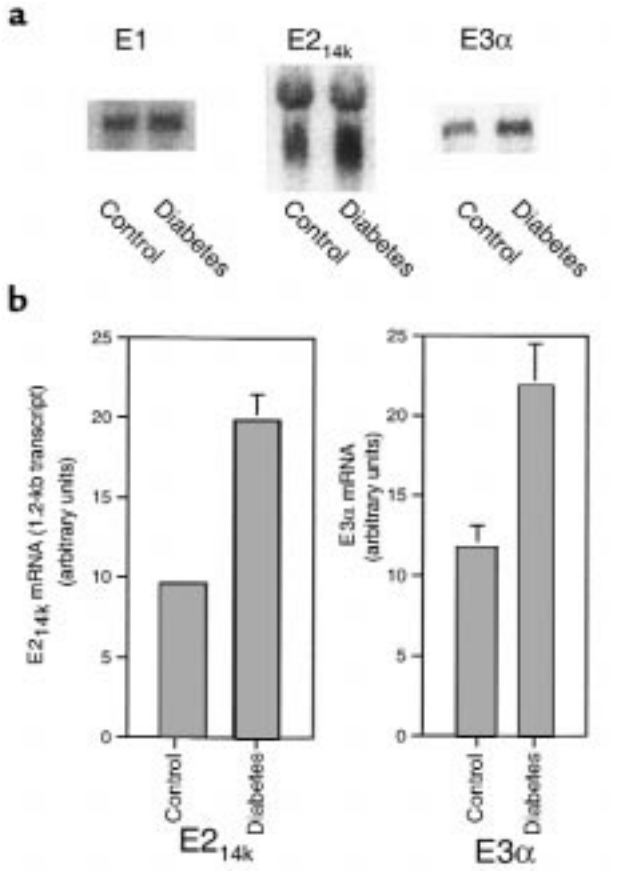

Figure 3
Muscles from diabetic rats contain higher levels of $E2_{14k}$ or E3α , but not E1 mRNA. (**a**) Northern blots. Total RNA (15 μg) pooled from 6 muscles of control or diabetic rats (for the E1 and E3α blots) or from a single control and diabetic rat (for $E2_{14k}$ blot) were electrophoresed, transferred to nylon membranes, and hybridized with specific E1, $E2_{14k}$, or E3α probes as described in Methods. (**b**) Quantification of $E2_{14k}$ or E3α transcripts in muscles from control and diabetic rats. $E2_{14k}$: Mean ± SD of mRNA content for the 1.2-kb $E2_{14k}$ transcript in individual muscles from 4 diabetic and control rats. E3α: Mean ± SD of mRNA content for the single 6.0-kb E3α transcript in individual muscles from 6 diabetic and control rats. Quantities of $E2_{14k}$ and E3α mRNA were normalized for gel-loading differences using the 18S rRNA band. The differences in $E2_{14k}$ and E3α mRNA were statistically significant ($P < 0.05$) by a 2-tailed Student's *t* test.

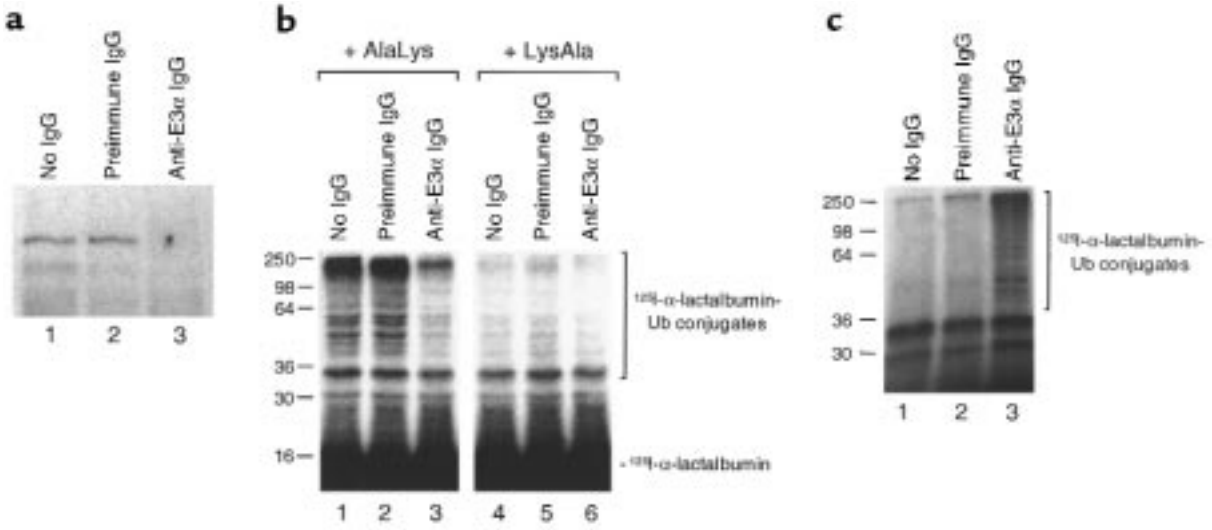

Figure 4

Depletion of E3α from rabbit reticulocyte extract by an anti E3α antiserum. Rabbit reticulocyte fraction II (50 μg) was mixed with buffer, preimmune IgG (3 μg), or anti-E3α IgG (3 μg), and rotated at 4°C for 30 hours. Protein A agarose (3 μL) was added to each sample and incubated an additional 3 hours at 4°C. After sedimentation of the agarose beads, the supernatant and washed beads were separately analyzed. (**a**) Anti-E3α immunoblot of postimmunoprecipitation supernatants. Supernatants of the above reactions were subjected to 8% SDS-PAGE, probed with anti E3α IgG at a dilution of 1:2000. Treatment of the extract with anti E3α IgG specifically removed a 200-kD immunoreactive band. (**b**) E3α activity was depleted from the postimmunoprecipitation supernatants after treatment with anti-E3α IgG. Supernatants of the above reactions were supplemented with AMPPNP (2 mM), Ub (25 μM), Ub aldehyde (1 μM), E1 (0.1 μM), $E2_{14k}$ (5 μM), and ^{125}I-α-lactalbumin (~0.75 μM). In the presence of LysAla (2 mM), a competitive inhibitor of E3α, no Ub-conjugation activity is seen (lanes 4–6). In the presence of the isomeric dipeptide, AlaLys (2 mM), which does not inhibit E3α, ^{125}I-α-lactalbumin-Ub conjugates were formed after buffer addition (lane 1) or after immunoprecipitation with preimmune IgG (lane 2). However, after immunoprecipitation with anti-E3α IgG, most of the E3α activity was abolished. (**c**) Pellets after anti-E3α IgG immunoprecipitation contained E3α activity. Washed protein A agarose beads from the above reactions (4-fold increase in amounts) were supplemented with AMPPNP (2 mM), Ub (25 μM), E1 (0.1 μM), $E2_{14k}$ (10 μM), and ^{125}I-α-lactalbumin (~0.75 μM). Only the beads that contained anti-E3α IgG supported formation of ^{125}I-α-lactalbumin-Ub conjugates.

activity as the endogenous enzymes. Because purification often results in a partial loss of enzyme activity, the relatively small increases in Ub-conjugation rates observed in these in vitro experiments probably underestimate the actual effect of increased synthesis of E3α or $E2_{14k}$ in the cell. In any case, these experiments suggest that even small increases in $E2_{14k}$ or E3α production (e.g., < 50%) in the diabetic animals can cause a 40–50% increase in ubiquitination, as was seen after streptozotocin treatment.

Levels of $E2_{14k}$ and E3α mRNA increase in the diabetic muscles. Because Ub conjugation by the N-end rule pathway was increased in muscle extracts from diabetic rats, we measured whether the genes for the components of the N-end rule pathway are induced in diabetic animals. As found previously (36), muscles from streptozotocin-treated rats contained twice as much of the $E2_{14k}$ 1.2-kb transcript as did control muscles (Figure 3), whereas the abundance of the $E2_{14k}$ 1.8-kb transcript was unchanged. A similar, specific increase in this 1.2-kb $E2_{14k}$ transcript has been seen in muscles undergoing atrophy due to sepsis (29), cancer (21), and fasting (26).

We also measured the mRNA levels of E3α, the Ub-protein ligase that functions together with $E2_{14k}$, and E1, the Ub-activating enzyme that charges E2 with Ub. The gene for E3α, also known as *UBR1* in yeast, has recently been cloned from mouse and humans (39). In muscle it is expressed as a single 6.0-kb transcript. Using the 5′ 1 kb of the mouse *UBR1* gene as a probe for E3α, we found that the E3α mRNA was also elevated about 2-fold in muscles from diabetic rats (Figure 3). These results demonstrate coordinate regulation of the 2 specific components of the N-end rule pathway. By contrast, mRNA levels for the Ub-activating enzyme, E1, which is required for all cellular ubiquitination reactions, was not increased in the muscles from diabetic rats (Figure 3a).

Preparation and characterization of anti-E3α antibody. To determine if increase in the level of E3α mRNA leads to an increase in E3α protein in muscles of diabetic rats, we prepared a polyclonal antibody to a 35-kD NH_2-terminal fragment of E3α that was expressed and purified in *E. coli*. A polyclonal anti-E3α IgG was obtained that reacted with a 200-kD protein in the rat muscle extract, in good agreement with the predicted molecular weight for the E3α gene product.

To establish that this 200-kd immunoreactive band was indeed E3α, we immunodepleted E3α from a rabbit reticulocyte extract (Figure 4).After immunodepletion, the 200-kD E3α band was no longer present in the supernatant (Figure 4a). Furthermore, when the E3α activity in the immunodepleted extracts was measured by assaying Ub conjugation to ^{125}I-α-lactalbumin (Figure 4b) in the presence of a large excess of recombinant E1 and $E2_{14k}$, E3α activity was much lower than in the untreated extracts or in extracts treated with preimmune IgG and protein A (Figure 4b, lanes 3 vs. 1 and 2). As expected, there was no formation of α-lactalbumin Ub conjugates in reticulocyte extracts in the presence of the dipeptide LysAla, a competitive inhibitor of E3α, either

before or after immunodepletion (6, 15) (Figure 4b, lanes 4–6). Thus, Ub conjugation to this substrate is by E3α rather than some other Ub-conjugating activity. It is also noteworthy that the immunoprecipitate retained E3α activity; i.e., it was capable of catalyzing Ub conjugation to α-lactalbumin in the presence of added Ub, AMPPNP, E1, and $E2_{14k}$ (Figure 4c).

Content and activity of E1, $E2_{14k}$, and E3α protein in muscle extracts. The anti-E3α antiserum, as well as specific antiserum to E1 and $E2_{14k}$, were used to compare the levels of these proteins in muscles from the insulin-deficient and control rats. No significant differences were detected in the content of E1, $E2_{14k}$, or E3α proteins in the extracts from diabetic muscles and controls (Figure 5a). To ensure that the immunoblot analysis was performed in a linear detection range, known amounts of $E2_{14k}$ or partially purified E3α were detected with the same antibodies (Figure 5b). This standardization indicates that 2-fold changes in protein level should be easily detectable.

Because no increase in the content of these proteins was demonstrable in the gastrocnemius from insulin-deficient rats, we determined if the activity of E1, $E2_{14k}$, or E3α, nevertheless, might be increased in these extracts through some posttranslational modification such as phosphorylation. E1 and E2 activity was assayed by measuring the formation of E1-Ub and E2-Ub thiolester adducts. These linkages are labile but can be seen by SDS-PAGE in the absence of reducing agents (50). The muscle extracts from diabetic and control rats showed similar abilities to activate Ub and to form E2 complexes; i.e., the patterns and intensities of E1-^{125}I-Ub and E2-^{125}I-Ub were indistinguishable (Figure 6a, lanes 2 vs. 3) and, specifically, the $E2_{14k}$-Ub complexes were of similar intensities (Figure 6a, arrowhead).

In addition, the activity of E3α was analyzed by measuring Ub-conjugation to α-lactalbumin using $(NH_4)_2SO_4$ precipitates (40%) of muscle extracts. Any difference in the rate of Ub conjugation to α-lactalbumin in the 2 extracts are due to E3α, because endogenous $E2_{14k}$ had been removed (Figure 6b, lanes 2 and 3 vs. lanes 4 and 5) and equivalent, excess amounts of $E2_{14k}$, E1 and ATP were added. In control experiments the ubiquitination of α-lactalbumin by these preparations was completely inhibited by LysAla, an inhibitor of E3α (6, 15) (data not shown). In these assays no consistent difference in E3α activity was seen in the diabetic and control extracts (Figure 6b, lanes 4 vs. 5), in accord with the measurements of E3α protein.

Discussion

The biochemical mechanisms by which skeletal muscles undergo rapid protein loss in response to endocrine signals and cytokines have been of intense interest, largely because of the debilitating effects of muscle wasting that accompanies numerous severe diseases (e.g., cancer, renal failure, sepsis). Many recent studies have suggested that the primary mechanism for this increased muscle-protein breakdown in diverse catabolic states is activation of the Ub-proteasome pathway (16). We demonstrate here that in muscles from acutely diabetic rats, both Ub-protein conjugates, the critical intermediates in this pathway, and rates of their formation are increased. These changes can account for the overall increase in proteolysis observed in these muscles (5). Recently, we also demonstrated that in 3 other experimentally induced catabolic states where muscle-protein breakdown is enhanced (i.e., septic rats, tumor-bearing rats, and rats treated with high doses of T_3 or T_4), there was also an increase in Ub conjugation in muscle extracts (34). In all these cases, this response occurs in large part through activation of the N-end rule pathway. This conclusion was based on our findings that a dominant inhibitor of E3α (C88S $E2_{14k}$) reduced the rate of Ub conjugation in the extract of diabetic muscle to below control levels and that mRNA for this E3 enzyme (E3α) and its protein cofactor, $E2_{14k}$, were elevated. Thus, activation of Ub conjugation appears to be a common adaptation in the development of muscle atrophy due to diverse causes. These findings strongly support the proposal (16, 17, 34) that there is a final, common cellular mechanism responsible for activating muscle protein degradation triggered by diverse physiological or pathological factors.

The physiological importance of the N-end rule pathway has long remained elusive because E3α recognizes selectively protein substrates with large, bulky, or charged NH_2-terminal residues, and more than 80% of intracellular proteins are either N-α-acetylated or begin with methionine (51). Deletion of the E3α homologue in yeast, UBR1, had no clear physiological consequences (52), and no physiological substrates with these abnormal amino termini have been identified in yeast or mammals. On the other hand, our recent experiments have demonstrated a major role for $E2_{14k}$ and E3α in overall Ub conjugation and protein degradation in muscle

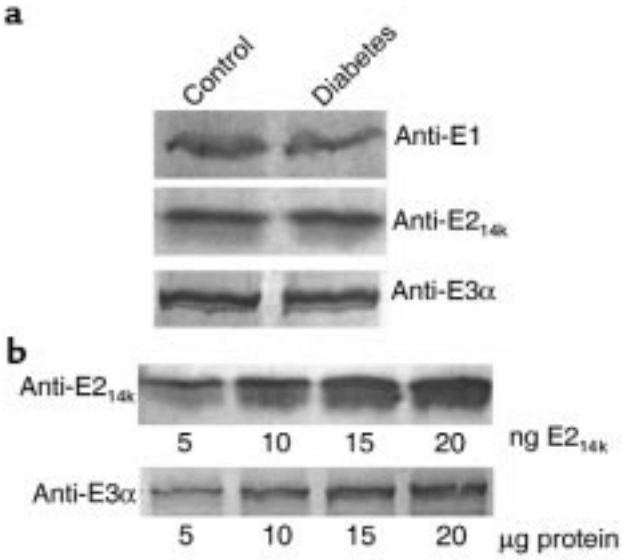

Figure 5
Protein content of E1, $E2_{14k}$, and E3α are similar in extracts from diabetic and control rats. (**a**) Immunoblots from control and diabetic muscle extracts. Muscle protein (50 μg of a crude extract for the anti-$E2_{14k}$ blot, and 50 μg muscle extract fraction II for the anti-E1 and anti-E3α blots) from control or diabetic rats was subjected to SDS-PAGE (9% for the anti-E1 blot, 15% for the $E2_{14k}$ blot, and 8% for anti-E3α blot) and probed with the appropriate polyclonal antibody. (**b**) Immunoblots of known quantities of $E2_{14k}$ and E3α. Increasing quantities of $E2_{14k}$ and E3α were subjected to immunoblot analysis as in **a** above.

extracts from normal rats, but not in cultured HeLa cells (6). Its special role in skeletal muscle may be related to a particular role in regulating overall proteolysis in muscle as a potential source of amino acids.

The finding that $E2_{14k}$ and E3α are important in the enhanced protein degradation observed in these diverse forms of muscle atrophy raises the fundamental question of how a large fraction of muscle proteins can be substrates for the N-end rule pathway. All muscle proteins are synthesized with an NH_2-terminal methionine and then, in most cases, have their NH_2-termini acetylated; both should prevent recognition by this system. One possibility is that in these catabolic states, muscle proteins are modified to expose novel NH_2-terminal residues (e.g., perhaps by endoproteolytic cleavage or deacylation) that are recognized by E3α. Alternatively, E3α might recognize other properties of protein substrates. In fact, yeast UBR1 is able to recognize some substrates (e.g., Gα and CUP9), that contain an NH_2-terminal methionine, presumably through interaction with internal regions of the proteins (53, 54). An important goal of future work will be to identify the polypeptides that undergo ubiquitination by $E2_{14k}$ and E3α in normal and atrophying muscle.

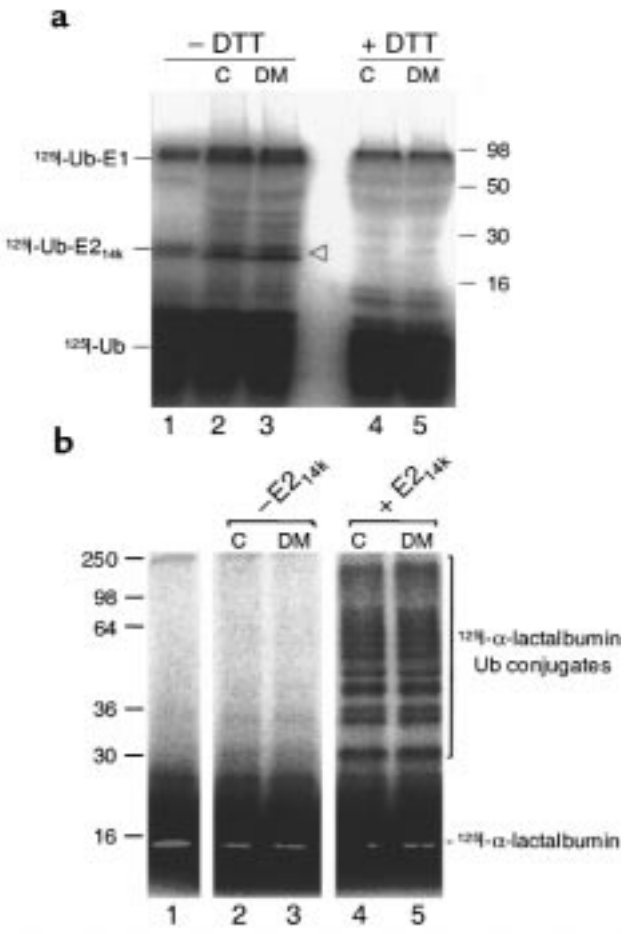

Figure 6
E1, $E2_{14k}$, and E3α activity is similar in extracts from diabetic and control rats. (**a**) E1/E2 assay. To measure formation of E1-Ub and E2-Ub adducts, fraction II of muscle extracts (100 μg) from diabetic (lanes 3 and 5) or control (lanes 2 and 4) rats were mixed with AMPPNP (2 mM) and ^{125}I-Ub (~2.5 μM) and incubated at room temperature for 10 minutes. To distinguish thiolesters from Ub-conjugated proteins, half the reaction was mixed with sample buffer in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of DTT (100 mM final concentration) and analyzed on 15% SDS-PAGE. Lane 1 contains ^{125}I-Ub (~2.5 μM), E1 (0.66 μM), $E2_{14k}$ (0.47 μM), and AMPPNP (2 mM) only. Bands present in lanes 2 and 3 but not lanes 4 and 5 reflect ^{125}I-Ub-E2 thiolester adducts. Arrowhead marks position of ^{125}I-Ub- $E2_{14k}$. The set of E2s and their abundance are the same in the extracts from the control and diabetic animals. (**b**) E3α activity in extracts of control and diabetic animals. To measure E3α activity, muscle extracts from control and diabetic animals were depleted of E2s by precipitation with $(NH_4)_2SO_4$ (40% wt/vol final concentration). The precipitated material after resuspension and dialysis was supplemented with AMPPNP, E1, ^{125}I-α-lactalbumin, and $E2_{14k}$ (lanes 4 and 5). The appearance of ^{125}I-α-lactalbumin-Ub conjugates was strictly dependent on addition of $E2_{14k}$, and no difference in rates of conjugation was seen in the 2 groups (lanes 4 and 5).

Before these studies, the rate-limiting component in the Ub-proteasome pathway, either in the degradation of specific polypeptides or tissue proteins, had not been systematically studied. We have found that both $E2_{14k}$ and E3α are rate limiting for Ub conjugation in normal muscle, as had been suggested by earlier findings (6). $E2_{14k}$ and E3α not only function together in the N-end rule pathway but their expression is coordinately regulated because mRNA for both enzymes increased in the diabetic muscles at a time when other genes (rRNA, E1) remained constant. Because E1 is required to activate ubiquitin before all its conjugation reactions, it is unlikely that E1 would be rate limiting for Ub conjugation, and, in fact, its addition to muscle extract did not augment protein degradation (6).

Surprisingly, in muscles from these insulin-deficient rats, despite the 2–3-fold increase in Ub protein conjugates, the 40–50% increase in the rate of Ub conjugation by the N-end rule pathway, and the 2-fold elevations in mRNA for $E2_{14k}$ and E3α, no consistent differences in the corresponding levels or activities of these enzymes could be demonstrated. These results emphasize the potential danger in extrapolating from measurements of increases in mRNA levels to enhanced protein expression, as has often been done in studies of the Ub-proteasome pathway in atrophying muscle (5, 8, 21–29). Whereas it is possible that $E2_{14k}$ or E3α protein levels might rise at a later time during the development of diabetes, at the time these experiments were performed (3 days after streptozotocin administration), the adaptations responsible for the major loss of muscle protein in diabetic animals are functioning because protein degradation in incubated muscles was elevated by approximately 40%, the animals had already lost more than 20% of their initial body weight, and Ub conjugation rates had already increased (36). Another example of an increase in mRNA without an increase in protein content is branched chain ketoacid dehydrogenase in the muscle of acidotic animals (55). Perhaps the high levels of mRNA for these enzymes may indicate an increased rate of their synthesis, which may be necessary just to maintain high levels of the enzymes in atrophying muscles in the face of accelerated protein breakdown.

The simplest explanation for the apparent discordance between our findings of an increase in the overall rate of Ub conjugation and both mRNA for $E2_{14k}$ and E3α in the diabetic extracts and the lack of demonstrable

increases in the amount of $E2_{14k}$ or E3α protein may stem from the lack of sufficient sensitivity in the assays used to measure these proteins or their activities. Detection of mRNA is more sensitive and requires less manipulation of the tissue than the enzymatic assays used here. In fact, immunoblot analyses can easily miss 30% differences in protein content. In addition, there is evidence in mammals of an additional isoform of UBR1 of similar molecular weight (39). If this additional form exists and is recognized by our antibody, it would be even harder to demonstrate increases in one form of E3α if the other does not change. Finally, a failure to detect small increases in $E2_{14k}$ and E3α seems quite possible in light of the data in Figure 2, which shows that small increases in $E2_{14k}$ and E3α act in an additive or synergistic manner in promoting Ub conjugation. Whereas we may have been unable to detect small increases in $E2_{14k}$ or E3α individually in muscle extracts from insulin-deficient animals, when coupled together in vivo, changes in overall Ub-conjugation rate might become detectable.

If $E2_{14k}$ or E3α changes are not responsible for the enhanced Ub conjugation and protein degradation in diabetic rats, then another explanation for these changes must exist. Ubiquitin isopeptidase activity, which is known to disassemble polyubiquitin chains (56), could, in theory, be decreased in the muscles from diabetic rats. In fact, such regulation has been proposed to be involved in memory formation in Aplysia (57). Differences in isopeptidase activity do not appear to occur in extracts from diabetic rats, however, because the addition of the isopeptidase inhibitor, ubiquitin aldehyde (58), increased Ub conjugation rates to a similar extent in extracts from control or diabetic muscles (Stewart Lecker, unpublished results). Finally, an unidentified enzyme or cofactor could be more active or induced by diabetes to act with $E2_{14k}$ and E3α to enhance Ub conjugation. Proteins that increase the efficiency and processivity of Ub-conjugation reactions (e.g., Rbx1 and homologues) have recently been found to enhance Ub conjugation to G1 cyclins (59), and a new factor termed E4 can act with E1, E2, and the Ub protein ligase to elongate Ub chains on particular protein substrates (60).

Finally, in the N-end rule pathway, there are enzymes that can modify certain polypeptides so they can be ubiquitinated by E3α. For example, arginyl tRNA protein transferase (ATE1) can add arginine to the NH_2-termini of polypeptides that bear Asp or Glu residues (61), and NH_2-terminal amidase (NTA1) can deamidate NH_2-terminal Asn and Gln residues to produce Asp and Glu, respectively (62). If these enzymes were more active or abundant in extracts from diabetic muscles, then higher rates of Ub conjugation might be seen. However, we found that α-lactalbumin, a protein that does not need further modification to be recognized by E3α (because of its NH_2-terminal lysyl residue) (46) was ubiquitinated at a faster rate in muscle extracts from diabetic rats, indicating that the enzymes that ubiquitinate α-lactalbumin (i.e., $E2_{14k}$ and E3α) are the ones changing in the diabetic extracts.

In summary, even with the new experimental tools introduced here, it is difficult to dissect critical adaptations when the magnitude of the overall changes occurring is small (20% decrease in muscle weight over 3 days and 40% change in Ub-conjugation rate) and may be multifactorial, i.e., involving smaller changes in multiple proteins. Regardless, a 20% reduction in muscle mass can be highly debilitating and, if progressive, would cause an insurmountable loss of tissue protein in the organism as a whole.

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