Ubiquitin-conjugating enzyme E2\textsubscript{14k}/HR6B is dispensable for increased protein catabolism in muscle of fasted mice

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Adegoke, Olasunkanmi A. J., Nathalie Bédard, Henk P. Roest, and Simon S. Wing. Ubiquitin-conjugating enzyme E2\textsubscript{14k}/HR6B is dispensable for increased protein catabolism in muscle of fasted mice. Am J Physiol Endocrinol Metab 283: E482–E489, 2002. First published April 30, 2002; 10.1152/ajpendo.00097.2002.—Activated skeletal muscle proteolysis in catabolic states has been linked to an upregulation of the ATP-ubiquitin-dependent proteolytic system. Previous studies suggested that the N-end rule pathway is primarily responsible for the bulk of skeletal muscle proteolysis. The activity of this pathway is dependent on the 14-kDa ubiquitin-conjugating enzyme E2\textsubscript{14k} (HR6B) and the ubiquitin protein ligase Ubr1. To address the requirement of E2\textsubscript{14k} in muscle proteolysis, we examined muscle protein metabolism in wild-type (WT) mice and mice lacking the E2\textsubscript{14k} gene (KO). There were no significant effects of genotype. The absence of any significant effect of loss of E2\textsubscript{14k} function was not due to a compensatory induction of the closely related isoformal HR6A. Total intracellular concentration of E2\textsubscript{14k} and HR6A in the WT mice was 290 ± 40 nM, but the level in the KO mice (reflecting the level of HR6A) was 110 ± 9 nM. This value is about threefold the apparent Michaelis-Menten constant (K\textsubscript{m}) of E2\textsubscript{14k} (∼40 nM) for stimulating conjugation in muscle extracts. Because the HR6A isoform has a K\textsubscript{m} of 16 nM for stimulating conjugation, the HR6A levels in the muscles of KO mice appear sufficient for supporting conjugation mediated by this pathway during fasting.

ubiquitin conjugation; starvation; muscle wasting; proteasome; muscle incubation

SKELETAL MUSCLE IS THE MAIN REPOSITORY of body proteins. Increased skeletal muscle protein catabolism occurs in many diseased and malnourishmental states. For example, in cancer, sepsis, and diabetes, increases in muscle proteolytic rates of up to 50% are described (24, 35, 48, 51). Because some of these conditions are also associated with suppression of muscle protein synthesis (15, 43, 46, and reviewed in Ref. 7), loss of muscle mass of up to 40% can occur (38, 51).

The ubiquitin system is the main cytosolic proteolytic system in eukaryotes (reviewed in Refs. 12, 55). Proteins to be degraded by this pathway are first covalently conjugated through the ε-amino residue of a lysine residue of the substrate to the carboxyl group of the terminal glycine residue of ubiquitin (reviewed in Refs. 22, 36). Studies with inhibitors of distinct intracellular proteolytic pathways have shown that, in experimental tumor implantation (8, 53), starvation (59), sepsis (24, 48, 51), diabetes (11, 35, 38), and muscle denervation (48), increased skeletal muscle protein catabolism is attributable largely to activation of the ubiquitin-dependent proteolytic system.

The conjugation of ubiquitin to proteins requires the serial actions of at least three classes of enzymes. Ubiquitin is first activated by ubiquitin-activating enzyme (E1), leading to the formation of a thiol ester bond between the carboxy-terminal glycine of ubiquitin and the active-site cysteine of the enzyme. The thiol ester-linked ubiquitin is then transferred to one member of a family of ubiquitin-conjugating enzymes (E2), which also transiently carries the ubiquitin as a thiol ester intermediate. The E2 then interacts with a member of the ubiquitin-protein ligase (E3) family. E3s play important roles by binding substrates. They fall into one of two broad classes, the HECT (homology to E6-AP carboxy terminus) domain or RING finger-containing E3s. Members of the HECT family include E6-AP and Rsp5/Nedd4. The RING finger-containing E3s may be monomeric enzymes (e.g., Ubr1/E3α, Rad5, Mdm2, c-Cbl) or members of stable protein complexes such as the anaphase-promoting complex or the Skp1-Cullin-F-box protein (36). Proteins to be degraded usually require polyubiquitination, in which a chain of at least four ubiquitin residues is attached to the protein. Polyubiquitinated proteins are subsequently recognized and degraded by the 26S proteasome, a 2,500-kDa barrel-shaped multisubunit protein complex.

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There appears to be only one major E1, as its inactivation leads to significant defects in both yeast and mammalian cells (17, 34). However, E2s exist as a large family, with 11 members in Saccharomyces cerevisiae (12, 36, 55). The E2s all contain a common core domain of ~150 amino acids and may, in addition, carry NH2- and/or COOH-terminal extensions (12, 36). These differences in primary structure confer specificities on the activities of these enzymes.

Each of these E2s appears to interact with one or more E3s, resulting in different pathways of ubiquitin conjugation, each recognizing one or more protein substrates (22, 36). The earliest described pathway was the N-end rule pathway (4, 50). This rule relates the metabolic stability of a protein to the identity of its NH2-terminal residue. This pathway requires UBC2 or the 14-kDa E2 (E214k) as the E2 and Ubr1/E3α as the recognition component of the pathway. Ubr1/E3α possesses different binding sites, one of which recognizes basic amino acids at the NH2 terminus whereas another recognizes hydrophobic amino acids. A third site recognizes substrates through features other than their NH2-terminal residue. E3α/Ubr1 specifically binds E214k/UBC2 at still another site, suggesting a model in which this E3 promotes conjugation by spatially approximating the substrate and the E2 charged with ubiquitin.

The N-end rule pathway is thought to be the main pathway of skeletal muscle protein ubiquitination (44, 45). In support of this, we and others have shown that increased proteolysis in skeletal muscle during catabolic states such as fasting (54, 56), tumor implantation (8, 14, 49, 53), sepsis (25, 51), diabetes (5, 6, 11, 29, 35), administration of proteolysis-inducing factor (30, 31), head trauma and burn injuries (16, 33), glucocorticoid administration (2), and disuse atrophy (47) is associated with two- to sevenfold increases in the gene expression of the ubiquitin-conjugating enzyme of the N-end rule, E214k. Others have reported that about twofold increases in the gene expression of E3α in muscle from diabetic and septic animals (18, 29, and reviewed in Ref. 26). In vitro ubiquitination assays with soluble extracts from normal and atrophying muscles also revealed that specific inhibitors of E3α or dominant negative forms of E214k attenuate protein ubiquitination in those extracts (44). Although these findings are highly suggestive, the significance of this pathway in muscle protein turnover in vivo remains to be firmly established.

A direct approach would be to examine in transgenic animals the effects of deletion of components of the N-end rule pathway on skeletal muscle proteolysis. E214k is the rat/rabbit homolog of the S. cerevisiae DNA repair protein UBC2 (58). Two closely related human isoforms of UBC2, HR6A and HR6B, have been previously described (27). HR6B is identical to E214k. Genetic inactivation of HR6B/E214k has been reported. Mice lacking this enzyme are infertile, exhibiting several defects in spermatogenesis (41). Because we have already shown that E214k/HR6B expression increased in skeletal muscle upon fasting and is suppressed by insulin and insulin-like growth factor I (IGF-I) (56, 57), we tested, in E214k/HR6B knockout animals, whether the increased skeletal muscle protein catabolism seen in fasting would be attenuated. Our data indicate that this conjugating enzyme is nonessential for this catabolism.

**MATERIALS AND METHODS**

**Animals.** The inactivation of the HR6B/E214k gene in the mouse has been described previously (41). Sibling wild-type and knockout mice were obtained by breeding heterozygotes. At 7–10 wk, mice were starved for 48 h, as previous studies in rats indicate significant loss of muscle mass and upregulation of E214k mRNA after such a deprivation (56). After the mice were killed, tissues were isolated and analyzed as described in the following sections. To measure dry carcass weights, the heads, paws, and tails were amputated, and the skin and visceral tissues in the abdominal and thoracic cavities were removed. The remaining carcass was then dried in an oven at 60°C for 3–4 days, at which time tissue weight remained constant. All experiments were performed in accordance with the Canadian Council on Animal Care Guidelines and were authorized by the institutional Animal Policy and Welfare Committee.

**Muscle incubations.** For in vitro measurements of proteolysis, soleus muscles were dissected and mounted on inert plastic supports derived from plastic tubing (Tygon). Incubation was carried out in Krebs-Ringer bicarbonate buffer (in mM: 120 NaCl, 5 KCl, 1 MgSO4, 1 KH2PO4, 25 NaHCO3) supplemented with 5 mM glucose, 170 μM leucine, 100 μM isoleucine, 200 μM valine, 250 μg/ml cycloheximide, and 4 μg/ml insulin, as previously described (59). Muscles were preincubated in 2 ml of buffer for 30 min. Incubation was then resumed in fresh medium and continued for 2 h. Because skeletal muscle neither synthesizes nor degrades tyrosine, the release of this amino acid, as measured fluorometrically (52), into the incubation medium serves as an index of protein degradation.

**Northern hybridization.** RNA was prepared from muscle by the guanidinium thiocyanate-CsCl method (3) and hybridization carried out as previously described (40, 56). Briefly, 10 μg of RNA were resolved on 1% formaldehyde-containing agarose gels followed by transfer to nylon membranes and cross-linking with ultraviolet light. Membranes were hybridized with a 32P-labeled 354-bp probe corresponding to bases 538–892 of the mHR6B cDNA, a region in the 3’-untranslated region of mHR6B/E214k (27). The probe from the HR6A cDNA (for HR6A Northern) corresponds to bases 275–584 of the mHR6A cDNA (27). After overnight hybridization, membranes were washed and then subjected to autoradiography. To correct for RNA loading, membranes were stripped and reprobed with a 125I-labeled 18S ribosomal RNA probe.

**Western blotting.** Endogenous levels of HR6A and HR6B/E214k proteins were determined by Western blotting as described previously (40). Proteins (50 μg) from soluble gastrocnemius muscle extracts were separated by 15% SDS-PAGE and transferred onto 0.1-μm polyvinylidene difluoride membrane. Membranes were blocked for 1 h in 5% skim milk protein in TTNS (25 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween 20), rinsed thoroughly in TTNS, and then incubated sequentially for 1 h each in primary antibody (9 μg/ml affinity-purified anti-HR6B/E214k raised against the entire enzyme) and secondary antibody (~80 μg/ml 125I-labeled goat anti-rabbit IgG) with washing in between. Antibodies were diluted in TTNS containing 2.5% BSA. Signals were quantified with a phosphoimager or by autoradiography followed by quantitative densitometry of appropriate bands on X-ray...
film. To permit absolute quantification of the sum of the HR6A and HR6B/E2\textsubscript{14k} proteins, purified HR6B/E2\textsubscript{14k} standards that had been quantitated by absorbance spectrophotometry were included on the gels. Because the antibody was raised against the entire E2\textsubscript{14k} protein and because the two proteins are 95% identical in amino acid sequence, the antibody recognizes both HR6A and HR6B/E2\textsubscript{14k} isoforms and does so equally well (data not shown). From the measurements of E2 levels per unit protein and the total protein per muscle, total E2 per muscle was calculated. Intracellular concentrations were then calculated by estimating intracellular cytoplasmic volume. Cytoplasmic volume was estimated at 0.5 ml/g tissue wet wt. This is based on previous data that indicate that intracellular aqueous volume is 0.65 ml/g tissue wet wt (13). Because these E2s are excluded from organelles (Golgi, mitochondria, endoplasmic reticulum, etc.), we assumed that cytoplasmic volume represented ~80% of the published total aqueous volume. In some studies, an antibody that was raised against a peptide corresponding to the COOH-terminal end of HR6B/E2\textsubscript{14k} and previously found to be specific for this particular isoform (41) was used. To measure levels of ubiquitinated proteins in muscle, the tissue was homogenized in 5 ml/g of 2% SDS, 50 mM Tris-Cl, pH 7.5, and 1 mM dithiotreitol (DTT), and the lysate was spun briefly to collect all of the solution at the bottom of the tube. After addition of β-mercaptoethanol to 5% (vol/vol), the lysates were boiled for 10 min and then stored frozen until use. Aliquots (50 μg of proteins) were run on a 10% SDS-PAGE gel, transferred onto nitrocellulose membrane, and blotted as above but with a monoclonal anti-ubiquitin antibody specific for ubiquitinated proteins (FK2, International Bioscience) followed by \textsuperscript{125}I-labeled goat antimouse secondary antibody.

Ubiquitination assays. The chloramine-T method was used to label bovine ubiquitin with Na\textsuperscript{125}I to a specific activity of 6,000 cpm/pmol. Unincorporated \textsuperscript{125}I was removed by passing the reaction products over a Sephadex G\textsubscript{25} column.

HR6B/E2\textsubscript{14k} was expressed in Escherichia coli and purified as previously described (39). HR6A was similarly subcloned into the pET11d expression vector (Novagen), expressed, and purified using the same protocol as for HR6B/E2\textsubscript{14k}. Quantitation of enzymatic activity was by thiol ester assays (37).

Gastrocnemius muscle homogenates were prepared from wild-type and knockout mice. Muscles were homogenized in 5 ml/g of 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 10 μg/ml pepstatin A with the use of a Polytron tissue homogenizer. Homogenates were spun in a microfuge at 16,000 × g for 10 min at 4°C. The supernatants were stored at −80°C until used in assays.

To determine the kinetic parameters of HR6A- or HR6B/E2\textsubscript{14k}-dependent conjugation in muscle homogenates, 50 μg of muscle proteins were mixed with different amounts of purified E2 enzyme in a 20-μl reaction in the presence of 50 mM Tris-HCl, pH 7.5, 2 mM MgCl\textsubscript{2}, 1 mM DTT, 2 mM 5′-adenylylimidodiphosphate (AMPPNP), 3 μM ubiquitin aldehyde, and 5 μM \textsuperscript{125}I-labeled ubiquitin. (The ATP analog AMPPNP supports conjugation of ubiquitin to proteins but does not support proteasome-mediated hydrolysis of ubiquitinated proteins. Ubiquitin aldehyde inhibits deubiquitinating enzymes that can remove ubiquitin from proteins.) Reactions were started with the addition of \textsuperscript{125}I-ubiquitin and, after 17 min, stopped with SDS-PAGE sample buffer. Pilot studies indicated that rates of conjugation were linear over this time period. Reaction products were then resolved on 10% SDS-PAGE (which separates free from conjugated ubiquitin), and the amount of ubiquitin protein conjugates formed in each reaction was determined by drying the gel and counting the radioactivity in each lane. For determination of the apparent Michaelis-Menten constant (K\textsubscript{m}), data from conjugation assays were fitted to a nonlinear regression function that corrects for endogenous conjugation due to other E2s, by using the equation f = c + V\textsubscript{m}[S]/(K\textsubscript{m} + [S]), where f is the reaction rate, c is the basal rate of conjugation in the absence of the E2 being tested, [S] is the concentration of the E2, V\textsubscript{m} is the rate at saturating [S], and K\textsubscript{m} equals [S] at one-half V\textsubscript{m}.

Statistical analyses. One-way analysis of variance or Student’s t-test was used to compare means. In experiments examining the effects of genotype and nutritional states (starved or fed), a 2 × 2 analysis of variance was employed and means were separated by the Student-Newman-Keuls test.

RESULTS

Effects of fasting on body mass, visceral tissues, and skeletal muscle. Gross phenotypes of HR6B/E2\textsubscript{14k} knockout mice have been reported previously (41). These mice appear normal except for defects in spermatogenesis in the testes. Here, we examined the responses of these mice to food deprivation. In the fed state (Table 1), there were no effects of loss of E2\textsubscript{14k} on body weight or weights of most of the visceral tissues. However, the weights of the liver and small intestine were found to be ~10% higher in the E2\textsubscript{14k} knockout mice. In response to fasting, the losses of body and tissue masses were similar in wild-type and knockout mice. This was true also in the liver and the small

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fed</th>
<th>Starved</th>
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<tr>
<td>HR6B/E2\textsubscript{14k} Wild Type</td>
<td>22.7 ± 0.5</td>
<td>16.6 ± 0.5</td>
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<tr>
<td>HR6B/E2\textsubscript{14k} Knockout</td>
<td>17.6 ± 0.5</td>
<td>12.8 ± 0.5</td>
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<tr>
<td>P Values*</td>
<td>NS</td>
<td>0.001</td>
</tr>
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Values are means ± SE; n = 6–8 mice per group. E2\textsubscript{14k}, 14-kDa ubiquitin-conjugating enzyme; NS, not significant. Wet weights of liver and intestine were significantly larger in knockout mice than in wild-type mice in fed and starved states. Except for the stomach, tissue weights were decreased upon fasting in wild-type and knockout mice. *There were no statistically significant interactions between genotype and starvation in any of the parameters examined.
Table 2. Carcass and skeletal muscle weights and muscle protein contents of HR6B/E2\textsubscript{14k} wild-type and knockout mice in response to fasting

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Knockout</th>
<th>Gene</th>
<th>P Values*</th>
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<td></td>
<td>Fed</td>
<td>Starved</td>
<td>Fed</td>
<td>Starved</td>
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<tr>
<td>Wet carcass, g</td>
<td>8.7 ± 0.3</td>
<td>6.7 ± 0.2</td>
<td>8.6 ± 0.2</td>
<td>6.9 ± 0.2</td>
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<tr>
<td>Dry carcass, g</td>
<td>2.8 ± 0.09</td>
<td>2.0 ± 0.08</td>
<td>2.8 ± 0.08</td>
<td>2.1 ± 0.08</td>
</tr>
<tr>
<td>Gastrocnemius, mg</td>
<td>238 ± 7</td>
<td>201 ± 6</td>
<td>242 ± 7</td>
<td>205 ± 4</td>
</tr>
<tr>
<td>Gastrocnin protein</td>
<td>0.145 ± 0.01</td>
<td>0.122 ± 0.01</td>
<td>0.151 ± 0.01</td>
<td>0.139 ± 0.01</td>
</tr>
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</table>

Data are means ± SE; n = 6–8 mice per group. *There were no statistically significant interactions between genotype and starvation in any of the parameters examined. Gastrocnemius (Gastroc) protein is expressed in mg protein/mg muscle wet weight.

Gene       Starvation

Fed Starved Fed Starved Gene Starvation

Wet carcass, g 8.7 ± 0.3 6.7 ± 0.2 8.6 ± 0.2 6.9 ± 0.2 NS<br>Dry carcass, g 2.8 ± 0.09 2.0 ± 0.08 2.8 ± 0.08 2.1 ± 0.08 NS<br>Gastrocnemius, mg 238 ± 7 201 ± 6 242 ± 7 205 ± 4 NS<br>Gastrocnin protein 0.145 ± 0.01 0.122 ± 0.01 0.151 ± 0.01 0.139 ± 0.01 0.08<br>0.013

Effects on rates of muscle proteolysis. Despite the aforementioned observations, subtle differences at the level of muscle protein degradation might not be easily noticed in the measurements described. Because the N-end rule pathway appears to be involved in mediating the ubiquitination of the bulk of muscle proteins before their degradation by the proteasome (44), we determined whether the loss of E2\textsubscript{14k}, the conjugating enzyme of this pathway, would lower rates of muscle proteolysis. We carried out these measurements in the soleus muscle, which has adequate tendon sizes in the mouse to permit reproducible stretching to resting length in in vitro incubations (the importance of which is reported in Ref. 9) and thereby permit reliable measurements of rates of proteolysis. Although fast-twitch muscles appear more sensitive to fasting than slow-twitch muscles such as the soleus (59), the use of the soleus in the mouse is probably less susceptible to this effect because it has a mixture of both fast- and slow-twitch fibers (23). Rates of proteolysis were similar in muscles from wild-type and knockout mice in the fed state (Table 3). This was also true in the fasted state. However, the significance of the measurements in the fasted state is less clear, because older animals had to be used for these studies to yield muscles that remained large enough after fasting to technically permit reliable stretching during the incubation. Unfortunately, older animals show less of an increase in proteolysis upon fasting (21, 32); accordingly, we were unable to detect a significant increase in the rate of proteolysis in the muscles from wild-type animals.

Effects on ubiquitination of skeletal muscle proteins and expression of the ubiquitin-conjugating enzymes E2\textsubscript{14k}/HR6B and HR6A. Although we could not detect differences between the two genotypes in overall rates of proteolysis, it remained possible that conjugation of ubiquitin to proteins might be affected by the loss of this E2. Therefore, we measured steady-state levels of ubiquitinated proteins in the muscles in the fed and fasted states (Fig. 1). As previously shown in rats (60), there is an increase in levels of ubiquitinated proteins upon fasting. However, despite the absence of HR6B/E2\textsubscript{14k} in the knockout mice, levels of ubiquitinated proteins were similar in the fed state and rose similarly upon fasting.

Because the inactivation of E2\textsubscript{14k} did not yield any apparent effects on overall muscle mass, muscle protein content, rates of proteolysis, or levels of ubiquitinated proteins, we decided to confirm that expression of this E2 rises upon fasting in the mouse muscle as in the rat (Fig. 2). RNA blot analysis with an E2\textsubscript{14k}/HR6B probe showed that, in some knockout mice, faint signals persist that are likely due to some cross-reactivity of the probe with the HR6A transcripts (Fig. 2A). Consistent with this, heterozygous mice show levels that are intermediate between those found in wild-type and knockout mice (data not shown). Immunoblot analysis with antibody specific to E2\textsubscript{14k}/HR6B (41) con-

Table 3. Rates of proteolysis in soleus muscles of HR6A/E2\textsubscript{14k} wild-type and knockout mice are similar in fed and starved states

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<th>Wild Type</th>
<th>Knockout</th>
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<tr>
<td>Rate of proteolysis, nmol tyrosine·mg\textsuperscript{-1}·2 h\textsuperscript{-1}</td>
<td>0.297 ± 0.02</td>
<td>0.343 ± 0.02</td>
<td>0.309 ± 0.02</td>
<td>0.301 ± 0.02</td>
</tr>
<tr>
<td>Muscle weight, mg</td>
<td>9.59 ± 0.6</td>
<td>8.78 ± 0.5</td>
<td>9.37 ± 0.6</td>
<td>8.62 ± 0.5</td>
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Values are means ± SE. Soleus muscles from fed or starved mice of either genotype (n = 6–8) were isolated and incubated in vitro, as described in MATERIALS AND METHODS. Proteolysis was measured as a release of tyrosine into the medium. In fed or fasted states, there were no statistically significant differences in the rates of proteolysis (genotype, P = 0.4; starvation, P = 0.32; interaction, P = 0.2) or muscle weights (genotype, P = 0.7; starvation: P = 0.2; interaction, P = 1) between wild-type and knockout mice.
firmed the absence of protein expression in the knockout mice (Fig. 2C). Upon fasting, there was indeed an increase in E2\textsubscript{14k} mRNA levels in skeletal muscle of wild-type mice, indicating that the lack of effect of gene inactivation was not due to a different response in mice compared with rats (Fig. 2B). No increase was seen in the faint signal in the knockout mice, also supporting the notion that the signal is due to some cross-reactivity with another transcript.

We next determined whether the lack of effect of inactivation of the E2\textsubscript{14k}/HR6B gene was due to a compensatory increase in protein levels of the closely related isoform HR6A. We therefore measured total levels of E2\textsubscript{14k}/HR6B and HR6A proteins (Table 4) by use of polyclonal antibodies generated against the complete E2\textsubscript{14k}/HR6B protein and that detect HR6A protein in a quantitatively similar extent (data not shown). Indeed, the combined levels of these two enzymes were reduced by 60% in the knockout mice, arguing that the lack of effects of the gene inactivation are not due to a compensating increase in HR6A protein levels. Finally, Northern hybridization using DNA probes specific for the HR6A gene showed that there was no induction of this gene in the basal state or in response to fasting in knockout animals (Fig. 2D).

![Figure 1](http://ajpendo.physiology.org/)

**Fig. 1.** The increase in steady-state levels of ubiquitinated proteins in muscle upon fasting is not affected by inactivation of the HR6B/E2\textsubscript{14k} (14-kDa ubiquitin-conjugating enzyme) gene. Wild-type or knockout mice that were either fed as usual or fasted for 48 h (n = 9–12 mice per group) were killed and the gastrocnemius muscles removed. After homogenization in SDS-containing buffer, equal amounts of protein were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. After hybridization with anti-ubiquitin antibody and \textsuperscript{125}I-labeled goat anti-mouse secondary antibody, the membranes were exposed to X-ray film. Autoradiography signals were quantified with a densitometer. Top: representative blot. Bottom: quantitation of samples showing means ± SE.

![Figure 2](http://ajpendo.physiology.org/)

**Fig. 2.** Expression of HR6B/E2\textsubscript{14k} and HR6A in gastrocnemius muscle of wild-type and knockout mice in fed and 2-day-fasted states. A: levels of HR6B/E2\textsubscript{14k} mRNA increase upon fasting in wild-type mice: representative blot. B: quantitation of HR6B/E2\textsubscript{14k} mRNA levels in wild-type and knockout mice in fed and fasted states (P < 0.05, n = 10–12 mice per group). In wild-type mice, mean level in fasted animals was significantly different from that in fed animals. C: HR6B/E2\textsubscript{14k} protein is not induced in response to 2 days of fasting and is absent in knockout mice. Muscle proteins from the indicated mice were analyzed by Western blot analysis using antibody specific to the HR6B/E2\textsubscript{14k} isoforms. D: levels of HR6A mRNA are similar in wild-type and knockout mice and are not induced upon fasting.
Levels of the HR6A enzyme isoform appear sufficient to maintain overall E214k/HR6B- and HR6A-dependent ubiquitination in skeletal muscle. Because we had previously shown that E214k/HR6B and HR6A isoforms of UBC2 together are responsible for ~50% of the rate of conjugation in muscle extracts (39), we were surprised that the loss of HR6B/E214k did not affect steady-state levels of ubiquitinated proteins. We therefore measured the dependence of conjugation in these muscle extracts on concentrations of E214k/HR6B and HR6A. As seen in Table 4, the apparent $K_m$ values of HR6B/E214k for stimulating conjugation are ~40 nM and are similar in muscle extracts of wild-type and knockout mice. Such values are low compared with the estimated muscle concentrations of enzyme both in wild-type (288 nM) and in knockout (114 nM) mice. Thus, if HR6A has a $K_m$ similar to E214k/HR6B for stimulating conjugation, the rates of conjugation would likely not decrease to a significant extent. In fact, the apparent $K_m$ values of HR6A for stimulating conjugation are ~20 nM and are similar in muscle extracts of wild-type and knockout mice (Table 4).

**DISCUSSION**

These studies are the first to evaluate the role of E214k in vivo in a condition of skeletal muscle atrophy and show that this enzyme is not essential for mediating the increase in muscle protein catabolism upon fasting. Interestingly, however, there were small increases in the weights of the liver and small intestine in the knockout mice. These observations are consistent with some findings that indicate that the ATP-ubiquitin proteolytic system may be important in the regulation of intestinal mass and protein content (1, 42). We are currently exploring further the basis for these differences.

The lack of effect of inactivation of the E214k/HR6B gene on protein catabolism in skeletal muscle was surprising, because numerous studies suggest strongly that the N-end rule pathway of ubiquitin conjugation plays an important role in mediating the activation of proteolysis in skeletal muscle atrophying in various catabolic conditions. In particular, increased HR6B/E214k expression has been reported in a wide array of catabolic conditions (see introductory remarks for details). Most of these studies demonstrated increased expression of the mRNA. Levels of E214k/HR6B protein have been less widely reported on, as antibodies generally are unable to resolve E214k/HR6B from the closely related but unregulated form HR6A. We have not seen increased levels of total HR6B and HR6A in muscle upon fasting (data not shown). Our studies using antibodies specific to E214k/HR6B suggest that E214k/HR6B levels in muscle are similar in the fed and fasted states (Fig. 2C), so the increased mRNA levels are likely required to maintain levels of this enzyme in the face of overall net protein catabolism in this tissue. All of these previous studies together argued that, whether it is to raise or maintain levels, the induction of E214k/HR6B plays some role in mediating the increased rates of proteolysis seen in these tissues. In the absence of any effect of the gene inactivation, it remains mysterious why the induction of this mRNA is so frequently seen in such a variety of catabolic conditions.

The ability of these knockout mice to activate ubiquitin conjugation and protein catabolism in response to fasting may be explained in several ways. First, our data suggest that, despite the much lower total E214k/HR6B and HR6A levels in the muscle of knockout mice, the residual levels are not limiting and are sufficient to maintain near-maximal rates of ubiquitin conjugation (Table 4). Indeed, total HR6B and HR6A levels appear to be similarly high in most other tissues, exceeding widely the apparent $K_m$s for supporting overall conjugation in extracts of those tissues (39). Thus examination of mice lacking both HR6A and HR6B would appear necessary to further examine the role of these E2s. Interestingly, although total HR6A and HR6B levels are similarly high in the testis, loss of HR6B alone does result in defective spermatogenesis (41). Thus, although levels of HR6A may be sufficient to support overall conjugation, there may be specific E3s in male germ cells that have lower affinities for HR6B or both isoforms and require higher levels to mediate ubiquitination. Alternatively, it remains possible that concentrations of these E2s are different in various subcellular compartments or locations and so may be limiting at some of these intracellular sites.

The lack of effect of loss of E214k/HR6B is consistent with the recent report (28) that inactivation of the Ubr1/E3α gene in mice also has no major effect on

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**Table 4. Combined intracellular concentrations of HR6B/E214k and HR6A and kinetic parameters for the ability of HR6B/E214k and HR6A to support conjugation of ubiquitin to substrates in muscle extracts from wild-type and knockout mice**

<table>
<thead>
<tr>
<th></th>
<th>HR6B/E214k-Dependent Conjugation</th>
<th>HR6A-Dependent Conjugation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Knockout</td>
</tr>
<tr>
<td>$K_m$, nM</td>
<td>43 ± 10</td>
<td>39 ± 12</td>
</tr>
<tr>
<td>$V_{max}$, fmol Ub/min</td>
<td>92 ± 21</td>
<td>164 ± 37</td>
</tr>
<tr>
<td>Endogenous HR6B/E214k conc, nM</td>
<td>288 ± 41</td>
<td>114 ± 9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Assays were done as described in MATERIAL AND METHODS. HR6A, closely related isoform of HR6B/E214k; $K_m$, Michaelis-Menten constant; $V_{max}$, maximum velocity; Ub, ubiquitin, conc, concentration. Note that because the antibody used in determining enzyme concentrations recognizes both HR6A and HR6B (E214k) proteins, the values are for combined HR6B/E214k in wild-type but for only HR6A protein in knockout mice. *Comparison between wild-type and knockout mice, $P < 0.05$, $n = 11$ (wild type) or 12 (knockout).
muscle mass in the fed state. Muscle mass was slightly smaller but proportionately so to the decrease in overall body size in these animals. However, the existence of the closely related isoforms Ubr2 and Ubr3, as well as the embryonic lethality of Ubr1/Ubr2 knockout mice, also makes it difficult to decide conclusively whether the N-end rule pathway is involved in the activation of muscle proteolysis.

Alternatively, these data may argue that the activation of ubiquitin conjugation seen in such conditions occurs by a non-N-end rule but ubiquitin-dependent mechanism or that other pathways of proteolysis are activated. Although we cannot rule out these possibilities, we did not observe any differences in rates of proteolysis in muscles of wild-type and knockout mice measured in the presence of inhibitors of lysosomal and calcium-dependent proteases (data not shown). Of interest, the induction of two other E3s in atrophying skeletal muscles has been recently reported (10, 20). The precise E2s with which these E3s interact remain unknown. One of these E3s possesses an F-box, suggesting that they likely interact with a member of the UBC4 family of E2s or with CDC34. The other contains a RING finger (10) and so may interact with one of several E2 families, including E2-14k/HR6A/HR6B. Importantly, inactivation of either of these E3s in mice results in decreased atrophy in response to denervation (10). However, neither gene inactivation completely suppressed wasting, which in denervation has been shown to be due solely to activated proteolysis (19). Thus both and possibly additional pathways of ubiquitin conjugation and potentially non-ubiquitin-dependent proteolytic pathways may contribute synergistically to mediate this process. Thus it appears that there is not one common pathway of proteolysis in atrophying skeletal muscles and that the mechanisms of muscle protein wasting and their regulation are more complex than previously envisioned.

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REFERENCES


