Hyperthermia stimulates energy-proteasome-dependent protein degradation in cultured myotubes

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Luo, Guang-Jiu, Xiaoyan Sun, and Per-Olof Hasselgren. Hyperthermia stimulates energy-proteasome-dependent protein degradation in cultured myotubes. Am J. Physiol. Regulatory Integrative Comp. Physiol. 278: R749–R756, 2000.—Previous studies suggest that elevated temperature stimulates protein degradation in skeletal muscle, but the intracellular mechanisms are not fully understood. We tested the role of different proteolytic pathways in temperature-dependent degradation of long- and short-lived proteins in cultured L6 myotubes. When cells were cultured at different temperatures from 37 to 43°C, the degradation of both classes of proteins increased, with a maximal effect noted at 41°C. The effect of high temperature was more pronounced on long-lived than on short-lived protein degradation. By using blockers of individual proteolytic pathways, we found evidence that the increased degradation of both long-lived and short-lived proteins at high temperature was independent of lysosomal and calcium-mediated mechanisms but reflected energy-proteasome-dependent degradation. mRNA levels for enzymes and other components of different proteolytic pathways were not influenced by high temperature. The results suggest that hyperthermia stimulates the degradation of muscle proteins and that this effect of temperature is regulated by similar mechanisms for short- and long-lived proteins. Elevated temperature may contribute to the catabolic response in skeletal muscle typically seen in sepsis and severe infection.

ubiquitin; skeletal muscle; temperature

ONE OF THE METABOLIC HALLMARKS of sepsis is the catabolic response in skeletal muscle, mainly reflecting increased protein breakdown, in particular myofibrillar protein breakdown (15, 17). Although glucocorticoids and the proinflammatory cytokines tumor necrosis factor and interleukin-1 are important mediators of sepsis-induced muscle cachexia (24, 28, 29), previous studies suggest that the elevated temperature (fever) that typically accompanies sepsis and severe infection may contribute to the increase in muscle proteolysis (14). Thus when incubated rat muscles or cultured myotubes were subjected to hyperthermia, protein degradation increased (3, 22), and this effect of high temperature was especially pronounced in muscles from septic rats (14).

Intracellular protein degradation is regulated by multiple proteolytic pathways, including lysosomal, calcium-dependent, and energy-proteasome-dependent mechanisms (1, 15). Although previous studies suggest that hyperthermia stimulates nonlysosomal, calcium-independent proteolysis in incubated rat muscles (3) and increases the chymotrypsin-like activity of the 26S proteasome in cell-free fractions of C2C12 myotubes (22), the role of the proteasome-dependent pathway in temperature-induced muscle proteolysis needs to be further defined. For example, it is not known if the increase in protein degradation caused by elevated temperature is energy dependent or can be blocked by specific proteasome inhibitors. In addition, the differential effects of high temperature on long-lived and short-lived muscle proteins and the involvement of different proteolytic pathways in temperature-induced changes in the degradation of different classes of proteins are not known.

In the present study, the role of different proteolytic pathways in the response to hyperthermia of both long-lived and short-lived proteins was determined in cultured L6 myotubes. This was done by using specific metabolic inhibitors, including the proteasome inhibitor β-lactone (7), and by incubating the myotubes in normal or energy-depleting medium. Results suggest that hyperthermia stimulates degradation of both long-lived and (to a lesser extent) short-lived proteins and that the effect of high temperature on the degradation of both classes of proteins is energy and proteasome dependent.

MATERIALS AND METHODS

Cell culture. L6 rat skeletal muscle cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown and maintained as described in detail recently (25) and were used between passages 2 and 8. Cells were seeded in 24-well culture plates (2.5 × 10⁶ cells/well) or 10-cm dishes (2.5 × 10⁶ cells/dish). Experiments were performed when ~90% of the cells had formed myotubes. Cell viability was assessed by determining trypan blue dye (5%) exclusion.

The L6 myoblast cell line was originally developed by Yaffe (27) from trypsin-suspended thigh muscle cells of newborn rats. During culture, the cells differentiate into multinucleated fibers that become cross-striated and develop contractility, thus resembling mature muscle cells. L6 muscle cells were used in previous studies examining the regulation of protein turnover, and results from those experiments suggest that the response in the myotubes to various treatments, including treatment with dexamethasone, calcium, and hor-
mones, is similar to the response seen in vivo or in incubated intact muscles (9, 25, 26). Thus, although results obtained in vitro in cultured cells always need to be interpreted with caution, regulatory mechanisms of protein turnover in L6 myotubes are likely to reflect mechanisms in muscle cells in vivo.

Protein degradation. Protein degradation was measured by determining the release of TCA-soluble radioactivity from proteins labeled with L-[3,5-3H]tyrosine (New England Nuclear, Boston, MA) as described previously by Hong and Forsberg (19) and with modifications described in detail recently (25). For determination of long-lived protein degradation, myotube proteins were labeled with 1 μCi/ml of L-[3,5-3H]tyrosine for 48 h, and for measurement of short-lived protein degradation, myotubes were labeled with 2 μCi/ml of L-[3,5-3H]tyrosine for 1 h. Cells were then washed twice with Hank’s balanced salt solution, and nonradioactive DMEM containing 2 mM tyrosine was added to the myotubes. Protein degradation was determined by measuring the release of TCA-soluble radioactivity during 6 h incubation at 37, 39, 41, or 43°C. Protein degradation was expressed as the percentage protein degraded per 6 h, calculated as described in detail elsewhere (25). The role of different proteolytic pathways in the regulation of protein degradation was assessed by treating the myotubes with inhibitors of the individual proteolytic pathways as described in RESULTS. The involvement of energy-dependent protein degradation was determined by adding 2-dinitrophenol (0.2 mM) to the culture medium. This treatment resulted in a substantial reduction of myotube ATP levels in a recent study from our laboratory (25).

To validate the method used here to measure protein degradation, control experiments were performed in which protein breakdown was assessed by determining the amount of free tyrosine released from the myotubes. Myotubes were incubated for 6 h at 37 or 41°C in tyrosine-free DMEM containing 0.5 mM cycloheximide. This was analogous to the method used to measure protein breakdown in incubated intact rat muscles (17, 24). Cycloheximide was present in the medium to prevent reincorporation of released tyrosine into protein during incubation. At the end of incubation, tyrosine was determined in the medium by high performance liquid chromatography, and cell protein was measured as described previously (17, 24).

Protein synthesis. Myotubes were cultured as described above for 6 h at 37, 39, 41, or 43°C. L-[3,5-3H]tyrosine (2 μCi/ml; 2 mM) was added to the medium during the last 1 h of incubation, and protein synthesis was assessed by determining the amount of radioactivity incorporated into TCA-precipitated proteins as described in detail recently (25).

mRNA levels. When mRNA levels were determined, myotubes were cultured in 10-cm dishes to increase the amount of tissue. Cells were cultured for 6 h pair-wise at 37 or 41°C, where after mRNA levels for ubiquitin, the proteasome subunit C7, m- and μ-calpain, and the muscle-specific calpain p94 and cathepsin B were determined by dot blot hybridization. RNA was extracted by the guanidinium thiocyanate-pheno1-chloroform method (5) using an RNA STAT-60 kit (Tel-Test “B,” Friendswood, TX). Dot blot hybridization was carried out under stringent conditions [50% formamide, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 56°C]. The cDNA probes were labeled with digoxigenin (DIG)-11-dUTP (Boehringer Mannheim, Indianapolis, IN) by random labeling. RNA was quantified by spectrophotometry and denatured by boiling for 5 min. Three amounts of RNA (20, 10, and 5 μg) from each sample were blotted on a nylon membrane (Boehringer Mannheim) using a Minifold II slot-blot filtration manifold (BioRad, Hercules, CA) and fixed to the membrane by ultraviolet cross-linking for 5 min. Prehybridization was carried out at 56°C for 4 h in a buffer consisting of 50% formamide, 7% SDS, 50 mM sodium phosphate (pH 7.0), 2% blocking reagent (Boehringer-Mannheim), 5× SSC, and 0.1% N-laurolysarcosine. Hybridization was carried out overnight at 56°C in the same buffer with 25 ng/ml DIG-labeled cDNA probe. After hybridization, the membranes were washed twice in 2× SSC, 0.1% SDS for 5 min at room temperature and twice in 0.1 SSC, 0.1% SDS for 15 min at 68°C. Chemiluminescence was detected by using 4-DIG alkaline phosphatase-conjugated Fab fragment (37.5 mU/ml; Boehringer Mannheim) and the substrate CDP-Star (0.25 mM; Boehringer Mannheim). The membranes were then exposed to X-ray film (X-Omat, Eastman-Kodak, New Haven, CT) for 1–4 h, and the signal intensities were determined by densitometry. The ratio between the signal intensities at 41 and 37°C was calculated for the 5, 10, and 20 μg RNA dots, and the mean of these calculations was used for each experiment. The cDNA probes were obtained as described previously (25).

Statistics. Results are presented as means ± SE. Two-way analysis of variance followed by Tukey’s test was used for statistical analysis, except in paired experiments, when paired Student’s t-test was used.

RESULTS

Degradation of long-lived proteins. Under basal conditions (37°C), long-lived proteins were degraded at a rate of ~10% per 6 h, similar to previous reports from our and other laboratories (19, 25). Subjecting the cultured L6 myotubes to hyperthermia resulted in increased protein degradation, with a maximal effect of ~30% noted at a temperature of 41°C (Fig 1). In most subsequent experiments, protein degradation was measured at 37 or 41°C. Cell viability determined by trypan blue dye exclusion was >98% at all temperatures tested. The increase in protein degradation at higher
temperatures noted here is similar to results in previous studies in which intact incubated rat muscles (3, 14) or cultured mouse skeletal muscle cells (22) were subjected to hyperthermia.

To validate the method used here to measure protein degradation, control experiments were performed in which protein breakdown was assessed as release of free tyrosine from myotubes incubated in the presence of 0.5 mM cycloheximide. When myotubes were incubated for 6 h at 37 or 41°C, the release of tyrosine was 0.61 ± 0.08 and 0.83 ± 0.06 nmol/mg protein, respectively (n = 6 paired observations; P < 0.05 by Student's paired t-test). This increase in protein degradation (36%) was somewhat more pronounced than the increase noted in the [3H]tyrosine experiments (compare with Fig. 1). Because the results in the control experiments were expressed as nanomoles tyrosine per milligram protein, it is possible that the results were influenced by reduced amount of protein at the high temperature. Results were therefore also calculated as nanomoles tyrosine per well and were 2.11 ± 0.06 and 2.46 ± 0.05 nmol tyrosine/well at 37 and 41°C, respectively (+17%, P < 0.05 by Student's paired t-test).

To assess the involvement of different proteolytic pathways in the hyperthermia-induced proteolysis, we next determined the effect of elevated temperature on protein degradation in the presence of different protease inhibitors. First, the role of lysosomal protein degradation was assessed by treating the cells with 100 µM leupeptin or 10 mM methylamine, substances and concentrations that have been shown in other experiments to block lysosomal protein degradation (3, 20). The effect of hyperthermia (41°C) was almost identical in the presence or absence of either of these substances, indicating that lysosomal activity was not essential for the effect of hyperthermia on protein degradation (Fig. 2). Protein degradation was reduced by 10–15% by leupeptin and methylamine at both temperatures, suggesting that basal protein breakdown in the myotubes was partly regulated by lysosomal activity.

To determine the role of calcium-dependent proteolysis in the response to hyperthermia, we next examined the effect of elevated temperature on protein breakdown in the absence of calcium, in the presence of 100 µM of the calpain inhibitor E-64 (23), or with a combination of these conditions. The effect of hyperthermia (41°C) was almost identical in normal and calcium-free medium and in medium with E-64, suggesting that hyperthermia-induced increase in protein degradation was independent of calcium and calpain activity (Fig. 3). The lower protein breakdown rates noted at both temperatures in myotubes incubated without calcium suggest that basal protein degradation was at least in part calcium dependent.

The role of the proteasome in temperature-induced increase in myocyte protein degradation was assessed by incubating cells in energy-depleting medium or in medium containing 100 µM N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LNNL) or 100 µM β-lactone. These treatments substantially reduced basal protein degradation rates, consistent with previous experiments using incubated intact rat muscles (8, 18). The effect of hyperthermia (41°C) on protein breakdown was abolished in myotubes treated with LNNL, β-lactone, or energy-depleting medium (Fig. 4), suggesting that the increase in protein degradation induced by elevated temperature is caused by an energy-proteasome-dependent mechanism.

![Fig. 2](http://apreghy.org/physiology/ajpregu/vol75/i11/f2.jpg)  

degradation of long-lived proteins in cultured L6 myotubes subjected to hyperthermia (41°C; hatched bars) or cultured at 37°C (open bars). Effect of hyperthermia was tested in presence of lysosomal protease inhibitors leupeptin (Leup; 100 µM) or methylamine (Methy; 10 mM) or in absence of protease inhibitor (Ctr). n = 6 for each group. *P < 0.05 vs. 37°C; †P < 0.05 vs. corresponding control group.

![Fig. 3](http://apreghy.org/physiology/ajpregu/vol75/i11/f3.jpg)  

degradation of long-lived proteins in cultured L6 myotubes subjected to hyperthermia (41°C; hatched bars) or cultured at 37°C (open bars). Effect of hyperthermia was tested in presence of 100 µM of calpain inhibitor E-64 or calcium-free medium or in the absence of protease inhibitor (Ctr). n = 6 for each group. *P < 0.05 vs. 37°C; †P < 0.05 vs. corresponding control group.
Degradation of short-lived proteins. To study the effect of hyperthermia on the degradation of short-lived proteins, myotube proteins were labeled for 1 h with $[^3H]$tyrosine, where after the release of TCA-soluble radioactivity was measured over 6 h as described previously (25). As expected, the basal breakdown rate of short-lived proteins was several times higher than that of long-lived proteins and was ~70% per 6 h. In previous reports as well, the breakdown rate of short-lived proteins determined in a similar fashion as here was ~5–7 times higher than the breakdown rate of long-lived proteins (12, 13). It should be noted that the technique used here to measure protein breakdown gives an average of breakdown rates for multiple individual proteins, and because the breakdown rates between individual proteins can vary substantially, results need to be interpreted with caution. Hyperthermia stimulated the degradation of short-lived proteins and similar to the response of long-lived proteins, a maximum effect was seen at 41°C (Fig. 5). The relative effect of hyperthermia on the breakdown of short-lived proteins, however, was smaller than that seen for long-lived proteins. Thus at 41°C, the degradation of short-lived proteins was increased above basal level by ~5% compared with an ~20–30% increase of long-lived protein degradation (compare with Fig. 1).

The role of different proteolytic pathways in the hyperthermia-induced breakdown of short-lived proteins was tested in a similar way as described above for long-lived proteins. Addition of leupeptin (100 µM) or methylamine (10 mM) to the culture medium did not prevent the increase in protein degradation seen at 41°C, suggesting that the effect of hyperthermia did not require lysosomal proteolytic activity (Fig. 6). The effect of hyperthermia on the degradation of short-lived proteins was also maintained in the presence of E-64 and in the absence of calcium in the medium, suggesting that the effect of hyperthermia was not caused by calcium-dependent or calpain-mediated protein breakdown (Fig. 7). To test the role of energy-proteasome-dependent proteolysis for the temperature-induced increase in short-lived protein degradation, myotubes were next treated with β-lactone or energy-depleting

Fig. 4. Degradation of long-lived proteins in cultured L6 myotubes subjected to hyperthermia (41°C; hatched bars) or cultured at 37°C (open bars). Effect of hyperthermia was tested in presence of proteasome inhibitors N-acetyl-L-leucinyll-leucinal-L-norleucinal (LLnL; 100 µM) or β-lactone (β- Lac; 100 µM) or energy blocker DNP (0.2 mM) or in absence of protease inhibitor (Ctr). n = 6 for each group. *P < 0.05 vs. 37°C.

Fig. 5. Effect of different temperatures on short-lived protein degradation in cultured L6 myotubes. n = 6 for each data point. *P < 0.05 vs. 37°C.

Fig. 6. Degradation of short-lived proteins in cultured L6 myotubes subjected to hyperthermia (41°C; hatched bars) or cultured at 37°C (open bars). Effect of hyperthermia was tested in presence of lysosomal protease inhibitors leupeptin (100 µM) or methylamine (Methy; 10 mM) or in absence of protease inhibitor (Ctr). n = 6 for each group. *P < 0.05 vs. 37°C.
medium. Basal protein breakdown rates were reduced by β-lactone, suggesting that the proteasome participates in the regulation of short-lived muscle proteins (Fig. 8). The effect of hyperthermia on short-lived protein degradation was abolished in the presence of β-lactone or energy-depleting medium, consistent with the concept that energy-proteasome-dependent proteolysis regulates temperature-induced degradation of short-lived muscle proteins.

Protein synthesis and myotube protein levels. To test whether the effect of hyperthermia was specific for protein degradation, we next examined the influence of elevated temperature on protein synthesis in the myotubes. As seen in Fig. 9, hyperthermia resulted in a "dose-dependent" increase in protein synthesis. The effect of hyperthermia on protein synthesis was less pronounced than that seen on protein degradation and was not statistically significant until the temperature was raised to 43°C.

Because both protein synthesis and breakdown rates were increased at high temperature, it was important to determine the overall catabolic effect of hyperthermia. This was done by measuring cell protein levels after incubating myotubes at 37 or 41°C for 6 h. Cell protein levels were reduced from 9.32 ± 0.11 to 8.69 ± 0.12 mg/well when cultured at 37 and 41°C, respectively (n = 6 paired observations; P < 0.05 by Student's paired t-test).

mRNA levels. To examine whether changes in proteolytic activity at high temperature were accompanied by changes in the gene expression of enzymes and various components of the different proteolytic pathways, mRNA levels for cathepsin B, m- and µ-calpain, p94, ubiquitin, and the proteasome subunit C7 were determined by dot blot hybridization. Steady-state levels of the mRNAs examined here were not altered significantly by elevated temperature (Fig. 10 and Table 1). It should be noted that Fig. 10 depicts the results from one experiment, and Table 1 provides the results from multiple experiments (n = 6 for the different transcripts).

DISCUSSION

The present results confirm previous reports of increased protein degradation in cultured muscle cells...
Fig. 10. Dot blot analysis of RNA from L6 myotubes cultured at 37 or 41°C. Result from 1 experiment for each transcript is shown. Quantitation of mRNA levels from multiple experiments is provided in Table 1.

(22) and intact incubated rat muscles (3, 14) subjected to hyperthermia. The results are important from a clinical standpoint because they suggest that fever, typically accompanying sepsis and severe infection, may contribute to the catabolic response seen in these conditions. The present finding of increased protein synthesis in myotubes at elevated temperatures differs from incubated intact rat muscles in which protein synthesis was not affected by hyperthermia (3, 14). The reason for this apparent discrepancy is not known but may be different regulation of protein synthesis in incubated intact rat muscles and cultured myotubes. The effect of hyperthermia on protein synthesis in cultured myotubes has not been reported previously.

Although the influence of hyperthermia on protein degradation in cultured myotubes was examined in a previous study by Morita et al. (22), the two reports differ in several important aspects. In the present study, the effect of hyperthermia on the degradation of both long- and short-lived proteins was examined, whereas in the report by Morita et al. (22), only long-lived proteins were studied. The overall catabolic effect of hyperthermia was determined here by including measurements of protein synthesis and cell protein levels, whereas in previous experiments (22), only protein degradation was measured. The involvement of proteasome and lysosomal protein breakdown was assessed by measuring proteasome and cathepsin activities at 37 and 40°C in the study by Morita et al. (22), whereas in the present report, the contribution of different proteolytic pathways to changes in actual protein breakdown rates was determined by using specific protease inhibitors.

The sensitivity of short-lived proteins to hyperthermia noted here is a novel finding. Although it is not known from the present experiments which specific proteins were degraded at an increased rate at the elevated temperature, there is evidence that many regulatory proteins are short lived (2), whereas the myofibrillar proteins probably make up the major part of long-lived proteins in muscle cells (4).

The main purpose of the present experiments was to determine the proteolytic mechanism(s) involved in temperature-dependent increase in muscle protein degradation. This was accomplished by using blockers of individual proteolytic pathways. Results from those experiments suggest that the hyperthermia-induced increase in both long-lived and short-lived protein degradation is energy-proteasome dependent and not dependent on lysosomal or calcium-activated proteolysis. A similar conclusion was reached in two previous reports as well (3, 22), but the present study expanded earlier experiments by using specific inhibitors of different proteolytic pathways and by examining the regulation of both long-lived and short-lived proteins.

Energy-proteasome-dependent proteolysis typically involves degradation of ubiquitinated proteins (1, 16, 21). Although in a recent study, mRNA levels for the proteasome subunits RC4 and RC8 and for the 19S subunits S4 and S7 as well as for ubiquitin were not increased in myotubes at high temperature (22), it is still possible that the increase in protein degradation noted here reflected degradation of ubiquitinated proteins by the 26S/20S proteasome. We recently found that increased activity in the ubiquitin-proteasome

<table>
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<tr>
<th>Enzymes</th>
<th>mRNA Level</th>
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<tbody>
<tr>
<td>Ubiquitin</td>
<td>114 ± 10</td>
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<tr>
<td>C7</td>
<td>99 ± 3</td>
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<tr>
<td>μ-Calpain</td>
<td>103 ± 6</td>
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<tr>
<td>m-Calpain</td>
<td>118 ± 7</td>
</tr>
<tr>
<td>p94</td>
<td>108 ± 2</td>
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<tr>
<td>Cathepsin B</td>
<td>87 ± 7</td>
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Results are expressed as means ± SE of ≥6 experiments for different transcripts in %mRNA levels at 37°C. mRNA levels were determined as mean of 41/37°C ratio for 5, 10, and 20 µg RNA dot for each experiment.
pathway is not necessarily accompanied by increased mRNA levels for the various components of this proteolytic pathway (25). In the present study, mRNA levels for ubiquitin and the proteasome subunit C7 were not influenced by elevated temperature, despite increased proteasome-dependent proteolysis, further supporting the concept that changes in proteolytic activity are not always accompanied by changes in the gene expression of enzymes and other components of the proteolytic pathway.

The relative increase in protein breakdown at high temperature was much smaller in the short-lived than in the long-lived protein pool, suggesting that short-lived protein degradation is less sensitive to regulation than long-lived protein breakdown. In other studies as well, evidence was found that the degradation of long-lived and short-lived proteins in muscle cells was differentially regulated. For example, glutamine inhibited the breakdown of long-lived but not short-lived proteins in cultured muscle cells (30). A similar lack of effect on short-lived protein degradation was found for insulin and insulin-like growth factor-I (12, 13).

Interestingly, the differential regulation of short-lived and long-lived proteins is not unique to the muscle cell. For example, in cultured L-132 human lung cells, depletion of polyamines resulted in inhibited degradation of long-lived proteins but increased degradation of short-lived proteins (6). Although most treatments result in a differential regulation of short- and long-lived protein degradation, the two classes are regulated in a similar fashion in some cell types and after certain stimuli. Thus in growing fibroblasts, an energy-dependent nonlysosomal process was responsible for the degradation of both short- and long-lived proteins (10).

Although the results in the present and a previous study (22) suggest that temperature-induced muscle protein breakdown is regulated by the proteasome, the mechanism by which hyperthermia induces increased proteasome-dependent proteolysis remains to be determined. A well-described consequence of hyperthermia is the induction of heat-shock proteins and it may be speculated that one or several of the heat-shock proteins are involved in the regulation of protein degradation during hyperthermia. Results in a previous study suggest that upregulated activity of the ubiquitin-activating enzyme E1 may be an additional mechanism of increased protein degradation at high temperature (11).

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