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Increased mRNA levels for components of the lysosomal, Ca^{2+}-activated, and ATP-ubiquitin-dependent proteolytic pathways in skeletal muscle from head trauma patients

(protein breakdown/cathepsin/calpain/proteasome)

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ABSTRACT **The cellular mechanisms responsible for enhanced muscle protein breakdown in hospitalized patients, which frequently results in lean body wasting, are unknown. To determine whether the lysosomal, Ca^{2+}-activated, and ubiquitin-proteasome proteolytic pathways are activated, we measured mRNA levels for components of these processes in muscle biopsies from severe head trauma patients. These patients exhibited negative nitrogen balance and increased rates of whole-body protein breakdown (assessed by [^{13}C]leucine infusion) and of myofibrillar protein breakdown (assessed by 3-methylhistidine urinary excretion). Increased muscle mRNA levels for cathepsin D, m-calpain, and critical components of the ubiquitin proteolytic pathway (i.e., ubiquitin, the 14-kDa ubiquitin-conjugating enzyme E2, and proteasome subunits) paralleled these metabolic adaptations. The data clearly support a role for multiple proteolytic processes in increased muscle proteolysis. The ubiquitin proteolytic pathway could be activated by altered glucocorticoid production and/or increased circulating levels of interleukin 1β and interleukin 6 observed in head trauma patients and account for the breakdown of myofibrillar proteins, as was recently reported in animal studies.**

Many intensive care patients experience a rapid loss of body proteins. Tracer studies have shown, in burned (1) or septic (2) patients, increased whole-body protein breakdown. Elevated muscle proteolysis is largely responsible for this increase, as shown by enhanced urinary 3-methylhistidine excretion, an index of myofibrillar protein breakdown (3). However, none of the methods to measure proteolysis *in vivo*, namely, whole body tracer studies, 3-methylhistidine excretion, or tracer balance across the forearm (a method allowing direct measurement of muscle proteolysis) (4), gives any information on the cellular mechanisms responsible for increased muscle proteolysis.

There are three well characterized intracellular proteolytic systems in mammalian skeletal muscle. The lysosomal pathway, which mostly degrades soluble and extracellular proteins, is not involved in the degradation of myofibrillar proteins (5–7) and contributes little to overall protein breakdown in muscles incubated under optimal conditions (7–9). The Ca^{2+}-dependent proteinases (calpains) degrade cytoskeletal but not myofibrillar proteins (5–7) and are mostly involved in limited proteolysis of some specific target proteins (10). The third, and most recently identified system, is the ATP-ubiquitin-dependent pathway in which proteins to be degraded are first covalently linked to multiple ubiquitin chains in a several-step process requiring ATP, the ubiquitin-activating enzyme E_1, and one of the ubiquitin-conjugating enzymes (E_2). One of the ubiquitin protein ligases (E_3) is sometimes required for substrate recognition and ubiquitylation (11). Ubiquitylation targets the proteins for hydrolysis by a multienzymatic complex (the 26S proteasome), the functioning of which also requires ATP (11). This system classically catalyzes the selective breakdown of abnormal and short-lived regulatory proteins (11) but is also likely to be the primary system for degradation of the bulk of myofibrillar proteins according to recent data in rodents (6, 7). However, while increased activities of lysosomal enzymes have occasionally been shown in the muscle of cachectic (12) or injured (13) patients, the role of the two other systems, and particularly of the ATP-ubiquitin proteolytic pathway, has never been directly studied in human muscle.

Therefore, to assess which proteolytic systems are activated in muscle of intensive care patients, we studied severely head-injured patients because they are clinically known to experience rapid muscle loss (14). We have demonstrated that both whole body and skeletal muscle myofibrillar protein breakdown were increased in these patients. These metabolic adaptations correlated with enhanced expression of critical components of the lysosomal, Ca^{2+}-dependent, and ATP-ubiquitin-dependent proteolytic pathways in muscle biopsies.

MATERIALS AND METHODS

Subjects. The protocol was approved by the Ethical Committee of Clermont-Ferrand and informed written consent was obtained from the volunteers and from the patients' families. Six head-injured patients and five control subjects were studied. The control subjects were healthy young volunteers [age = 27 ± 3 years (mean ± SEM); body mass index = 23 ± 2 kg/m^2; four men and one woman], matched for age, weight, and height with the patients (age = 28 ± 4 years; body mass index = 20 ± 1 kg/m^2; four men and two women). The patients had exclusive and severe head traumas, as indicated by a Glasgow coma scale score between 3 and 8 (normal = 15) at admission. They had no prior disease and received a standardized treatment combining artificial ventilation that was adjusted to maintain normoxia and hypocapnia, sedation with phenoperidin and flunitrazepam, preventive treatment of epilepsy with phenytoin and of stress ulcer with sucralfate, and artificial nutrition. Patients requiring inotropic drugs, steroids, or barbiturates or who developed a sepsis (elevated blood white cell count and/or fever and/or bacteremia) during the course of the experiment were excluded from the study. Artificial nutrition initially consisted of intravenous glucose

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Abbreviations: KIC, α-ketoisocaproate; IL-1β, interleukin 1β; IL-6, interleukin 6; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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(1.5 g per kg per day) followed by enteral nutrition initiated between days 2 and 4. From day 5 and until completion of the study, all the patients received a continuous nasogastric feeding at a fixed rate of 1500 ml/day (Nutrison E$^+$ Nutricia, The Netherlands) together with 5% (wt/vol) intravenous glucose (250 ml/day) providing 39 ± 2 kcal per kg per day and 1.4 ± 0.09 g of protein per kg per day (i.e., 1.6 kcal and 0.06 g of protein per kg per h).

Study Protocol. Patients were studied on day 8 after admission. They received a primed (6.9 μmol/kg) continuous (0.17 ± 0.02 μmol per kg per min) infusion of L-[1-^{13}C]leucine for 10 h through a central venous catheter. L-[1-^{13}C]Leucine (99% atom percent excess) was obtained from Tracer Technologies (Sommerville, MA) and tested for apyrogenicity and sterility prior to use. Blood samples for measurement of plasma leucine and α-ketoisocaproate (KIC) ^{13}C enrichments were taken through an intra-arterial line, prior to and at 30-min intervals during the last 2 h of infusion. At the end of the tracer infusion, a 30- to 60-mg muscle biopsy was taken from a vastus lateralis muscle, after incision of the skin and aponevrosis using a Wecester–Blake clamp. The tissue was immediately frozen in liquid nitrogen and kept at −80°C until analysis. Enteral nutrition and other routine treatments were continued throughout the study. In addition to routine intensive care measurements, blood and urine samples were also taken to measure plasma interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor (TNF) (days 4 and 8), 24-h urinary 3-methylhistidine and cortisol (days 4 and 8), and urinary nitrogen excretion (days 5–7).

Control subjects were studied in a similar manner. They were instructed to follow a meat-free diet, providing 40 kcal per kg per day and 1.5 g of protein per kg per day, for 3 days prior to the study. They received an identical 10-h tracer infusion except that the tracer infusion rate was lower (0.10 ± 0.01 μmol per kg per min) and that arteriolized blood was obtained from a hand vein placed in a heated box. Enteral nutrition was administered as small meals given every 20 min, to mimic continuous feeding, and provided 1.4 kcal and 0.06 g of protein per kg per h (equivalent to 34 kcal and 1.4 g of protein per kg per day). Muscle biopsies were performed under local anesthesia with 2% Xylocain (Astra, Sweden). Twenty-four-hour urinary 3-methylhistidine excretion was measured on the study day. Nitrogen balance was not performed.

Analytical Methods. Plasma leucine and KIC ^{13}C enrichments were determined by gas chromatography–mass spectrometry as described (15) by monitoring the ions of mass/charge ratios 303/302 and 302/301 of the *tert*-butyldimethylsilyl derivatives of leucine and KIC, respectively. Urinary nitrogen was measured by pyrochemiluminescence, 3-methylhistidine was measured by liquid chromatography, cortisol was measured by standard RIA, and cytokines were measured by immunoradiometry with TNF, IL-1β, and IL-6 human monoclonal antibodies (IRMA, Medgenix, Fleurus, Belgium).

Northern Blot Analysis. Total RNA was extracted from the muscle biopsies as described by Chomczynski and Sacchi (16). Eight micrograms of total RNA was electrophoresed in 1% agarose gels containing formaldehyde. RNA was electrophoretically transferred to a nylon membrane (GeneScreen, NEN) and covalently bound to the membrane by UV-crosslinking. Results are only given for five of six patients and five controls, because when all samples were spotted on the same membrane, the sixth patient was not yet included in the study. The membranes were hybridized with cDNA probes encoding chicken polyubiquitin (17), rat 14-kDa ubiquitin-conjugating enzyme E2 (18), HC2 and HC8 human proteasome subunits (19), and human m-calpain (20) and cathepsin D (21). The hybridizations were performed at 65°C with [^{32}P]cDNA fragments labeled by random priming as described (9). After washings at the same temperature, the filters were autoradiographed for 3–48 h at −80°C with intensifying screens on Hyperfilm-MP films (Amersham). After stripping of the different probes, the filters were reprobed with a cDNA fragment encoding the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (22) to confirm that changes seen were not due to nonspecific changes in all mRNAs or to uneven loading. Densitometric signals for ubiquitin, the 14-kDa E2, and HC2 proteasome subunit were quantified by using digital image processing and analysis (NIH IMAGE version 1.43); values were normalized by using the corresponding GAPDH values to correct for variations in RNA loading.

Calculations and Statistical Analysis. Nitrogen balance was calculated as the difference between nitrogen intake and urinary nitrogen excretion (there were no feces from days 5 to 7). No correction was made for miscellaneous losses. Steady states for leucine and KIC ^{13}C enrichments and concentrations were obtained over the last 2 h of infusion (five time points), as assessed by a coefficient of variation <5% and a slope not significantly different from zero in each experiment. Total leucine flux, an index of whole-body protein turnover, was calculated as the ratio of isotope infusion rate (corrected for isotopic purity) divided by the plasma [^{13}C]KIC enrichment, which is representative of the enrichment of the intracellular leucine pool (23). This value includes the tracer infusion. Endogenous leucine rate of appearance, an index of whole body proteolysis, was equal to total leucine flux minus the tracer infusion and minus the enteral leucine intake. A similar calculation was also done with [^{13}C]leucine enrichment.

All the results are expressed as the mean ± SEM. Statistically significant differences between the patients and control groups were determined by an unpaired Student's *t* test for all values, except mRNA levels, for which a Mann–Whitney *U* test was used.

RESULTS

All patients were in negative nitrogen balance (−6.9 ± 1.6 g of N per day; range, −3.3 to −12.7 g of N per day). Whole-body protein turnover (i.e., leucine flux) and protein breakdown (i.e., endogenous leucine production) were higher (both, $P < 0.01$) in patients (3.52 ± 0.10 and 2.51 ± 0.09 μmol per kg per min) than in controls (2.36 ± 0.15 and 1.38 ± 0.15 μmol per kg per min), as shown in Fig. 1. Similar results were obtained when [^{13}C]leucine enrichments were substituted for [^{13}C]KIC enrichments (data not shown), the KIC/leucine enrichment

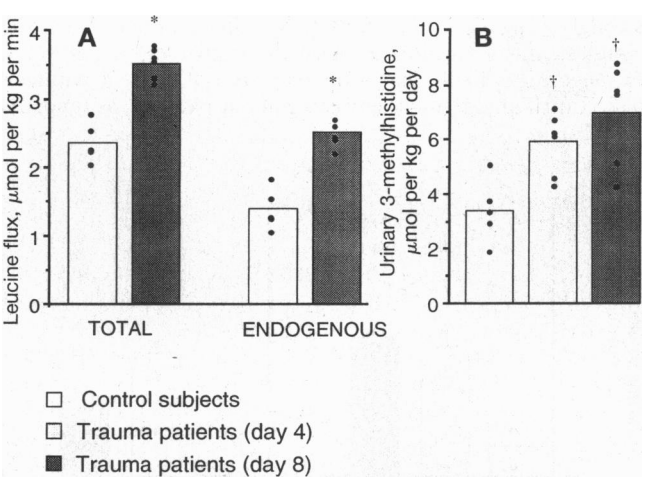

FIG. 1. Whole-body protein kinetics in head-injured patients at days 4 and 8 and in control subjects. Total leucine flux and endogenous leucine flux, measured by isotope dilution, are indexes of whole-body protein turnover and breakdown, respectively. (*A*) Total leucine flux and endogenous leucine flux. (*B*) Urinary 3-methylhistidine (myofibrillar protein breakdown). *, $P < 0.001$; †, $P < 0.01$, vs. controls.

ratios being 0.68 ± 0.03 and 0.73 ± 0.05 in the patients and in the controls, respectively ($P > 0.05$). Urinary 3-methylhistidine excretion was elevated in patients at days 4 (5.94 ± 0.51 μmol per kg per day) and 8 (6.98 ± 0.83 μmol per kg per day) (both, $P < 0.01$) compared with controls (3.40 ± 0.58 μmol per kg per day) (Fig. 1). Urinary cortisol excretion was also markedly elevated at days 4 and 8 (272 ± 56 and 551 ± 120 nmol/day, respectively) (normal values for the assay = 30–100 nmol/day). As shown in Fig. 2, plasma TNF concentration was unchanged, while plasma IL-1β, and particularly IL-6, were elevated in patients at day 4 or 8 ($P < 0.02$).

Northern blot analysis and hybridizations showed that mRNA levels for cathepsin D and m-calpain rose in the muscles from head trauma patients compared to healthy volunteers, suggesting an activation of both lysosomal and Ca^{2+}-dependent proteinases (Fig. 3). These changes occurred without any significant variation in GAPDH expression (1778 ± 276 and 1786 ± 369 arbitrary densitometric units in controls and patients, respectively), as also reported in other muscle wasting conditions (8, 9, 30). Recent observations indicate that the ATP-ubiquitin-dependent proteolytic pathway plays a major role in muscle wasting in animal models (24). Fig. 3 also shows that mRNA levels for both transcripts of ubiquitin were elevated in the muscles from head trauma patients (+110%, $P < 0.05$; Fig. 4). Since ubiquitin has various roles in nonproteolytic functions (25–27), the RNA blots were also probed with the cDNA of the 14-kDa E2 that functions in E3-dependent ubiquitin conjugation and protein breakdown (18) and with cDNAs encoding the HC2 and HC8 subunits of the 20S proteasome, which is the proteolytic core of the 26S proteasome that degrades ubiquitin conjugates (11). We found an increased expression of these other components (14-kDa E2, +105%, $P < 0.02$; HC2, +171%, $P < 0.02$; HC8, +113%, $P < 0.05$) of the ATP-ubiquitin-dependent proteolytic pathway (Figs. 3 and 4; data not shown for the HC8 proteasome subunit).

DISCUSSION

Head-injured patients exhibited negative nitrogen balances, enhanced rates of whole body protein breakdown, and a sustained rise in urinary 3-methylhistidine excretion, an index of muscle myofibrillar protein breakdown (3). An increased expression for critical components of the lysosomal, Ca^{2+}-dependent, and ATP-ubiquitin-dependent proteolytic processes in muscle biopsies paralleled these adaptations, thus, strongly suggesting that muscle wasting resulted from the simultaneous activation of these three proteolytic pathways. However, it is likely that a reduced rate of protein synthesis also contributed to muscle wasting in our patients, as reported (28, 29).

Very little information is available on the nature of proteolytic systems responsible for muscle wasting in humans. To our knowledge, the lysosomal pathway is the only degradative system that has been reported to play a role in cachexia in humans. Lundholm *et al.* (12) reported an involvement of cathepsins in muscle wasting in cancer patients. Increased cathepsin B and D activities were also reported in needle muscle biopsies from patients with severe accidental trauma (13). This latter observation is supported by the increased expression of cathepsin D reported herein. By contrast, the role of either the Ca^{2+}-dependent or the ubiquitin-proteasome proteolytic process has never been studied in human muscle, presumably because measuring their activity requires large muscle samples. In the present study, the increased expression of m-calpain and proteasome subunits suggests an involvement of at least two nonlysosomal proteolytic pathways in increased skeletal muscle protein breakdown. However, our observations do not indicate whether the changes in mRNA levels reflect increased transcription or alterations in mRNA processing and transport or in degradation rates. The precise significance of changes in mRNA levels for components of proteolytic systems is presently unknown, although they generally correlate with changes in muscle protein breakdown measured by alternative methods (e.g., *in vitro* techniques) or with proteolytic activities (for review, see ref. 24).

A coordinate stimulation of the ATP-ubiquitin-dependent proteolytic pathway with either the Ca^{2+}-dependent (9) or the lysosomal process (8) or both (7) prevails in different types of muscle wasting. These observations suggest that these pathways may serve to eliminate different classes of cellular proteins (7). However, many observations in animal studies demonstrate that neither the lysosomal nor the Ca^{2+}-dependent proteinases are responsible for the loss of myofibrillar proteins. (*i*) Inhibitors of lysosomal acidification (e.g., methylamine and chloroquine) or function (e.g., E64 and leupeptin that inhibit the cysteine proteinases cathepsins B, H, and L and the Ca^{2+}-dependent proteinases) do not affect 3-methylhistidine release by incubated muscles (5–7). (*ii*) The lysosomal and the Ca^{2+}-dependent proteolytic pathways do not contribute significantly to increased protein breakdown in a variety of situations characterized by enhanced proteolysis such as starvation (8), acidosis (30), or cancer (9). (*iii*) Evidence for activation of either lysosomal or Ca^{2+}-dependent proteinases is lacking in many instances of muscle wasting (8, 9, 24, 31). By contrast, myofibrillar protein breakdown requires ATP (6, 7), and enhanced degradation of contractile proteins is associated with increased expression of ubiquitin (6). Elevated mRNA levels for ubiquitin (8, 9, 30, 32, 33), the 14-kDa E2 (9, 18, 24), and/or proteasome subunits (9, 24, 30, 32) are also observed in several muscle wasting conditions in rodents. An accumulation of ubiquitin-protein conjugates was also

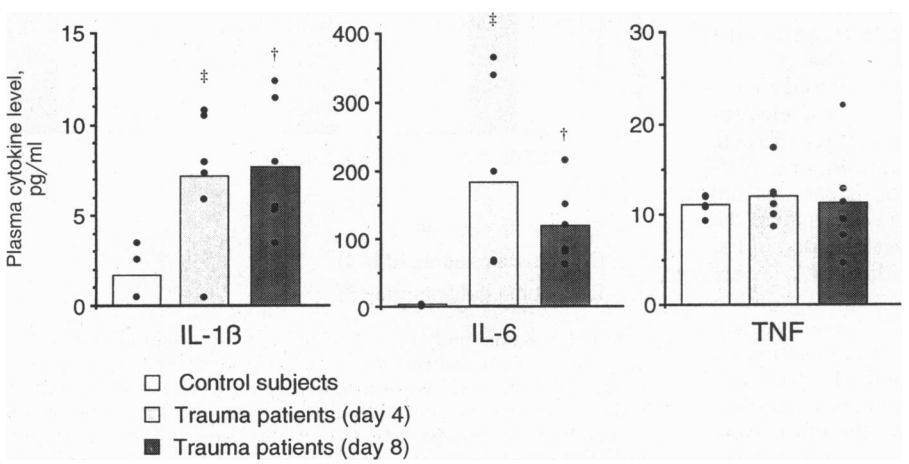

FIG. 2. Plasma levels of cytokines in control subjects and in head-injured patients at days 4 and 8 (radioimmunoassay). †, $P < 0.01$; ‡, $P < 0.02$, vs. controls.

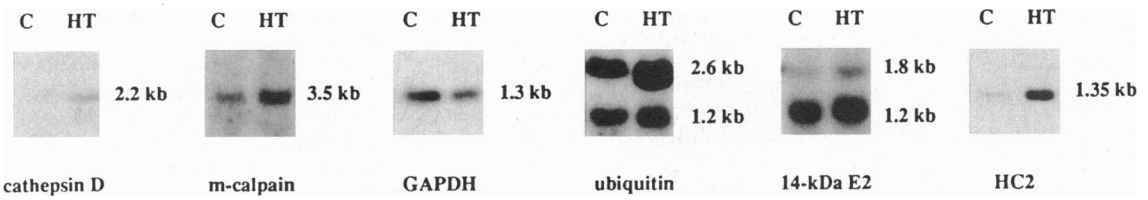

FIG. 3. Representative Northern blots for cathepsin D, m-calpain, GAPDH, ubiquitin, 14-kDa E2, and the proteasome HC2 subunit in vastus lateralis muscle biopsies from a single head trauma patient and control subject. RNA was isolated and electrophoresed through a denaturing 1% agarose gel, transferred to nylon membranes, and consecutively hybridized with the different ^{32}P-labeled cDNAs. C, control volunteer; HT, head trauma patient. The size of the transcript(s) is given in kb. The two 14-kDa E2 transcripts arise from different polyadenylylation sites (18). The 1.8-kb transcript, which corresponds to the major mRNA species in the rat muscle, is barely detectable in humans. However, the 1.2-kb transcript was mainly affected by head trauma, as observed in the muscles from fasted (18) and tumor-bearing (9) animals.

reported in the muscles from starved, denervated, and tumor-bearing rats (33, 34). Furthermore, proteasome preparations may degrade contractile proteins (35, 36), and the accumulation of ubiquitin-protein conjugates occurred primarily in the myofibrillar fraction (34). (*iv*) It was recently shown that the ubiquitin proteolytic pathway, which is primarily believed to catalyze the elimination of short-lived and abnormal proteins (11), is also involved in the degradation of the bulk of long-lived cellular proteins (37). Thus, although our data preclude the identification of substrates of a given proteolytic system, the clear overexpression of multiple components of the ubiquitin proteasome proteolytic pathway in muscle biopsies from head trauma patients strongly suggests that this pathway is involved in the breakdown of contractile proteins in humans.

The increased whole body and muscle proteolysis observed in the head trauma patients could be due to several factors. (*i*) Our patients had been immobilized for 8 days and disuse is an important factor leading to muscle atrophy. An *in vivo* human study demonstrated that atrophy of an immobilized muscle solely results from decreased protein synthesis (38). By contrast, muscle wasting seen after section of the sciatic nerve (7) or after simulated weightlessness (39) in rats results primarily from increased protein breakdown. It is noteworthy that the enhanced breakdown of proteins in these conditions resulted from the coordinate activation of lysosomal, Ca^{2+}-dependent, and ATP-ubiquitin-dependent proteolytic systems (7, 39), as shown here. (*ii*) The high circulating levels of cortisol in our patients may also have contributed to increased protein breakdown. Recent studies have shown that glucocorticoids are necessary for increased expression of ubiquitin in fasted animals (8) or of proteasome subunits in acidotic rats (40), and dexamethasone administration also resulted in increased mRNA levels for the 14-kDa E2 in rat skeletal muscle (41). (*iii*)

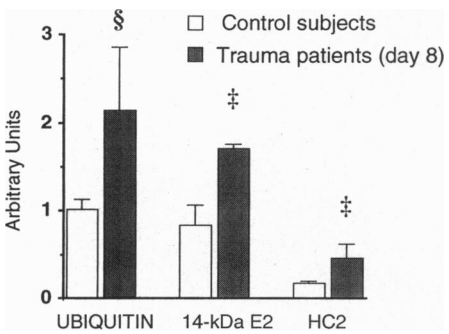

FIG. 4. Effects of head trauma on abundance of mRNAs encoding critical components of the ubiquitin-proteasome proteolytic pathway from vastus lateralis muscles. RNA was isolated and electrophoresed through a denaturing 1% agarose gel, transferred to nylon membranes, and hybridized with ^{32}P-labeled cDNAs encoding ubiquitin, the 14-kDa E2, or the proteasome HC2 subunit and GAPDH. Densitometric signals were normalized by using the corresponding GAPDH values to correct for variations in RNA loading. Values are the mean ± SEM ($n = 5$). §, $P < 0.05$; ‡, $P < 0.02$, vs. controls.

Several cytokines, namely TNF, IL-1β (42), or IL-6 (43) have been reported to increase protein breakdown, in skeletal muscle. In particular, IL-1β is a possible signal for enhanced ATP-ubiquitin-dependent proteolysis in muscle (24), as well as IL-6 in myotubes (44). Thus, the high circulating levels of both IL-1β and IL-6 in head trauma patients may contribute to the activation of the ubiquitin proteolytic pathway in skeletal muscle. (*iv*) Nutrition of the patients and controls was not exactly identical; the controls received slightly (but not significantly) less energy (≈5 kcal per kg per day) and were fed for a shorter period (10 h) for practical reasons. However, these minor differences presumably do not account for the dramatic modifications observed. Although the patients did not receive steroids and β-agonists, which are known for their catabolic and anabolic properties, respectively, we also cannot exclude that other drugs might have affected protein metabolism. Thus, the respective roles of rest, glucocorticoids, cytokines, and other factors as mediators of the catabolic muscle response in trauma require further investigation.

In conclusion, we showed that the mRNA levels for several proteinases or cofactors involved in protein breakdown are coordinately increased in the muscles from head-injured patients who exhibit enhanced whole-body and myofibrillar muscle protein breakdown. These data suggest that these mRNA levels in human muscle biopsies, may be used as sensitive indicators of increased protein breakdown since currently available techniques for measuring *in vivo* rates of protein breakdown do not provide any information about specific proteinases that are responsible for muscle wasting. To our knowledge, our observations are also the first to suggest an involvement of the ATP-ubiquitin-dependent proteolytic pathway in muscle wasting in hospitalized humans and, therefore, support further study of the role of this specific proteolytic pathway in muscle atrophy due to injury.

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