Enhancement of Myofibrillar Proteolysis Following Infusion of Amino Acid Mixture Correlates Positively with Elevation of Core Body Temperature in Rats

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Summary  Administration of an amino acid (AA) mixture stimulates muscle protein synthesis and elevates core body temperature (Tb), as characteristically found under anesthetic conditions. We tested the hypothesis that not only AA given, but also AA produced by degradation of endogenous muscular protein are provided for muscle protein synthesis, which is further reflected in Tb modifications. Rats were intravenously administered an AA mixture or saline in combination with the anesthetic propofol or lipid emulsion. We measured plasma 3-methylhistidine (MeHis) concentrations as an index of myofibrillar protein degradation, rectal temperature and mRNA expression of atrogin-1. MuRF-1 and ubiquitin in gastrocnemius and soleus muscles of rats following 3 h infusion of test solutions. Tb did not differ significantly between conscious groups, but was higher in the AA group than in the saline group among anesthetized rats. Plasma MeHis concentrations were higher in the AA group than in the saline group under both conditions. Plasma MeHis levels correlated positively with Tb of rats under both conditions. AA administration decreased mRNA levels of atrogin-1 and ubiquitin in gastrocnemius muscle and all mRNA levels in soleus muscle. These results suggest that AA administration enhances myofibrillar protein degradation and that the change is a determinant of Tbs modification by AA administration. However, the mechanisms underlying AA administration-associated enhancement of myofibrillar proteolysis remains yet to be determined.

Key Words  methylhistidine, hypothermia, anesthesia, muscle, protein metabolism

Amino acid (AA) administration or protein ingestion elevates blood temperature in healthy human subjects (1, 2). The conversion into a positive heat balance by AA administration is clinically applied to the intraoperative management of core body temperature (Tb) (3–6). Hypothermia is common during surgery due to impairment of thermoregulatory responses by anesthetic administration (7), representing one of the major causes of postoperative complications (8, 9). Given that AA administration is utilized for intraoperative thermal management, understanding the precise mechanisms by which AA induces heat accumulation in the body is crucial.

Energy expenditure is increased after AA administration compared to glucose or lipid administration, as energy derived from ingested AAs or stored nutrients is more utilized with either AA incorporation into protein or direct oxidation (10). Skeletal muscle makes a major contribution to total body protein turnover. We have previously reported that AA administration stimulates muscle protein synthesis in both conscious and anesthetized rats (11). In general, not only AA administered, but also AA liberated by endogenous protein breakdown would be utilized in part for protein synthesis. Muscle protein breakdown along with muscular protein synthesis may also affect Tbs modification after AA administration. However, no studies have described whether administration of AA alone affects muscle protein breakdown under anesthetic and conscious conditions and thus whether Tbs is modified.

The 3-methylhistidine (MeHis) generated by biological degradation of muscular actin and myosin is not reutilized as a material for muscular protein and is not reabsorbed by the renal tubule. Levels of urinary MeHis excretion or plasma MeHIs are thus used as indices of myofibrillar protein breakdown. Plasma MeHis concentrations can reflect acute changes in the degradation of myofibrillar protein (12, 13). Measurement of plasma MeHis concentrations would thus be appropriate to elucidate relationships between degradation of myofibrillar protein and comparable early stages of nutritional changes after AA administration.

Skeletal muscle contains multiple proteolytic systems (e.g., lysosomal and non-lysosomal Ca2+- and ATP-ubiquitin-dependent pathways), each of which could be involved in metabolic protein turnover. The ubiquitin proteolytic pathway appears primarily responsible for the increased degradation of contractile components.
such as myofibrillar proteins, in skeletal muscle under various catabolic conditions (14). The ubiquitin-proteosome system first requires the targeting of specific protein substrates for degradation, which is fulfilled by the activity of a hierarchical cascade containing E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligating) enzymes (15). The gene expression of the two muscle-specific E3 ligases: muscle RING finger (MuRF)1 and muscle atrophy F-box (MAFbx: also known as atrogin-1) is extremely well studied from its relevance to muscular protein degradation following nutrient ingestion (16–18), but the precise mechanisms and physiological roles have remained to be determined. Furthermore, the contributions of the proteolytic pathway for situations in which AA mixture alone is provided have yet to be addressed.

The aim of the present study was to elucidate whether AA administration enhances myofibrillar protein degradation in anesthetized and conscious rats and whether such degradation is associated with T₃ modification. Furthermore, to elucidate the mechanism underlying changes in myofibrillar protein degradation by AA administration, we also examined mRNA expression profiles of components of the ubiquitin-proteosome system in rat skeletal muscles.

MATERIALS AND METHODS

Animals and surgery. Male Sprague-Dawley rats from Charles River Japan (Yokohama, Japan) weighing 250–310 g were maintained under conditions of constant humidity and temperature (22±2°C) on a 12:12 h light-dark cycle. Rats were provided with ad libitum access to standard diet and water. The following surgical and experimental procedures were approved by the Committee on the Care and Use of Laboratory Animals at Otsuka Pharmaceutical Factory, Inc. (Tosu-shima, Japan).

Infusion protocol. Infusion of the test solutions and anesthetic was conducted as described previously (11). The day before the experiment, a silicon catheter was inserted into the jugular vein and threaded proximally 2.5 cm from the tip in rats under diethyl-ether anesthesia. Saline was infused via the implanted catheter continuously at a rate of 1 mL/h to prevent blood coagulation. Food was then withheld for 18 h, but rats were allowed ad libitum access to water. In the experiment, simultaneous infusion of test solutions (AA mixture (Amiparen®; Otsuka Pharmaceutical Factory) or saline (Otsuka Pharmaceutical Factory)) and anesthetically propofol (Diprivan® 1%; Astra Zeneca, Osaka, Japan) or lipid emulsion (Intralipids®; Otsuka Pharmaceutical Factory) was started and continued for 3 h. The composition of the AA mixture is shown in Table 1. Lipid emulsion Intralipids® was administered to conscious rats because the solvent for propofol consists of the same amount of lipid in terms of percentage and ingredient (soybean oil), thus eliminating any effect of lipid treatment. Conscious or anesthetized rats received an intravenous bolus injection of propofol or lipid emulsion over 5 s at a bolus volume of 1.5 mL/kg via the catheter. Respectively, subsequently, the catheter was immediately joined to 2 plastic tubes via a Y-connector and test solutions were infused at a rate of 14 mL/kg/h via a single vinyl tube. Rats were simultaneously administered propofol or lipid emulsion via the other tube from a different pump by sequential infusion at a rate of 4.5 mL/kg/h (0–60 min) and 2.25 mL/kg/h (60–180 min).

Measurement of rectal temperature. At 3 h after starting the infusion of test solution, T₃ in rats was measured using a rectal probe thermocouple thermometer (Physiotemp, model BAT-12, accuracy ±0.1°C; Sensoarte, Clifton, NJ, USA). The lubricated probe was inserted ~3.5 cm until a stable temperature reading was obtained after sodium pentobarbital (50 mg/kg) was administered via bolus injection through a catheter. Blood was collected from the abdominal artery with a heparinized syringe, after which the soleus and gastrocnemius muscles were rapidly excised. The blood was centrifuged at 1,800 × g for 20 min at 4°C to obtain plasma and tissues were immediately frozen in liquid nitrogen and stored at −80°C.

Measurement of plasma creatinine and MeHIs. Plasma creatinine concentrations were measured using the creatininase-HMMPS method (I-Type Creatinine M; Wako Pure Chemical Industries, Ltd., Osaka, Japan). For determination of plasma concentrations of MeHIs, the plasma sample was mixed with ethanol and then hydrolyzed with 6 N HCl at 110°C for 2 h, then concentrations of 3-MeHIs were measured by high-performance liquid chromatography (2690 Alliance separation module, Waters, Milford, MA) with a CD-C18 column (2.0× 150 mm, 3 μm; Intakt Co., Kyoto, Japan) using a mobile phase of 50 mM SDS/acetonitrile/phosphate at 610: 390: 3 at a flow rate of 0.2 mL/min. An ultraviolet detector (2487, Waters) was used for detection at

| Table 1. Composition of amino acid mixture: Amiparen®. |
|---------------------------------|-----------|
| Amino acids | g/dL |
| L-Leucine | 1.40 |
| L-Isoleucine | 0.80 |
| L-Valine | 0.80 |
| L-Lysine | 1.05 |
| L-Threonine | 0.57 |
| L-Tryptophan | 0.20 |
| L-Methionine | 0.39 |
| L-Phenylalanine | 0.70 |
| L-Cystine | 0.10 |
| L-Tyrosine | 0.05 |
| L-Arginine | 1.05 |
| L-Histidine | 0.50 |
| L-Alanine | 0.80 |
| L-Proline | 0.50 |
| L-Serine | 0.30 |
| Glycine | 0.59 |
| L-Aspartic acid | 0.10 |
| L-Glutamic acid | 0.10 |
| Total concentration | 10.00 |
213 nm. Concentrations of MeHis were determined by comparing peak height of samples with those of external standards.

RNA isolation and real-time PCR. Total RNA was extracted from the skeletal muscles using a tagman Rneasy® Mini kit (QIAGEN, Hilden, Germany). Total RNA samples obtained from rat tissues were diluted to 10 μg/mL with RNase-free water containing 50 μg/mL of yeast tRNA (Life Technologies, Rockville, MD, USA). Total RNA at 30 ng per 20 μL of reaction mixture was used for measurement of the target mRNA in each tissue. RT-PCR assays were performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the same conditions as in previous studies (19). Real-time PCR primers were designed for rat ubiquitin, atrogin-1, MuRF1 and β-actin, and the sequences of the forward primers and reverse primers were as follows:

- **rat ubiquitin**: 5'-TGACCAGCAGAGGCTCATCTT-3' and 5'-TGAGCCACAAGTTTGACGC-3'.
- **rat atrogin-1**: 5'-AGATCGCAACGGATTTGATC-3' and 5'-TGGGTAACATCGCAACAGC-3'.
- **rat MuRF1**: 5'-TGAGCCACAAGTTTGACGC-3' and 5'-CGA TGAATCCAGCTCTCTCTCT-3'.
- **rat β-actin**: 5'-ATCGTACAGATGGATGCAGA-3' and 5'-TAGACCAACCAATCCACACAG-3'.

The relative expression of each mRNA was calculated by the ΔCt method (where ΔCt is the value obtained by subtracting the Ct value of β-actin mRNA from the Ct value of the target mRNA), as employed in previous studies (19). Specifically, the amount of target mRNA relative to β-actin mRNA is expressed as 2^(-ΔCt). Data are expressed as the ratio of the target mRNA to β-actin mRNA. Studies were conducted in duplicate.

**Statistical methods.** Tests for statistical significance were performed using Statistical Analysis Software (ver-
Fig. 3. Effects of amino acid (AA) administration on the mRNA expression of atrogin-1 (A and D), MuRF-1 (B and E) and ubiquitin (C and F) in gastrocnemius (A, B and C) and soleus (D, E and F) muscles in conscious and anesthetized rats. These values were examined in rats after 3-h administration with either an AA mixture (AA; closed bars) or saline (SAL: open bars), either with the anesthetic propofol (right: Ane) or without anesthetic (left: Nor). Values represent mean±SE (n=10). Means not sharing a superscript are significantly different according to the Tukey-Kramer multiple comparisons test (p<0.05).

RESULTS

The T'b, plasma MeHis and creatinine concentrations of rats 3 h after the start of test solutions infusion are shown in Fig. 1A–C. T'b tended to be higher by 0.4°C on average in the conscious group given AA (Nor-AA group) than in the conscious group given saline (Nor-saline group), but no significant difference was found (Fig. 1A). In contrast, T'b of anesthetized rats was low compared to that of conscious rats. T'b was significantly higher in the anesthetized group given AA (Ane-AA group, 33.5±0.4°C; n=10) than in the anesthetized group given saline (Ane-saline group, 32.0±0.8°C; n=9). Plasma MeHis concentrations did not differ between Nor-saline and Ane-saline groups (Fig. 1B). By contrast, plasma MeHis concentrations were higher in AA groups compared to saline groups under both anesthetized and conscious conditions (p<0.05). Furthermore, increments of plasma MeHis levels were more prominent in the Ane-AA group than in the Nor-AA group, with a significant difference observed between these groups. Plasma creatinine concentrations did not differ among groups (Fig. 1C).

Figure 2A and B shows the correlation between rectal temperature and plasma MeHis concentrations of conscious and anesthetized rats 3 h after the start of test solutions infusion, respectively. Higher plasma MeHis concentrations were associated with higher rectal temperature under both the anesthetized (r=0.66, p<0.05, n=19) and conscious conditions (r=0.67, p<0.05, n=20).

Figure 3 shows the mRNA expression of atrogin-1 (A and D), MuRF-1 (B and E) and ubiquitin (C and F) in gastrocnemius (A–C) and soleus (D–F) muscles. Two-way ANOVA revealed significant effects of AA administration on levels of atrogin-1 and ubiquitin mRNA in gastrocnemius muscle and of all mRNAs in soleus muscle (p<0.05), whereas no mRNA but atrogin-1 in gastrocnemius muscle showed a significant difference in levels under the various conditions investigated.
nemius muscle was significantly affected by anesthetic treatment. The mRNA of atrogin-1 in soleus under both conditions and of atrogin-1 and ubiquitin in gastrocnemius muscle under the conscious condition became significantly lower in the AA group than in the saline group (p<0.05).

**DISCUSSION**

The present study revealed that plasma MeHis concentrations were higher in the rats given AA than in those given saline under both conscious and anesthetized conditions. Furthermore, plasma MeHis levels correlated positively with $T_b$ of rats under both conditions. On the other hand, AA administration decreased most, if not all, mRNA expression of ubiquitin, atrogin-1 and MuRF1 gene in skeletal muscle compared to Sal administration.

Myofibrillar protein breakdown is often examined using urinary MeHis excretion. However, collecting urine is generally difficult in anesthetized rats due to muscular relaxation in the urinary bladder resulting from anesthesia. Nagasawa et al. reported that the amount of MeHis released from isolated muscles into medium during a 2-h incubation period increases with starvation and corresponds to plasma MeHis concentrations (12). In cases where the experimental period is relatively short, as in the present study, and urine collection is difficult, discussion of plasma concentrations as an index of myofibrillar protein breakdown appears appropriate. In this study, plasma creatinine concentrations did not differ among groups, strongly suggesting that neither anesthetic nor the test solution affects the filtration ratio of substances like MeHis, which are not reabsorbed in the renal tubules. Plasma MeHis concentrations would thus reflect the amount of MeHis released from muscles.

Of note is the finding that AA administration enhanced myofibrillar protein breakdown under both anesthetized and conscious conditions. Results from several previous reports support the present finding about enhanced myofibrillar proteolysis following AA administration. Svanberg et al. noted that alterations in MeHis release from human arm and leg muscle tissues in response to increasing doses of AA mixture infusion showed an increasing trend (20). Furthermore, urinary MeHis output was elevated in response to increased dietary protein levels in rats under conditions of restricted food intake (21). Conversely, AAs, particularly branched chain AAs, are well known to inhibit myofibrillar proteolysis (22-24). Interestingly, Omstedt et al. observed that the better the quality of dietary protein that should result from differences in AA composition, the higher the MeHis excretion in rats (25). Therefore, the composition of AA given may account for previous discrepancies in AA effects on myofibrillar proteolysis. In fact, we used a commercially available AA mixture (Table 1) as Svanberg et al. did (20). Another plausible explanation is that insufficiency of non-protein energy might have caused the increment of plasma MeHis concentrations in this study. Hypocaloric AA mixture infusion leads to elevated urinary MeHis excretion compared to sufficient caloric nutrition containing the same amount of nitrogen (26).

Moreover, the urinary MeHis excretion in response to dietary protein ingestion is known to be positively correlated with net protein utilization in rats (25). This implies that AAs administered and AAs liberated from the proteolysed muscle tissue were used for protein synthesis. We have previously reported that muscle protein synthesis is stimulated in both anesthetized and conscious rats given AA mixture under a similar experimental design (11). Taken together, the present finding that plasma MeHis concentrations were increased by AA administration would thus not reflect any specific effect on myofibrillar protein breakdown, but could be the result of a general increase in muscular protein turnover.

Protein turnover is a substrate cycle that accounts for roughly 20% of thermo-neutral heat production (27). Increases in metabolic heat production by elevated protein turnover would be attributable to either incorporation of ingested or degraded AA into protein or their direct oxidation, as both reactions are accompanied by increases in energy expenditure (10). For example, it is known that in ad lib.-fed chicks exposed to 22°C, whole body protein synthesis and degradation were increased compared with those exposed to 30°C (28), possibly leading to the prevention of $T_b$ declines. Therefore, one might predict that increased rates of protein turnover after AA or protein ingestion would contribute to elevated thermogenesis. In fact, plasma MeHis concentrations that would reflect increased protein turnover as discussed above displayed good correlations with rectal temperature under both anesthetic and conscious conditions. Furthermore, it is well known that AA administration increases oxygen consumption and elevates $T_b$ under both conscious and anesthetized conditions (1-3, 6). These findings therefore indicate that increased protein turnover in rats given AA contributes to increased metabolic heat production, resulting in elevated $T_b$.

The present study also revealed that anesthetic administration alone did not affect plasma MeHis concentrations. Anesthetics generally induce a decrease in $T_b$, as seen in anesthetized rats in the present study, and $T_b$ of anesthetized rats should be above the thermoregulatory threshold for thermogenesis (9). Thermogenic responses against $T_b$ decline might therefore not have been evoked, which would be different from the above-mentioned thermoregulatory response (increased protein turnover) to cold under the conscious condition (28). This is supported by the finding that urinary MeHis excretion after cardiac surgery did not differ between patients randomized to intraoperative blood temperatures of 28 or 20°C (29). In addition, myofibrillar protein degradation (MeHis release) in white or red muscles incubated at different temperatures is unaffected by temperature (30). Taken together, these findings may imply that hypothermic contributions by anesthetic treatment to the regulation of myofibrillar
protein degradation are minimal. This suggests that changes in myofibrillar protein turnover offer a determinant of T₈ modification by AA administration and that T₈ does not adjust myofibrillar protein turnover.

We showed herein that AA administration down-regulates mRNA expressions of several ubiquitin-proteasome-proteolytic pathway components in skeletal muscle of both anesthetized and conscious rats. This observation is consistent with in vivo data showing several AAs, particularly leucine, isoleucine, glycine, suppress the expression of ubiquitin-proteasome-proteolytic related genes in skeletal muscle (23, 31, 32). Furthermore, activation of insulin signaling in skeletal muscle, particularly phosphorylation of protein kinase B (PKB) and forkhead transcription factor Foxo3, in general, suppresses mRNA expression of atrogin-1 and MuRF1 genes (33–36). In our previous study, PKB phosphorylation was clearly observed in both anesthetized and conscious rats given amino acids (11). This suggests that the decreases in mRNA expression of ubiquitin-proteasome-proteolytic related genes by AA administration likely result from the activation of insulin signaling, although Foxo3 phosphorylation has not been determined. However, decreases in mRNA expression of ubiquitin-proteasome-proteolytic related genes in skeletal muscle in this study did not exactly reflect plasma insulin levels (11). Although the reasons for this remain unclear, anesthetic treatment may affect unknown factors that prevent the insulin signaling pathway in the ubiquitin-proteasome pathway.

The ubiquitin-proteasome-proteolytic pathway among several proteolytic pathways is up-regulated in various catabolic states that cause muscle proteolysis (14, 15, 33, 34). Suppression of mRNA expression of the ubiquitin-proteasome-proteolytic related genes, in general, prevents catabolic response produced by catabolic insult (16, 17, 36). In contradiction to these finding, our data and other studies suggest that atrophic response in skeletal muscle can occur independently of ubiquitin-proteasome-proteolytic related gene expression (37–39). We speculate on some possible explanations for the dissociation between the effects of AA administration on the expression of mRNA associated with the ubiquitin-proteasome-proteolytic pathway and myofibrillar proteolysis. First, levels of mRNA expression of the genes and myofibrillar proteolysis were determined under non-stressed conditions in the current experiment. This implies that the role of gene expression of the ubiquitin-proteasome pathway in myofibrillar proteolysis is important just in cases where catabolic insult occurs. Second, other components involving the proteolytic pathway are actually rate-limiting, despite the fact that the activation of insulin signaling is prominent in rats given AAs (11). In fact, microarray profiling of human skeletal muscle revealed that increased plasma insulin concentrations elevate expression of 17 mRNAs for ubiquitin-conjugating enzymes and 11 mRNAs for the proteasome components (40). Third, down-regulation in the two ligases, especially MuRF1 in the current study, was not enough to decrease myofibrillar proteolysis. Mice lacking both atrogin-1 and MuRF1 exhibit the greatest protection from denervation-induced muscle loss compared to mice lacking only one of these genes (41). Fourth, the decreases in mRNA of the components in the ubiquitin-proteasome-proteolytic pathway did not lead to coordinate changes in their protein levels and/or activities during AA infusion, since factors controlling translation initiation are activated by AA administration in a similar model as reported (11).

In conclusion, the results shown herein strongly suggest that AA administration for 3 h enhances myofibrillar protein degradation, which is reflected in T₈ modification in both anesthetized and conscious rats. Taken together with our previous finding that AA administration enhances muscle protein synthesis in both situations, the current results imply that changes in myofibrillar protein turnover offer a determinant of T₈ modification by AA administration. In addition, our data suggest that decreases in ubiquitin-proteasome-proteolytic related gene expression examined herein do not necessarily predict a coordinate change in myofibrillar proteolysis. Hence, the precise mechanisms regulating the myofibrillar protein degradation by AA mixture administration remain yet to be determined.

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REFERENCES


