Contractile Protein Breakdown in Human Leg Skeletal Muscle as Estimated by [$^2H_3$]-3-Methylhistidine: A New Method

R.F. Vesali, M. Klaude, L. Thunblad, O.E. Rooyackers, and J. Wernerman

3-Methylhistidine urinary excretion and net balances across the leg or forearm have been used as markers of contractile protein breakdown in muscle tissue. Here we investigate whether infusion of labeled 3-methylhistidine and the measurement of the arteriovenous dilution of the tracer with unlabeled 3-methylhistidine will result in more consistent and precise measurements of 3-methylhistidine rates of appearance and consequently muscle contractile protein breakdown rates in comparison with conventional arteriovenous concentration difference measurements. Six healthy volunteers were studied in the postabsorptive state and received a primed continuous infusion of 3-[$^2H_3$-methyl]- methylhistidine and L-[ring-$^2H_5$]-phenylalanine for 4 hours. $^2H_3$-3-methylhistidine reached an isotopic steady state after 210 minutes in all subjects. Arteriovenous differences of 3-methylhistidine, measured by high-performance liquid chromatography (HPLC), showed both uptake and release from skeletal muscle, which is theoretically not likely to occur. The enrichment of $^2H_3$-3-methylhistidine was consistently lower in the femoral vein than in the artery, and therefore a constant net release of 3-methylhistidine from the leg was observed. The mean rates of appearance for 3-methylhistidine and phenylalanine were 0.44 ± 0.30 nmol × min^{-1} ×100 mL^{-1} and 11.2 ± 5.7 nmol × min^{-1} × 100 mL^{-1}, respectively. In summary, arteriovenous difference measurement of $^2H_3$-3-methylhistidine enrichment is more reliable than measurement of arteriovenous difference of unlabeled 3-methylhistidine. Consequently, measuring rates of appearance from leg muscle using labeled 3-methylhistidine resulted in more consistent and accurate values of contractile protein degradation rates in human skeletal muscle.

TO STUDY protein metabolism on the tissue level, it is necessary to have accurate in vivo assessments of protein synthesis and degradation simultaneously for tissue proteins, but also for specific proteins with specific functions. Available techniques give quantitative or sometimes semiquantitative as well as qualitative estimates. For measuring protein synthesis rates there are several techniques using the incorporation of a labeled amino acid into a protein or a protein pool of interest to obtain quantitative estimates. For degradation the situation is more problematic, and available quantitative techniques depend on mathematical modeling with a number of underlying assumptions and apply to total tissue protein only.

Measurement of urinary 3-methylhistidine secretion has been used as a marker of skeletal muscle (contractile) protein degradation.[1,2] Later, a tracer method to measure whole body 3-methylhistidine production rates for the same purpose was developed.[3] 3-Methylhistidine is formed by posttranslational methylation of histidine in the myofibrillar proteins, actin and myosin. Following degradation of these proteins, 3-methylhistidine cannot be reutilized (no tRNA for 3-methylhistidine is present) nor metabolized in humans, and is therefore quantitatively excreted in the urine. However, when employing this method, urine needs to be collected quantitatively and over a prolonged period of time,[4,5] which makes measurement of acute changes not possible. In addition, human subjects should refrain from eating meat for at least 3 days before the measurements, because meat contains 3-methylhistidine, which will also appear in the urine.[6] In addition, this approach has been criticized because contractile proteins located in other tissues (mainly the gut) could theoretically contribute to the urinary 3-methylhistidine secretion.[7] However, in humans this contribution seems to be minimal.[8,9]

To elude many of these potential problems, direct release of 3-methylhistidine from muscle has been measured using the arteriovenous balance technique.[6-8,10,11] The net balance of 3-methylhistidine over the leg or forearm will represent the actual degradation of contractile proteins. However, 3-methylhistidine concentrations in plasma are very low and even when adapting a sensitive high-performance liquid chromatography (HPLC) method,[12] the small arteriovenous difference cannot be detected reliably.

In this experiment we studied healthy volunteers in the postabsorptive state. The leg was used to represent skeletal muscle and degradation of contractile proteins was estimated by measurement of the efflux of 3-methylhistidine. 3-Methylhistidine labeled with a stable isotope was infused and the arteriovenous difference of tracer enrichment, ie, the rate of appearance, was measured, to calculate the efflux of 3-methylhistidine from the leg. Arteriovenous differences in 3-methylhistidine concentrations were determined in the same subjects. The hypothesis tested is that the arteriovenous difference of the labeled 3-methylhistidine can be measured more accurately than that of the 3-methylhistidine concentration, which will result in more consistent and reproducible measures of contractile protein degradation in leg skeletal muscle.

MATERIALS AND METHODS

Materials

L-[ring-$^2H_5$]-phenylalanine, 3-[$^2H_3$-methyl]-methylhistidine and L-[alpha,beta,beta,2,3,4,5,6-$^2H_8$]-phenylalanine were purchased from Cambridge Isotope Laboratory (Andover, MA). Tracer infusates were prepared by the local pharmacy and tested for sterility and pyrogenity.

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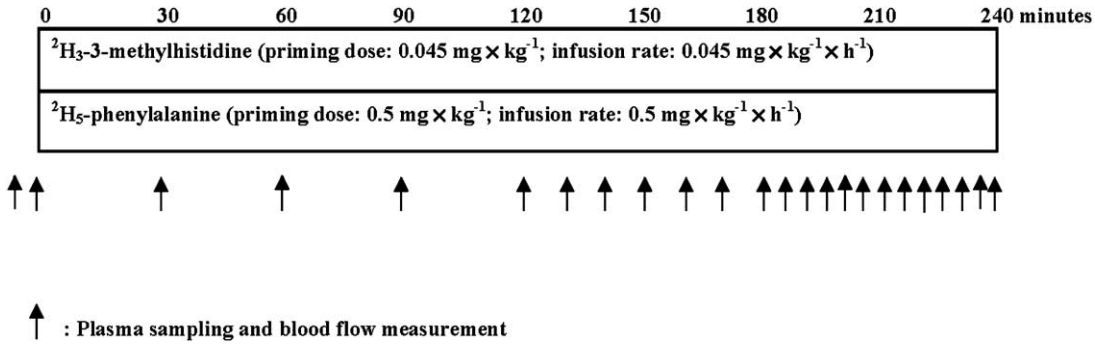

Fig 1. Experimental protocol of isotope infusion and blood sampling points. Phenylalanine and 3-methylhistidine tracers were infused in a forearm vein and blood samples were taken from the radial artery and the femoral vein. Blood flow was measured at the same time as blood samples were taken.

Subjects

Six healthy male volunteers were studied. Their mean age was 27 ± 5 years (range, 23 to 35), body weight was 83 ± 12 kg (range, 75 to 100), height was 184 ± 7 cm (range, 175 to 190), and body mass index was 25 ± 2 kg/m^2 (range, 22 to 28). Calf circumference of the leg used for blood flow measurements was 404 ± 23 mm (range, 370 to 425). The local ethical committee at Karolinska Institutet approved the study protocol and the subjects gave their written informed consent. All subjects were healthy as assessed by a physical examination and blood chemistry analysis. None of the subjects was taking any medication on a regular basis.

Study Protocol

The subjects reported to the research lab in the morning at 7:30 AM. No food was allowed after 8 PM the previous day. The experimental protocol is presented in Fig 1. A catheter was inserted in a forearm vein for phenylalanine and 3-methylhistidine tracer infusion. For blood sampling, catheters were inserted in the opposite forearm artery and in the femoral vein. Leg blood flow was measured in the same leg as the blood sampling using venous occlusion plethysmography. A prime continuous infusion of $^2H_5$-phenylalanine (priming dose, 0.5 mg/kg; infusion rate, 0.5 mg × kg^{-1} × h^{-1}) and $^2H_3$-3-methylhistidine (priming dose, 0.045 mg/kg; infusion rate, 0.045 mg × kg^{-1} × h^{-1}) was started and continued for 4 hours. During the first 2 hours, blood samples were taken every 30 minutes from the forearm artery and the femoral vein. Between 120 and 180 minutes, samples were taken every 10 minutes, and between 180 and 240 minutes, every 5 minutes. Plasma was separated from red blood cells by centrifugation at 600 × *g* for 15 minutes at 5°C. The plasma samples were frozen immediately and kept at −80°C until analysis.

Blood flow was measured with every blood sampling by venous occlusion plethysmography as described previously.[13,14] Briefly, an occlusion cuff was placed around the thigh 15 cm above the knee. The pressure used was 60 mm Hg. A single-strand mercury-in-rubber strain gauge was wrapped around the calf at the level of maximal circumference. The blood flow values expressed as milliliters per minute × 100 mL leg volume represent the mean of at least 9 separate readings. During the first 3 hours of the study, measurement of blood flow was performed during 7 minutes immediately after the blood sampling. Blood flow was measured continuously between 180 and 240 minutes, except for a short break during the period the blood samples were drawn. The multiple readings had a coefficient of variation (CV) of 16.6% ± 3.5% and the mean values of the blood flow between individuals had a CV of 10.2% ± 3.3%. The temperature of the room was constant between 20 and 21°C. Plasma flow was calculated from the blood flow and hematocrit.

Sample Analysis

For measurement of $^2H_3$-3-methylhistidine and $^2H_5$-phenylalanine enrichment and phenylalanine concentrations, the plasma samples were deproteinized in 1 mol/L perchloric acid, containing 20 μmol/L of $^2H_8$-phenylalanine as internal standard. Samples were prepared as previously described to determine the enrichment of phenylalanine.[15] Briefly, the amino acids in the perchloric acid extract were purified by cation exchange columns (AG-50 resin, Bio-Rad, Sundbyberg, Sweden). The eluent was dried over night. The residue was dissolved in N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) (Regis Technologies, Morton Grove, IL) and acetonitrile (1:1). The samples were heated for 30 minutes at 80°C to prepare the tert-butyldimethylsilyl (tBDMS) derivative of the amino acids. Mass-to-charge ratios (m/z) of 336, 341, and 344 for phenylalanine and 238 and 241 for 3-methylhistidine were measured on a quadruple gas chromatograph–mass spectrometer (GC-MS) (Agilent 5973n, Agilent Technologies, Stockholm, Sweden). The CVs for enrichment analysis were 3.6% and 2.8% for 3-methylhistidine and phenylalanine, respectively.

3-Methylhistidine concentration was analyzed by a HPLC method described previously.[12] Briefly, plasma samples were deproteinized in 3% sulfosalisylic acid-2-hydrate containing 80 μmol/L norvaline as internal standard. 3-Methylhistidine was analyzed using pre-column derivatization with ortho-phtaldialdehyde/3-mercaptopropionic acid (OPA/3-MPA) on a HPLC system (Alliance, Waters 2690, fluorescence detector Waters 474; Waters, Stockholm, Sweden). The CV for concentration analysis of 3-methylhistidine was 1.8 %.

Calculations

Net balances (NB) of amino acids across the leg were calculated as: NB = (C_A − C_V) × PF, where C_A and C_V are the amino acid concentrations (μmol/L) in the artery and the femoral vein, respectively, and PF is plasma flow. NB is expressed as nmol × min^{-1} × 100 mL^{-1} leg tissue.

In case of 3-methylhistidine, since there is no consumption (or utilization) of this compound in the skeletal muscle, the net balance of the labeled 3-methylhistidine tracer should be equal to zero. Therefore:

$$C_A \times E_A - C_V \times E_V = 0 \quad (1)$$

Thus:

$$C_V = C_A \times E_A/E_V \quad (2)$$

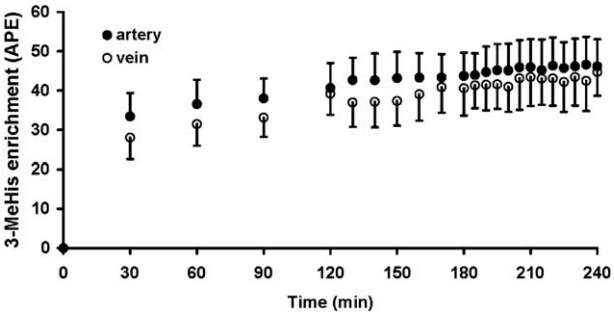

Fig 2. Arterial and venous isotope enrichment of $^2H_3$-3-methylhistidine (3-MeHis) in plasma expressed as atom percent excess (APE) during a constant infusion over 240 minutes in healthy volunteers (n = 6) in the postabsorptive state. Mean ± SD.

Where E_A and E_V are the tracer atom percent excess (APE) in artery and femoral vein, respectively.

Theoretically, the net release of 3-methylhistidine or its appearance rate (Ra) should be:

$$Ra = (C_V - C_A) \times PF \quad (3)$$

By substituting C_V in equation (2) into (3), we can reach the following equation:

$$Ra = (C_A \times E_A/E_V - C_A) \times PF = C_A \times (E_A/E_V - 1) \times PF$$

Ra is the rate of appearance (nmol × min^{-1} × 100 mL^{-1}).

For the phenylalanine tracer, the net isotopic labeled phenylalanine balance across the leg muscle, ie, $C_A \times E_A - C_V \times E_V$, is not equal to zero because a fraction of the artery-delivered phenylalanine tracer would disappear in the leg for protein synthesis Therefore, the rate of appearance for phenylalanine was calculated as:

$$Ra = C_V \times (1 - [E_V/E_A]) \times PF$$

Whole body rates of appearance (μmol × kg^{-1} × h^{-1}) were calculated as: F (E_i/E_p − 1), where E_i is the enrichment of the tracer infused, E_P is the enrichment in the artery at plateau, and F is the infusion rate of tracer.

Statistical Analysis

All data are given as means ± SD. Student's *t* test was used to determine whether fluxes were different from zero and multiple regression was used to determine correlation 3-methylhistidine and phenylalanine flux (Statistica, StatSoft Inc, Tulsa, OK).

RESULTS

The arterial and venous enrichments of $^2H_3$-3-methylhistidine increased up to 210 minutes in healthy volunteers (Fig 2). Isotopic steady-state was present for both arterial and venous plasma enrichment during the last 30 minutes in all subjects. Enrichment in the femoral vein was lower than in the artery for all 6 healthy volunteers, with a mean value during isotopic steady-state of 43.2 ± 7.2 APE and 46.2 ± 7.0 APE, respectively.

The mean plasma concentrations of 3-methylhistidine in the artery and femoral vein were 5.7 ± 0.4 and 5.6 ± 0.3 μmol/L, respectively. Arteriovenous differences of 3-methylhistidine were very variable, resulting in net balances showing both release and uptake of 3-methylhistidine (Fig 3). Net balances were not significantly different from zero.

Isotopic steady-state for $^2H_5$-phenylalanine was reached after 120 minutes. The enrichments of $^2H_5$-phenylalanine in the 6 healthy volunteers during the last 30 minutes were 7.2 ± 1.2 APE and 5.9 ± 1.2 APE in the artery and femoral vein, respectively. The mean value of phenylalanine plasma concentrations in the artery and femoral vein were 59.4 ± 8.3 and 63.6 ± 8.4 μmol/L, respectively.

The mean rates of appearances for 3-methylhistidine (Fig 4A) and phenylalanine (Fig 4B) during isotopic steady-state were significantly different from zero. The mean rates of appearance for 3-methylhistidine and phenylalanine were 0.44 ± 0.30 nmol × min^{-1} × 100 mL^{-1} and 11.2 ± 5.7 nmol × min^{-1} × 100 mL^{-1}, respectively.

Plasma flow did not change significantly during the study. The changes seen in amino acid fluxes between time points and subjects were mainly due to alterations in the arteriovenous differences.

Mean values for the whole body rates of appearance of 3-methylhistidine and phenylalanine during steady-state were 0.30 ± 0.08 μmol × kg^{-1} × h^{-1} and 36.6 ± 7.1 μmol × kg^{-1} × h^{-1}, respectively.

DISCUSSION

Theoretically, arteriovenous fluxes of 3-methylhistidine should always be negative, since protein-bound methylated histidine released during protein degradation cannot be reutilized for protein synthesis nor metabolized by humans. However, arteriovenous differences of 3-methylhistidine measured in the present study by HPLC are sometimes positive, indicating a net uptake of 3-methylhistidine, and even alternating between uptake and release within the same subject. In contrast, a consistent release of 3-methylhistidine was obtained by infusing a 3-methylhistidine tracer, measuring the arteriovenous difference of this tracer, and calculating the dilution of this tracer by unlabeled 3-methylhistidine over the leg (rate of appearance of 3-methylhistidine).

The main reason for obtaining more consistent results when using the tracer, is that the arteriovenous difference of the tracer can be measured more accurately than the arteriovenous difference in concentration of the tracee. The arteriovenous dif-

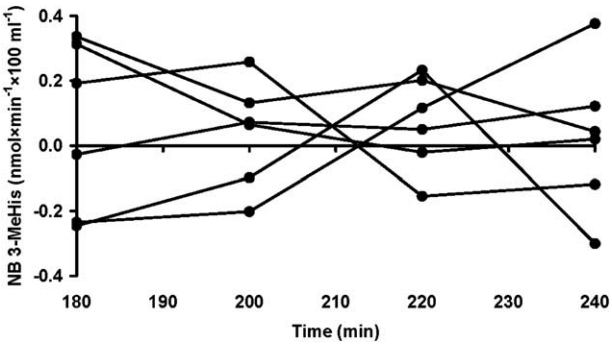

Fig 3. 3-Methylhistidine (3-MeHis) net balances (NB) across the leg in healthy volunteers (n = 6) in the postabsorptive state. Balances were not significantly different from zero.

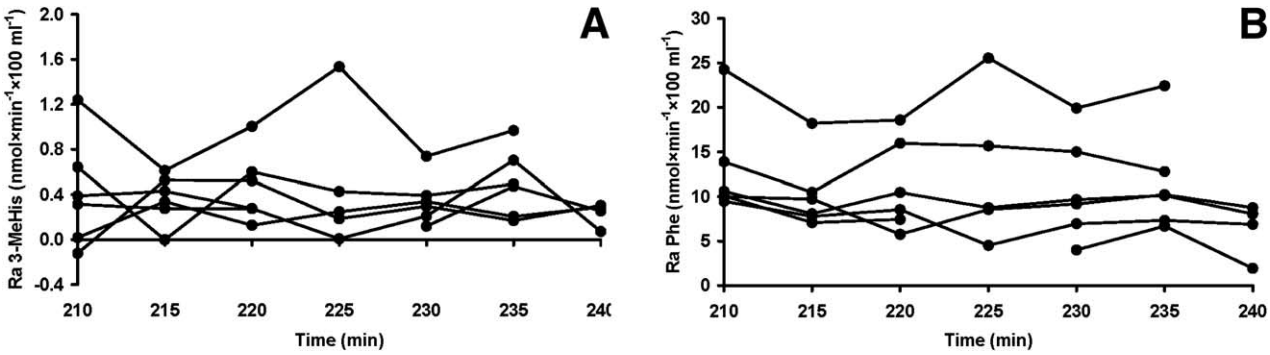

Fig 4. Rates of appearance (Ra) of (A) 3-methylhistidine and (B) phenylalanine from the leg at steady-state in healthy volunteers (n = 6) in the postabsorptive state. Mean rates of appearance for both amino acids were significantly different from zero.

ference of 3-methylhistidine concentrations was only 0.6% of the arterial concentration in the 6 subjects. This is several times lower than the analytical precision of the HPLC analysis, which was 1.8%. In comparison, the mean arteriovenous difference for the tracer was 6.4% of the arterial enrichment, which was higher than the analytical precision of 3.6% for the tracer measurement on the GC-MS.

Although, addition of a tracer made the measurement of 3-methylhistidine releases by leg muscle more consistent and less variable, the CV of the 7 measurements in each subject was still 52% ± 24%. Calculating the cumulative mean values showed that 4 measurements of the 3-methylhistidine rate of appearance need to be made during isotopic steady state to minimize the scatter of the values (Fig 5A and B). The cumulative mean was calculated by taking the mean of an increasing number of measurements until this mean value was stable. In addition, the small variation in the cumulative mean and especially the small variation in the cumulative standard deviation confirm that an isotopic steady-state was achieved for the labeled 3-methylhistidine.

The 3-methylhistidine whole body rate of appearance in the 6 subjects studied was 0.30 ± 0.08 μmol × kg^{-1} × h^{-1}. Assuming a physiological steady-state, this should be equal to the rate of disappearance and, since 3-methylhistidine is not metabolized, to urinary 3-methylhistidine secretions rates. The rate of appearance found in these 6 subjects amounted to 620 ± 155 μmol/d. This value is higher than 3-methylhistidine urinary secretion rates normally reported in the literature, which range from 210 to 426 μmol/d.[5,16,17] However, in the present study we studied young male subjects with a mean body weight of 87 kg, who were not controlled for dietary meat intake before the study. Since male subjects have a higher urinary 3-methylhistidine secretion than females[16] and dietary meat might increase the urinary secretion by up to 58%,[17] the subjects in the present study were likely to have a high 3-methylhistidine turnover. Whole body rate of appearance measured with a bolus injection of labeled 3-methylhistidine and compartmental modeling is reported to be 0.13 μmol × kg^{-1} × h^{-1}.[3] This is lower than measured in the present study due to the same reasons mentioned above. However, the present protocol was designed to accurately measure 3-methylhistine production over leg muscle and not to measure whole body production rates. Studies should be designed differently with strict dietary control if whole body measurements are of interest as well. For just measuring the leg muscle production rates, no interference from dietary behavior should be anticipated, which is an advantage.

Assuming a 3-methylhistidine content in human skeletal muscle of 4.2 μmol/g total protein,[18,19] the average net loss of contractile protein from leg muscle in the present study can be calculated to be 105 μg protein × min^{-1} × 100 mL^{-1} of leg

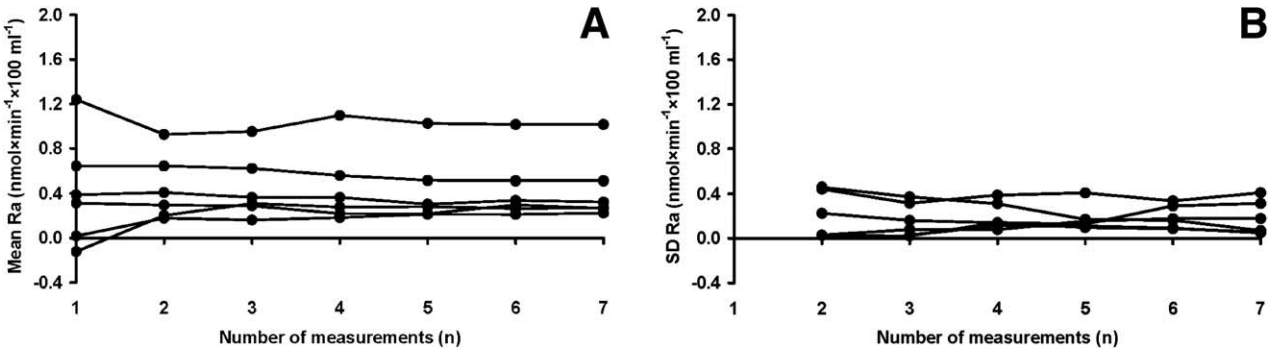

Fig 5. Cumulative values of (A) means and (B) standard deviations of 3-methylhistidine rate of appearance (Ra) at steady-state in healthy volunteers (n = 6) in the postabsorptive state. Cumulative mean and SD were calculated by taking the mean and SD of an increasing number of measurements, starting at 210 minutes.

tissue in the healthy young subjects. If 100 mL of leg tissue contains 68 g of muscle[20] which contains 13.6 g of protein, the fractional breakdown rates of contractile proteins in leg muscle of young male subjects can be calculated to be 0.046%/h. This value is close to the fractional synthesis rates of myosin heavy chain (0.047 %/h) and actin (0.075 %/h) measured previously in young healthy subjects.[21,22]

The 3-methylhistidine rate of appearance over the leg significantly correlated with the rate of appearance of phenylalanine ($R = 0.96$, $P < .005$). Phenylalanine is released from breakdown of all proteins, which to a large extent consist of contractile proteins in skeletal muscle. Therefore, this correlation suggests that the measurement of 3-methylhistidine rate of appearance is a reliable measure of protein breakdown rates in human skeletal muscle. The same blood flow value was used in the calculation for both measurements, but multiple regression analysis showed that the correlation was not dependent on blood flow. Still, this result needs to be confirmed in a larger group of subjects.

In summary, measurements of 3-methylhistidine rate of appearance from leg muscle using labeled 3-methylhistidine resulted in consistent and accurate values of contractile protein degradation rates in humans. As compared to measurement of concentration differences of 3-methylhistidine across the leg, this method offers clear advantages. This new technique can be recommended to study loss of protein mass and specifically contractile proteins from skeletal muscle in human pathology.

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