A Proteomics Analysis of the Effects of Chronic Hemiparetic Stroke on Troponin T Expression in Human Vastus Lateralis

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Stroke disability is attributed to upper motor neuron deficits resulting from ischemic brain injury. We have developed proteome maps of the Vastus lateralis to examine the effects of ischemic brain injury on paretic skeletal muscle myofilament proteins. Proteomics analyses from seven hemiparetic stroke patients have detected a decrease of three troponin T isoforms in the paretic muscle suggesting that myosin–actin interactions may be attenuated. We propose that ischemic brain injury may prevent troponin T participation in complex formation thereby affecting the protein interactions associated with excitation–contraction coupling. We have also detected a novel skeletal troponin T isoform that has a C-terminal variation. Our data suggest that the decreased slow troponin T isoform pools in the paretic limb may contribute to the gait deficit after stroke. The complexity of the neurological deficit on Vastus lateralis is suggested by the multiple changes in proteins detected by our proteomics mapping.

Key Words: Troponin T isoforms—Skeletal muscle proteome—2D gel electrophoresis—Stroke.

STROKE is the leading cause of chronic disability in the United States. The disability of stroke is widely attributed to the upper motor neuron deficits that result from ischemic brain injury. However, secondary biologic changes in hemiparetic skeletal muscle may also contribute to functional deficits. Little is known about skeletal muscle biochemical abnormalities after stroke. Studies on skeletal muscle pathology after stroke have produced variable conclusions (1–5). Recent studies report gross muscular atrophy in the thigh, as well as Vastus lateralis (V. lateralis) in chronic hemiparetic stroke patients (1).

Skeletal muscle alters its expression profile of major contractile proteins in response to pathology and environmental stimuli such as muscle use, loading, neural innervation patterns, external electrical stimulation, and exercise (6,7). Conditions associated with reduced muscular use; loading; and peripheral and central neural activation, such as prolonged bed rest, space flight, peripheral nerve, and spinal cord injury, result in a major shift of muscle phenotype toward a predominantly fast, type II myosin heavy chain (MHC) isoform (6–9). This shift from slow to fast MHC isoform predominance has been shown to increase fatigability and reduce oxidative capacity of the paralyzed quadriceps muscles following spinal cord injury (10–13). Skeletal muscle shift from slow to fast MHC predominance can be reversed by exercise (14) or low-frequency electrical stimulation (7,15,16).

Paretic V. lateralis has a major shift to predominantly fast MHC isoform expression that correlates with gait deficit severity (8). Consistent with this switch to fast MHC isoform predominance, the paretic limb has anaerobic metabolism and produces lactic acidosis during single limb exercise (5,17). The MHC fiber-type shift in the paretic limb is similar to the MHC shift observed in other muscle disuse, unloading, and spinal cord injury supporting a change of the molecular characteristics of the muscle contractile proteins in these disability conditions.

Although evidence for the role of troponins is mainly associated with cardiomyopathy (18–21), recent studies have suggested altered expression and release into the serum of skeletal muscle troponins associated with various skeletal muscle disorders (22), but few of them have focused upon conditions such as hemiparetic stroke that induce muscle atrophy and alterations of MHC expression. Troponins facilitate the interactions of myosin with actin by binding to calcium and regulating the action of tropomyosin during excitation–contraction coupling. Troponin T is the troponin subunit that binds to tropomyosin and interacts with troponin C, the Ca++-binding subunit. This Ca++-regulated interaction of troponin T with tropomyosin plays an important role in the calcium sensitivity of the actomyosin adenosine triphosphatase activity (23). There are distinct troponin T isoforms that each has markedly different calcium sensitivity, tolerance to acidosis (24,25), and patterns of expression under pathological conditions (26). To date, there are few studies investigating alterations of skeletal muscle troponin T expression in pathological conditions (22,27,28). In a rodent hind-limb suspension model, soleus muscle slow
troponin T expression is reduced (29,30), and this troponin T isoform reduction correlates with changes in muscle fiber calcium activation properties after unloading (27).

Proteome mapping (two-dimensional [2D] gel electrophoresis) has proven to be an excellent tool for the investigation of alterations in skeletal muscle protein expression levels in a variety of physiological conditions that affect muscle function. Studies of aged rat gastrocnemius used as a model for sarcopenia have demonstrated a perturbed pattern of protein expression, which reflects the decline in muscle strength in aging (21,31). Similarly, the application of proteomics mapping by 2D gel electrophoresis to the analysis of changes in protein expression associated with muscle adaptation to enhanced neuromuscular activity has identified coflin-I and transgelin as new markers of fast-to-slow muscle fiber transition (32). These and other studies have successfully described the global changes in skeletal muscle proteomics maps associated with specific physiological conditions. In our studies, we have developed proteome maps (2D gel electrophoresis) of the human \textit{V. lateralis} that have identified multiple differences in levels of protein expression in hemiparetic stroke patients that would provide insight in the effects of ischemic brain injury on the function of the paretic skeletal muscle.

In this study, we present evidence that the levels of expression of slow troponin T isoforms are decreased in paretic \textit{V. lateralis} compared with the nonparetic leg muscle. We identify multiple slow human skeletal troponin T isoforms by 2D gel electrophoresis and mass spectrometry (MS) and demonstrate a significant decrease of the slow skeletal troponin T isoform pool levels in the paretic \textit{V. lateralis} after stroke. Thus, we propose that the diminished levels of muscle slow troponin T protein isoforms may contribute to the gait deficit after stroke in the paretic limb.

METHODS

Patients and Muscle Biopsy Material

Men or women \((n = 7)\) at least 45 years of age with chronic hemiparetic gait deficits from an ischemic stroke were recruited for this study. Residual mild–moderate hemiparetic gait deficit was defined as gait asymmetry with reduced stance or reduced stance and increased swing only on the affected limb. All participants had preserved capacity for ambulation with or without assistive devices, such as ankle–foot orthoses, canes, or walkers. All participants had completed all conventional rehabilitation therapy more than 12 weeks and had stable neurological deficits for at least 8 weeks before they were eligible to enter the study. Chronic stroke was defined as more than 6 months after the index stroke to ensure stability of residual paretic gait deficits and to avoid any potential confounding by early neurological recovery or ongoing rehabilitation therapy on skeletal muscle. Study exclusion criteria included known muscle disease, anticoagulant medications, symptomatic congestive heart failure (New York Heart Association Class II), unstable angina, peripheral arterial occlusive disease (Fontaine Class II), pulmonary or renal failure, orthopedic or chronic pain conditions limiting mobility, dementia, untreated major depression, and aphasia (operationally defined as incapacity to follow two-point commands or provide adequate informed consent). Consecutive stroke patients who agreed to bilateral \textit{V. lateralis} muscle biopsies were enrolled and provided written informed consent. The University of Maryland Institutional Review Board approved this study.

Functional Measures: 30-ft Walk

The 30-ft floor walk is widely recognized as a valid index of mobility recovery after stroke that simulates home-based activities of daily functions. Participants were instructed to walk at a comfortable pace for 30 ft using their usual assistive device and/or orthoses at a pace that they would “walk across the room at home.” Walking was initiated from standing rest and ended 2 m beyond the 30-ft point to avoid the confound from end of walk slow down. The mean self-selected walking speed for the three trials was averaged.

Cardiovascular Fitness

Oxygen consumption was measured by indirect calorimetry during a graded exercise test on a motorized treadmill with open circuit spirometry. Briefly, participants walked at a constant velocity throughout the protocol; grade was initially set to 0%, increased to 4% after 2 minutes, and increased 2% every 2 minutes thereafter to maximal effort. Values for oxygen consumption were averaged in 30-second increments, and cardiovascular fitness was defined as the highest oxygen consumption value obtained for a full 30-second increment.

Knee Extension Strength

Quadriceps strength was measured by Medical Research Council manual muscle strength testing and by Kin-Com 125AP with isokinetic maximal concentric and eccentric knee extensor contractions at 30° per second on the paretic and nonparetic legs.

Knee Spasticity

Knee spasticity was based on the modified Ashworth spasticity scale.

Mid-Thigh Area by Computed Tomography Imaging

Bilateral thigh computed tomography (CT) imaging was performed using a PQ 6000 Scanner (GE Medical Systems, Piscataway, NJ) to quantify total thigh area muscle with Hounsfield units of 30–80 for the paretic and nonparetic legs.
Bilateral *V. lateralis* needle muscle biopsies were performed in untrained chronic hemiparetic stroke patients. Skeletal muscle punch biopsies were obtained under local anesthesia from the mid *V. lateralis*, approximately 12–13 cm above the patella on the anterolateral aspect of the thigh, using a 5-mm Bergström needle (Stille-Werner, Ronkonkoma, NY). Four 25-mg muscle specimens were frozen in isopentane precooled by liquid nitrogen and stored at −80°C. Direct comparisons of the paretic to the nonparetic limb muscles permitted determination of the molecular characteristics of paretic muscle that were attributable to abnormal pattern of motoneuron activation. The nonparetic limb muscle also served as an internal control tissue for individual confounds such as age, gender, body composition, illnesses, medications, and physical activity level.

**Protein Isolation and Gel Electrophoresis**

Total protein was isolated from frozen human muscle biopsy material (approximately 25 mg) by grinding the tissue with abrasive resin in urea/3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid;[3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) buffer (8 M urea, 4% CHAPS, supplemented with protease inhibitors: 1 μg/mL of antipain, chymostatin, leupeptin, pepstatin A, and 0.5 mM phenylmethylsulphonylfuoride) using the manufacturer’s protocol (PlusOne Sample Grinding Kit, Amersham Biosciences). Proteins were quantified using the BioRad Protein Assay kit with bovine serum albumin as a standard (33).

For single dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, proteins were mixed with 2× loading buffer (2% SDS, 5% β-mercaptoethanol, 20 mM Tris–HCl, pH 8.8) and resolved on 4–20% polyacrylamide gradient Tris–glycine gels (Bio-Rad Laboratories, Inc., Hercules, CA).

For 2D gel electrophoresis, proteins (125 μg) were incubated in a rehydration solution (8 M urea, 2% CHAPS, 0.5% immobilized pH gradient [IPG] buffer, bromophenol blue, 16 mM diithiothreitol [DTT]) for 30 minutes and then applied to 11-cm IPG strips (Amersham Biosciences). In preliminary experiments, 11-cm strips from pH 3–10 rehydrated and then focused for 17,500 V-hour. IPG strips were equilibrated in SDS equilibration buffer (50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 100 mM DTT) and placed onto 10%–20% Tris–glycine SDS-PAGE gels (Criterion, Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, the gels were stained with SYPRO Ruby Gel Stain (Molecular Probes, Carlsbad, CA) using the procedure recommended by the supplier or transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore Corporation, Billerica, MA) for Western analysis.

**2D Gel Image Analysis**

The 2D PAGE gels were stained with SYPRO Ruby Gel Stain, and a digital image obtained using the Fluorochrome 8900 Imaging System (Alpha Innotech, San Leandro, CA). The images were analyzed using the Phoretix 2D version 5.01 software (Alpha Innotech). Pool values were normalized within individuals using a protein that had been previously shown not to vary from hemiparetic to nonparetic muscle.

**Identification of Proteins Isolated From Gels**

Matrix-assisted laser desorption–ionization time-of-flight mass spectrometry sample preparation.—Individual protein bands were excised from 2D SDS-PAGE gels run simultaneously with the gels that were immunoblotted and analyzed by the Proteomics Core Facility at University of Texas Medical Branch. Gel samples were placed into separate 0.5-mL polypropylene tubes. Ammonium bicarbonate buffer (50 mM, 100 μL) was added to each tube, and the samples were incubated at 37°C for 30 minutes. After incubation, the buffer was removed, 100 μL of water was added to each tube, and the samples were incubated again at 37°C for 30 minutes. After incubation, the water was removed and 100 μL of acetonitrile was added to each tube to dehydrate the gel pieces. The samples were vortexed, and after 5 minutes, the acetonitrile was removed. Acetonitrile (100 μL) was again added to each of the sample tubes and vortexed and acetonitrile removed after 5 minutes. The samples were then placed in a SpeedVac for 45 minutes to remove excess solvent.

Lyophilized trypsin (20 μg; Promega Corp., Madison, WI) was added to 2 mL of 25 mM ammonium bicarbonate, pH 8.0. The trypsin solution was then vortexed and added to each sample tube in an amount (~10 μL) to just cover the dried gel. The samples are then incubated at 37°C for 6 hours.

After digestion, 1 μL of the sample solution was spotted directly onto a matrix-assisted laser desorption–ionization (MALDI) target plate and allowed to dry. Alpha-cyano-4-hydroxycinnamic acid (1 μL; Aldrich Chemical Co., Atlanta, GA) matrix solution (50/50 acetonitrile/water at 5 mg/mL) was then applied on the sample spot and allowed to dry. The dried MALDI spot was blown with compressed air (Deacon Laboratories, Inc., King of Prussia, PA) before inserting into the mass spectrometer.

**Mass Spectrometry**

Matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF–TOF) was used for
protein identification. Data were acquired with an Applied Biosystems 4800 MALDI-TOF/TOF Proteomics analyzer. Applied Biosystems software package included 4000 Series Explorer (v. 3.6 RC1) with Oracle Database Schema Version (v. 3.1.9.0), Data Version (3.80.0) to acquire both MS and MS/MS spectral data. The instrument was operated in positive-ion reflectron mode, mass range was 850–3,000 Da, and the focus mass was set at 17,000 Da. For MS data, 1,000—2,000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using a peptide mixture with reference masses 904.468, 1,296.685, 1,570.677, and 2,465.199.

Following MALDI MS analysis, MALDI-TOF–TOF was performed on several (1.27–29.34,35) abundant ions from each sample spot. A1-kV positive-ion MS/MS method was used to acquire data under post source decay conditions. The instrument precursor selection window was ±3 Da. For MS/MS data, 2,000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using each sample spot. Automatic external calibration was performed using reference fragment masses 175.120, 480.257, 684.347, 1,056.475, and 1,441.635 (from precursor mass 1,570.700).

Applied Biosystems GPS Explorer (v. 3.6) software was used in conjunction with Matrix Science Inc. (Boston, MA), to search the respective protein database using both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values and/or MASCOT protein scores. MS peak filtering included the following parameters: mass range 800–4,000 Da, minimum signal/noise (S/N) filter = 10, mass exclusion list tolerance = 0.5 Da, and mass exclusion list (for some parameters) included the following: selecting the enzyme as trypsin, maximum missed cleavages +1, fixed modifications included oxidation (M), precursor tolerance was set at 0.2 Da, MS/MS fragment tolerance was set at 0.3 Da, Mass = monoisotopic, and peptide charges were only considered as +1. The significance of a protein match, based on both the peptide mass fingerprint in the first MS and the MS/MS data from several precursor ions, is based on expectation values; each protein match is accompanied by an expectation value. The expectation value is the number of matches with equal or better scores that are expected to occur by chance alone. The default significance threshold is \( p < 0.05 \), so an expectation value of 0.05 is considered to be on this threshold. We used a more stringent threshold of \( 10^{-3} \) for protein identification; the lower the expectation value, the more significant the score.

### Table 1. Clinical and Demographic Data (n = 7)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 ± 2*</td>
<td>57–72</td>
</tr>
<tr>
<td>Gender (male:female)</td>
<td>5:2</td>
<td></td>
</tr>
<tr>
<td>Race (African American:Caucasian)</td>
<td>2:5</td>
<td></td>
</tr>
<tr>
<td>Body mass index</td>
<td>25 ± 3*</td>
<td>22–28</td>
</tr>
<tr>
<td>Latency since stroke onset (mo)</td>
<td>39 ± 44*</td>
<td>8–108</td>
</tr>
<tr>
<td>Hemiparetic side (right:left)</td>
<td>5:2</td>
<td></td>
</tr>
<tr>
<td>Ankle-foot orthosis:single-point cane:walker</td>
<td>3:6:1</td>
<td></td>
</tr>
<tr>
<td>30-ft Self-selected walking speed (mph)</td>
<td>1.24 ± 0.34*</td>
<td>0.82–1.81</td>
</tr>
<tr>
<td>Cardiovascular fitness (peak VO\textsubscript{2} [mg/kg/min])</td>
<td>14.5 ± 2.2*</td>
<td>11.2–17.8</td>
</tr>
</tbody>
</table>

**Note:** Values are mean ± SD.

### Immunoblot Analysis

Western analysis was performed as described previously (36). Specific antisera for slow skeletal troponin T and fast skeletal troponin T were obtained commercially (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies (Chemicon, Santa Cruz, CA) were used for detection in conjunction with SuperSignal chemiluminescent substrate (Millipore Corporation). For comparison of immunoreactive spots with SYPRO Ruby stained protein spots, Kodak X-Omat AR film was used to visualize specific antibody binding. For estimation of pool sizes, a digital image was obtained using the Fluorchem 8900 Imaging System (Alpha Innotech) and analyzed using the AlphaEase FC software (Alpha Innotech).

### Statistical Analysis

Skeletal muscle slow and fast troponin T pool levels were expressed as the absolute densitometry value and as the ratio of paretic to nonparetic pools. Paired \( t \) tests were used to examine the significance of differences of absolute troponin T values and the ratios of paretic to nonparetic troponin T. Significance was set at \( p < 0.05 \) for two-tailed \( t \) tests. Secondary variables included age, sex, latency index stroke, body mass index, strength, spasticity, mid-thigh cross-sectional area by CT, 30-ft self-selected walking speed, and cardiovascular fitness level. Pearson correlation analyses were used to determine the relationship between skeletal muscle troponin T values and the clinical and demographic features.

### Results

This study utilizes 2D gel proteome maps of the paretic and nonparetic \textit{V. lateralis} from chronic hemiparetic stroke patients to identify specific proteins whose expressions are changed with altered central nervous system activation and muscle atrophy. The demographics of the seven individuals recruited for this study are shown in Table 1. Individuals were between 57 and 72 years of age. Five were men, and two were women. Five had right hemiparesis, and two had left hemiparesis. The paretic leg had reduced cross-sectional area compared with the nonparetic leg by mid-thigh (paretic 78 ± 29 cm\(^2\) and nonparetic 93 ± 33 cm\(^2\)) in all six of seven.
individuals who had mid-thigh CT imaging. The walking speeds for these individuals with stroke were slow. The 30-ft self-selected walking speed was a mean of 1.24 ± 0.34 and range of 0.82–1.81 mph. Three used ankle–foot orthosis, six used a single-point cane, and one used a walker for ambulation. The cardiovascular fitness that was indexed by open circuit spirometry peak oxygen consumption was 14.5 ± 2.2 mL/kg/min.

**Slow Skeletal Troponin T Is Downregulated in Hemiparetic V. lateralis**

Analyses by 2D gel electrophoresis of total protein isolated from paretic and nonparetic *V. lateralis* reveal significant changes in protein pool levels in paretic compared with nonparetic muscle. There are a number of spots that show relative decreases in paretic muscle protein expression. MALDI-TOF–TOF analysis identifies that Spots 1, 2, 3, and 4 on the 2D gels correspond to human slow skeletal troponin T isoforms (Figures 1 and 2; Table 2). Three of these, Spots 1, 2, and 3 (Figure 1), were identified to be comparable in size (molecular weight [MW] 32,948) to the slow skeletal troponin T isoforms predicted by analysis of cloned, expressed troponin T messenger RNA (mRNA) species (37,38). The fourth spot (Spot 4 in Figure 1), also identified as a troponin T isoform, has a significantly lower MW compared with the slow troponin T isoforms reported in the literature.

The MALDI-TOF–TOF analyses identified all four spots as human slow skeletal muscle troponin T isoforms having a MW of 32,948 and theoretical pI 5.86 (Table 2). Although it has been shown that the products of alternative splicing yield isoforms missing amino acid residues 25–35 (MW 31,242; pI 5.64) and amino acid residues 25–35 and 205–220 (MW 30,096; pI 6.14), these isoforms were not specifically identified by the MS analyses. However, the mobility of the troponins in Figure 1 indicates that the approximate pI values of Spots 1–4 are 6.14, 5.86, 5.64, and 5.2, respectively. A comparison of these pIs with the theoretical pIs suggests that Spot 1, ~pI 6.1, may be the 30,096-kDa isoform; Spot 2, ~pI 5.9, may be the 32,948-kDa isoform, and Spot 3, ~pI 5.75, may be the 31,242-kDa isoform. The data in Table 2 clearly show a 100% confidence interval for the protein scores and protein expectation values ranging from $7.9 \times 10^{-15}$ to $1.6 \times 10^{-32}$ for all four of the troponins.

The identities of these spots are further confirmed to be slow skeletal troponin T isoforms by Western analysis using antisera specific to slow skeletal muscle troponin T applied to the 2D gel electrophoresis (Figure 2). The two higher MW isoforms, that is, Spots 1 and 2 in Figure 1 and the Spots within the dashed box and inset in Figure 2, which correspond to those identified in the literature, react strongly with the slow troponin T antisera. These data confirm the identity of these spots as human slow skeletal troponin T isoforms. An additional low-abundance slow skeletal troponin T isoform was detected by Western analysis but not detected on SYPRO Ruby stained gels (Spot 3 in the inset of Figure 2).

A novel, low-MW species (Spot 4 in Figure 1) was not detected on Western analysis using the slow troponin T antisera, which is a C-terminal–directed antibody. It was, however, detected through SYPRO Ruby gel staining and subsequently identified to be a slow skeletal troponin T isoform by MALDI-TOF–TOF analysis. These results are consistent with a truncated form of slow skeletal troponin T, missing the C-terminal end of the protein.
11-cm, pH 4–7, immobilized pH gradient strips for the first dimension and Vastus lateralis the antibody specific for the C-terminal region of troponin T. The circles, labeled Spots 1, 2, and 3, indicate the location of the three troponin T isoforms identified by MALDI-TOF analysis of a second identical gel stained with SYPRO Ruby. The inset is an enlarged image of the region shown in dashed lines. The circle labeled Spot 4 is not detected by the antibody specific for the C-terminal region of troponin T.

Identification of Other Proteins Downregulated in Hemiparetic V. lateralis

Several other spots whose levels decreased in the paretic muscle were also identified by MALDI-TOF–TOF analyses. These data are also shown in Table 2 and clearly indicate the complexity of the effects of neuronal damage on the level of muscle structural proteins. We identified, for example, that myosin light chain 6B (MYL6B; Figure 1A, Spot 5) was significantly downregulated in the paretic muscle. However, proteins in the same spot (Figure 1B, Spot 5) of the paretic 2D gel were identified as a mixture of heat shock protein beta-1 (HSPβ1) and growth factor receptor bound protein-2 (GRB2), labeled as Spots 5a and 5b in Table 2. Both of these proteins identified with very significant protein expectation scores. Because the mass spectrometry data only identify MYL6B in contralateral muscle and only HSPβ1 and GRB2 in paretic muscle, our results suggest that MYL6B may have been downregulated and that the HSPβ1 and GRB2 may have been induced in the paretic muscle. In addition, Spot 6 in Figure 1A and B, which was identified as myosin/myoglobin, is also downregulated in the paretic muscle (Table 2, Spots 6 and 6a).

In these studies, we also observed a significant level of patient-to-patient variation of protein levels between the paretic versus nonparetic muscle that were not statistically significant when combined with the data from the other cohort samples. The differences were, however, very significant within the single-patient samples. Although these variations are interesting with respect to the individual responses to stroke, these data require further study as to their significance to the overall characteristics of the paretic muscle.

Western Analysis of Troponin T Pool Levels in Paretic and Nonparetic V. lateralis

Recent evidence shows a shift from slow to fast MHC isoforms in V. lateralis from hemiparetic stroke patients (8).

Table 2. Matrix-Assisted Laser Desorption–Ionization Time-of-Flight Mass Spectrometry Identification of Protein Spots From Two-Dimensional Gel

<table>
<thead>
<tr>
<th>Spot</th>
<th>ID</th>
<th>Calculated MW (Da)</th>
<th>Protein Score*</th>
<th>Protein Score†</th>
<th>Total Ion Score‡</th>
<th>Protein Expectation Value§</th>
<th>Peptide Count∥</th>
<th>Theoretical pl</th>
<th>Apparent pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TNNT1_Hu</td>
<td>32,948</td>
<td>332</td>
<td>100</td>
<td>314</td>
<td>9.97631 × 10⁻³⁰</td>
<td>11</td>
<td>5.86</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>TNNT1_Hu</td>
<td>—</td>
<td>560</td>
<td>100</td>
<td>535</td>
<td>1.58114 × 10⁻⁵²</td>
<td>14</td>
<td>5.86</td>
<td>5.9</td>
</tr>
<tr>
<td>3</td>
<td>TNNT1_Hu</td>
<td>—</td>
<td>398</td>
<td>100</td>
<td>376</td>
<td>2.50594 × 10⁻³⁶</td>
<td>13</td>
<td>5.86</td>
<td>5.75</td>
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<tr>
<td>4</td>
<td>TNNT1_Hu</td>
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<td>183</td>
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<td>166</td>
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<tr>
<td>5</td>
<td>MYL6B_Hu</td>
<td>22,750</td>
<td>491</td>
<td>100</td>
<td>431</td>
<td>1.25594 × 10⁻⁴⁵</td>
<td>16</td>
<td>5.56</td>
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<tr>
<td>6</td>
<td>HSPβ1_Hu</td>
<td>22,769</td>
<td>152</td>
<td>100</td>
<td>142</td>
<td>9.97631 × 10⁻¹²</td>
<td>5</td>
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<td>—</td>
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<tr>
<td>7</td>
<td>GRB2_Hu</td>
<td>25,190</td>
<td>111</td>
<td>100</td>
<td>65</td>
<td>1.25594 × 10⁻⁰⁷</td>
<td>5</td>
<td>5.89</td>
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<tr>
<td>8</td>
<td>MYG_Hu</td>
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<td>227</td>
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<td>200</td>
<td>3.15479 × 10⁻¹⁹</td>
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<tr>
<td>9</td>
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<td>100</td>
<td>114</td>
<td>6.29463 × 10⁻⁰⁸</td>
<td>16</td>
<td>5.64</td>
<td>—</td>
</tr>
</tbody>
</table>

Notes: The NCBI Accession Numbers for Spots 1, 2, and 3 are P13805. MW = molecular weight.

* Protein score: The MOWSE (39) score calculated from mass spectrometry (MS) data by the MASCOT search engine for each protein matched for the MS peak list. The score is based on the probability that the peptide mass matches are nonrandom events. If the protein score is equal to or greater than the Mascot significance level calculated for the search, then the protein match is considered to be statistically nonrandom at the 95% confidence interval (CI). The protein score is $-10 \times \log(p)$, where $p$ is the probability that the observed match is random. Protein scores greater than 55 are significant ($p < 0.05$).

† Protein score CI%: The CI% for the protein score.

‡ Total ion score: Calculated from MS/MS data by weighing ion scores of individual peptides of a given protein.

§ Protein expectation value: The number of matches with equal or better scores that are expected to occur by chance alone for each protein score in a peptide mass fingerprint, and each ion score in an MS/MS search. It is directly equivalent to the E value in a Blast search result. The expectation value is 0.05 for a score that is exactly on the default significance threshold ($p < 0.05$). An increase of the score by 10 drips the expectation value to 0.005. The lower the expectation value, the more significant the score. Expectation value threshold was set at 0.001.

∥Peptide count: The number of peptides with unique sequences matching the selected protein.
To determine whether a similar shift in the troponin T expression pattern occurs in stroke patients, total protein from paretic and nonparetic *V. lateralis* from four patients was analyzed by 1D SDS-PAGE and Western immunoblot using antisera to specific slow and fast skeletal muscle troponin T (Figure 3A). The relative pool levels in a cohort of six chronic stroke patients, determined by this procedure, are presented in Table 3 and summarized graphically in Figure 3. The two major slow troponin T isoforms (Spots 1 and 2) identified on 2D gels have essentially identical MWs differing significantly only in pI. Therefore, on 1D SDS-PAGE slow skeletal troponin T antisera detect both isoforms as a single band. Table 3 illustrates that the nonparetic slow skeletal troponin T pool level varies widely from individual to individual. However, in every individual, the relative paretic slow skeletal troponin T pool is decreased compared with the nonparetic muscle (paretic/nonparetic slow troponin T ratio). The fast skeletal troponin T pool level similarly varies widely from individual to individual for both the paretic and the nonparetic muscles. Notably, there is no consistent direction for the fast skeletal troponin T in the paretic muscle pool level relative to the nonparetic muscle, that is, the fast troponin T paretic/nonparetic ratio increases in some individuals and decreases in other individuals.

Paired *t* tests were used to examine the significance of the differences of absolute fast and slow troponin T values in paretic compared with nonparetic muscle from each individual. Only the decrease in pool size of slow troponin T in paretic muscle was statistically significant (Figure 3). To confirm the results of our *t* test analyses, we also performed analyses of the variance and nonparametric analysis of the differences between absolute fast and slow troponin T values in the paretic and nonparetic legs. The results of these tests revealed the same level of significance as found with the paired *t* tests. We conclude that although our study involves a small sample size, the differences between the limbs appear to be robust and that there is no beta error present. Although the mean of the paretic/nonparetic ratio shows an increase in fast skeletal troponin T, this result is not significant.

**Comparison of Slow Skeletal Troponin T Pools Measured by Western Analysis and 2D Gel Electrophoresis**

The pool sizes of each slow skeletal troponin T isoform were determined for paretic and nonparetic muscle by Western blot analysis. The spot density data for the three high-MW slow troponin T isoforms on the 2D gels (Figure 2: Spots 1, 2, and 3) were directly compared with a single band representing these isoforms on the 1D SDS-PAGE/Western analysis of total protein. The data from all six paretic and nonparetic muscle samples show a decrease of the slow troponin T pool in paretic compared with nonparetic muscle (Table 4). Furthermore, similar reductions were observed in each individual for ratios measured by Western analysis or 2D gel electrophoresis (Table 4). These results confirm the Western analysis results and demonstrate that pool measurement using 2D gel electrophoresis has similar sensitivity and accuracy to Western analysis.

Finally, the magnitude of the pool size changes for three slow skeletal troponin T isoforms (Spots 1, 2, and 4) in paretic compared with nonparetic *V. lateralis* was determined by MALDI-TOF–TOF analysis from the 2D gel electrophoresis. The average ratio of paretic/nonparetic pools of each slow skeletal troponin T isoform from the six individuals (Table 4) is shown in Table 5. These data further confirm that the pool level of each of the three isoforms is decreased in paretic compared with nonparetic muscle.

**Relationship Between Troponin T Levels and Clinical and Demographic Features**

Our protein data suggest that the significant decrease in the levels of slow skeletal muscle troponin T in the paretic
limb compared with those of the nonparetic limb (Tables 3 and 5) correlates with certain clinical features of the paretic muscle. For example, the self-selected walking speed was related to the slow troponin I level in the paretic ($r = .60, F = 2.2, \Sigma = 0.21$) and the nonparetic legs ($r = .83, F = 9, \Sigma = 0.04$). The slow troponin level in the paretic leg was related to the latency after the index stroke ($r = .69, F = 3.5, \Sigma = 0.13$). On the other hand, a similar comparison of the ratio of the fast skeletal muscle troponin T in the paretic versus nonparetic limb, that is, no change in pool level, is consistent with no effect of the stroke on the function of these muscle fibers. However, although these correlations suggest these clinical features may be consequences of troponin level changes, further studies are needed to establish their significance to the functional outcomes characteristic of the stroke. There was no relation between fast troponin T and walking speed or stroke latency, and there was no correlation between either slow or fast troponin T levels and knee extension strength and spasticity, cardiovascular fitness, or mid-thigh CT area.

**DISCUSSION**

Stroke is a major cause of chronic disability in the United States. In this study, we examined the changes in skeletal troponin T pool levels in chronic hemiparetic *V. lateralis* and correlated these to observed functional deficits. The significant decrease of the three specific isoforms of slow skeletal troponin T in paretic leg muscle suggests that this may be a contributing factor to the muscle paresis. More specifically, the decreased slow skeletal troponin T protein pool levels in paretic compared with nonparetic *V. lateralis* suggests that the loss of neuronal function to the muscle as well as the ensuing muscle atrophy may affect processes such as mRNA production or protein turnover. The specificity of the consequences of this loss of neuronal function is evident by the fact that fast troponin T differences were not statistically significant. On the other hand, skeletal muscle has been shown to modulate its contractile properties by the expression of different troponin T isoforms under various pathological conditions (13,35). For example, it has been shown that different troponin T isoforms have markedly different tolerance to acidosis (24,25). The paretic leg muscle in untrained chronic, hemiparetic stroke patients has been shown to be relatively anaerobic in single limb exercise studies (5,40). Therefore, the lower anaerobic threshold in the paretic compared with nonparetic leg after stroke might compound the alteration in the paretic leg troponin T profile. Thus, we propose that a major consequence of the motor neuron dysfunction combined with muscle atrophy causes a severe decrease in pool levels of slow troponin T, thereby contributing to the skeletal muscle dysfunction. Furthermore, we propose that this may be a contributing factor to the observed gait deficit.

In humans, there is a single gene that encodes the multiple isoforms of slow skeletal troponin T (34,35,41). The multiple slow skeletal troponin T isoforms are produced, therefore, by alternative splicing of a single primary transcript (37,38). Thus, because the levels of each of the isoforms are decreased in the paretic muscle, the motor neuron defect may affect the mRNA level, thereby accounting for the decreased pool levels of each of the isoforms produced by alternative splicing. With respect to alternative splicing, there are two optional exon inserts that may give rise to up to four similarly sized isoforms (27,38,41). We observed three slow troponin T isoforms (Figure 1) on stained 2D gels and a possible fourth by Western analysis (Figure 2). The high-MW slow skeletal troponin T isoforms correspond to the isoforms predicted from cloned mRNA species (37,38). Furthermore, a comparison of the predicted pIs versus the approximate pIs indicted by their mobilities on the 2D gel electrophoresis that Spot 1, ~pI 6.1, may be the 30,096-kDa isoform; Spot 2, ~pI 5.9, may be the 32,948-kDa isoform.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Nonparetic Pool</th>
<th>Paretic Pool</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slow</td>
<td>643,734</td>
<td>206,976</td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td>860,440</td>
<td>958,320</td>
</tr>
<tr>
<td>2</td>
<td>Slow</td>
<td>277,277</td>
<td>108,864</td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td>2,613,240</td>
<td>2,179,190</td>
</tr>
<tr>
<td>3</td>
<td>Slow</td>
<td>926,471</td>
<td>822,185</td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td>2,222,970</td>
<td>1,220,700</td>
</tr>
<tr>
<td>4</td>
<td>Slow</td>
<td>289,492</td>
<td>68,292</td>
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<tr>
<td></td>
<td>Fast</td>
<td>547,680</td>
<td>766,220</td>
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<tr>
<td>5</td>
<td>Slow</td>
<td>717,612</td>
<td>464,373</td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td>1,223,280</td>
<td>1,567,670</td>
</tr>
<tr>
<td>6</td>
<td>Slow</td>
<td>831,467</td>
<td>727,637</td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td>86,930</td>
<td>11,200</td>
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</tbody>
</table>

**Table 4. Relative Slow Skeletal Troponin T Pool Comparison**

<table>
<thead>
<tr>
<th>Individual</th>
<th>Western</th>
<th>Two-dimensional Gel</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.32</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>3</td>
<td>0.89</td>
<td>0.79</td>
</tr>
<tr>
<td>4</td>
<td>0.24</td>
<td>0.27</td>
</tr>
<tr>
<td>5</td>
<td>0.65</td>
<td>0.56</td>
</tr>
<tr>
<td>6</td>
<td>0.88</td>
<td>0.87</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>0.92</td>
</tr>
</tbody>
</table>

**Table 5. Comparison of Slow Skeletal Troponin T Isoform Pools in Paretic and Nonparetic Muscle**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Paretic/NonParetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot 1</td>
<td>0.74</td>
</tr>
<tr>
<td>Spot 2</td>
<td>0.72</td>
</tr>
<tr>
<td>Spot 4</td>
<td>0.47</td>
</tr>
</tbody>
</table>

*Note: *Values are the mean ratio for six individuals.
isoform, and Spot 3, ~5.75, may be the 31,242-kDa isoform. The similarity of the theoretical versus approximate pI values provides further support for the conclusion that we may have identified troponin T isoforms.

The additional low-MW isoform identified by MALDI-TOF analysis on 2D gels is not recognized by antisera directed against a C-terminal peptide, indicating a C-terminal sequence variation. These observations suggest that we may have identified a human isoform of slow skeletal troponin T with a C-terminal variation. Our data (Table 5) show a decrease in the level of this isoform in paretic muscle similarly to the high-MW forms. Jin and colleagues (42) have reported a low-MW mouse slow skeletal troponin isoform, which differs at the N-terminal region. Thus, these studies as well as our observations suggest the existence of two isoforms, one that differs at the N-terminal end and the other at the C-terminal end. However, our studies do not rule out the possibility that the low-MW isoform (Spot 4) may be a product of proteolysis. The functional significance of these isoforms remains to be determined.

Although the literature strongly supports our identification of the three slow troponin T isoforms as products of alternative splicing, because we are working with samples from elderly patients (66 ± 2 years), it is possible that these isoforms are formed by age-associated protein modifications due to oxidations such as carbonylation and/or nitration. In our current studies, for example, we have shown that nitrations of creatine kinase increases dramatically in aged compared with young mouse skeletal muscle. These results strongly suggest that the troponins in the contralateral aged human V. lateralis used in these studies may be oxidatively modified and that this may also result in the formation of multiple isoforms. Because we do not have skeletal muscle samples from young participants, this comparison cannot be made. We are currently testing whether the troponins of aged human and mouse skeletal muscle are modified compared with young muscle.

Our observation of a significant decrease in slow skeletal troponin T pool levels in the hemiparetic V. lateralis is consistent with studies that showed decreased slow MHC pools in paretic muscle (8). These authors also report a corresponding increase in fast MHC pools, indicative of a switch from slow to fast muscle fiber types. Our data, however, do not show a statistically significant, corresponding increase in fast skeletal troponin T pool levels. This discrepancy may be due to the fact that the pathological condition of the paretic muscle caused by motor neuron dysfunction, for example, loss of electrical spike stimuli, may affect the normally tight regulation of fiber-specific proteins.

Although we have previously reported increased fast MHC proportions in hemiparetic leg muscle after stroke, no prior studies have examined the human skeletal muscle troponin profiles in either neurological disease or disuse syndromes. However, data from animal models of muscle disuse and peripheral denervation provide evidence that both MHC and troponin profiles are altered in a parallel fashion (43). Comparisons of gene expression patterns between normal and atrophied rat soleus skeletal muscle undergoing unloading atrophy (27,29) and rat extensor digitorum longus undergoing denervation atrophy (43) yield similar results. Analysis of the rat soleus muscle (27) complementary DNA expression library demonstrated an increase in fast skeletal troponin T (29). Western analysis showed a decrease in slow and an increase in fast skeletal troponin T (30), and 2D gel analysis showed a reduction in the level of slow skeletal troponin T. Finally, microarray data comparing control and denervated rat extensor digitorum longus showed increases in fast MHC and cardiac troponin T (43). Thus, expression of muscle-specific proteins, troponin T and MHC, is altered in atrophied skeletal muscle induced by chronic stroke and other models of muscle atrophy.

The severity of gait deficits indexed by self-selected walking speed in chronic paretic stroke patients has been correlated with the increased proportion of fast MHC seen in the paretic compared with nonparetic leg (8). Our data demonstrate that the decrease of slow skeletal troponin T pool levels is another significant correlation of altered skeletal muscle proteins and functional deficits. This pattern is consistent with the report that the proportion of slow troponin T in paretic compared with nonparetic muscle was associated with gait deficit severity indexed by self-selected walking speed (8). Although it is recognized that functional consequences are due to the neurological deficits in the muscle of hemiparetic patients, our data raise the question of whether and how the failure of neurological function results in the loss of the troponins as well as altering other proteins detected by our proteomics mapping. For example, the neurological deficit may involve the role of neuronal activity in the regulation of transcription of several genes, one of which is the troponin gene as well as the alternative splicing processes and/or the efficiency of translation of the mRNA. Our studies suggest that the integrity of the myoneural activity is essential for the maintenance of the proteomic profile of the muscle, which reflects the level or efficiency of muscle function. This is consistent with our recent observations that creatine kinase, which is essential for adenosine triphosphate homeostasis, is heavily modified by nitrations and carbonylation, which is indicative of oxidative damage. Our studies also show that the activity of creatine kinase is decreased in the paretic muscle, thus suggesting a change in energy metabolism (data not shown). Together these processes strongly suggest a perturbed physiological environment that reflects the decline in function of the paretic muscle.

Our cross-sectional study has emphasized that altered skeletal muscle troponin T profiles are related to deficit profiles after stroke (28). Our current focus on altered troponin T expression patterns does not exclude the possibility of alterations to other myofibrillar proteins such as those shown
in genomic analysis of protein expression in chronic low-frequency stimulation (32). The altered troponin T profiles in the present study raise the possibility that exercise interventions, functional electrical stimulation, or pharmacological therapy may have the potential to alter troponin T profiles in skeletal muscle following stroke.

The significant multiple protein changes detected by our 2D gel analyses demonstrate the complexity of the biochemical alterations between the paretic versus nonparetic muscle. In addition to the changes in troponin T expression, MS analyses of several other spots have shown that (a) there is a significant level of patient-to-patient variation of proteins whose expression levels are affected in the paretic muscle. For example, we observed significant differences between the paretic versus nonparetic muscle in a single sample set that were not statistically significant when combined with the data from the other samples; (b) interestingly, we identified that MYL6B was one of the proteins significantly downregulated in the paretic muscle. However, the protein in the same spot of the paretic 2D gel turned out to be HSPB1 and GRB2. Thus, we speculate that the MYL6B may have been downregulated in the paretic muscle and that HSPB1, a low-MW HSP22 and GRB2 are induced and coincidently migrate to the same spot. Alternatively, posttranslational modification of MYL6B may alter its mobility making it appear as though its pool level decreased. Interestingly, HSP27, which is detected on 2D gels at pH 5.5–6.4 in normal muscle, shifts to alkaline pHs in various myopathies with structural pathology of the desmin cytoskeleton (44). This shift may be due to posttranslational modification. We speculate that the mobility of a number of proteins identified to be downregulated by 2D gel electrophoresis may have shifted due to the modification. Importantly, because of the critical role troponin T plays in muscle function, our study contributes significantly to understanding a part of the molecular basis of skeletal muscle paralysis in hemiparetic stroke and neuromuscular disease. Our results contribute to a further understanding of the molecular characteristics of the paretic muscle and point to the significance and consequences of neuronal damage to the homeostasis and function of skeletal muscle.

Although we have used the nonparetic limb as the control condition, we acknowledge that other factors may affect the nonparetic, ipsilateral limb. For example, the upper motor neuron lesions of one hemisphere may influence ipsilateral motor unit activity, thereby affecting the overall proteomic maps. Secondly, the nonparetic leg spends more time in weight bearing during ambulation following stroke, suggesting that the nonparetic limb may receive more muscle loading compared with the paretic leg. Finally, as an alternative explanation to the data in Figure 3, it is possible that the overloading of the nonparetic limb may induce increases in the slow troponin. Further studies are needed to determine the molecular regulation of the troponin T profiles after stroke and other neurological disease conditions, their clinical relevance, and response to therapeutic interventions to improve muscle function.

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