Functional and biochemical characterization of soleus muscle in Down syndrome mice: insight into the muscle dysfunction seen in the human condition

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1Syracuse University, Department of Exercise Science, Syracuse, New York; 2State University of New York Upstate Medical University, Department of Neuroscience and Physiology, Syracuse, New York; 3Le Moyne College, Department of Biological Sciences, Syracuse, New York; 4University of Illinois at Chicago, College of Applied Health Sciences, Chicago, Illinois; and 5University of Missouri, Department of Nutrition and Exercise Physiology, Columbia, Missouri

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Cowley PM, Keslacy S, Middleton FA, DeRuisseau LR, Fernhalls B, Kanaley JA, DeRuisseau KC. Functional and biochemical characterization of soleus muscle in Down syndrome mice: insight into the muscle dysfunction seen in the human condition. Am J Physiol Regul Integr Comp Physiol 303: R1251–R1260, 2012. First published October 31, 2012; doi:10.1152/ajpregu.00312.2012.—Persons with Down syndrome (DS) exhibit low muscle strength that significantly impairs their physical functioning. The Ts65Dn mouse model of DS also exhibits muscle weakness in vivo and may be a useful model to examine DS-associated muscle dysfunction. Therefore, the purpose of this experiment was to directly assess skeletal muscle function in the Ts65Dn mouse and to reveal potential mechanisms of DS-associated muscle weakness. Soleus muscles were harvested from anesthetized male Ts65Dn and wild-type (WT) colony controls. In vitro muscle contractile experiments revealed normal force generation of nonfatigued Ts65Dn soleus, but a 12% reduction in force was observed during recovery from fatiguing contractions compared with WT muscle (P < 0.05). Indicators of oxidative stress and mitochondrial oxidative capacity were assessed to reveal potential mechanisms of DS-associated muscle weakness. Protein expression of copper-zinc superoxide dismutase (SOD1), a triplicated gene in persons with DS and Ts65Dn mice, was increased 25% (P < 0.05) in Ts65Dn soleus. Nontriplicated antioxidant protein expression was similar between groups. Lipid peroxidation was unaltered in Ts65Dn animals, but protein oxidation was 20% greater compared with controls (P < 0.05). Cytochrome-c oxidase expression was 22% lower in Ts65Dn muscle (P < 0.05), while expression of citrate synthase was similar between groups. Microarray analysis revealed alteration of numerous pathways in Ts65Dn muscle, including proteolysis, glucose and fat metabolism, neuromuscular transmission, and ATP biosynthesis. In summary, despite biochemical and gene expression differences in soleus muscle of Ts65Dn animals, the functional properties of skeletal muscle likely contribute a minor part to the in vivo muscle weakness.

low muscle strength, among other conditions (28). Considerable evidence shows persons with DS to have very low muscle strength and the presence of intellectual disability only partially explains this deficit (1, 2, 8, 11, 18, 24, 32, 34, 40). Persons with DS demonstrate up to a 40–70% decrement in knee extensor force-generating capacity compared with persons with intellectual disability but without DS, and persons without intellectual disability (1, 2, 8, 11, 18, 24, 32, 34, 40). This large discrepancy in muscle strength is comparable to the difference in muscle strength observed between healthy adults of 20 and 70 yr of age (29, 37). Not surprisingly, muscle weakness interferes with these individuals’ ability to perform tasks of daily living (8, 16, 39). Reductions in physical function limits opportunities for independent living, vocational opportunity and productivity, and economic self-sufficiency that ultimately lead to assisted living and lower quality of life in this population (17, 36, 49, 51). Furthermore, mobility impairments are predictive of mortality in adults with DS (13). Thus, understanding why persons with DS have such low muscle strength is an important area for research that has immense ramifications for these individuals’ social and medical care needs.

The Jackson Laboratory produced the first viable aneuploidy mouse model of DS (i.e., the Ts65Dn mouse) more than twenty years ago (19). Ts65Dn mice are segmental trisomic for mouse chromosome 16, which corresponds to roughly half of the genes on human chromosome 21 (21). The Ts65Dn mouse is the most extensively studied murine model of DS that displays a remarkable number of phenotypes expressed in the human condition, including structural and cognitive alterations of the brain, Alzheimer’s-like brain pathology, craniofacial alterations, and congenital heart defects (10, 33, 43, 44). Thus, the Ts65Dn mouse has considerable value in determining mechanisms of pathology in DS and for testing the efficacy of therapeutic treatments. Surprisingly, there has been little interest in the use of the Ts65Dn mouse to gain insight into the muscle weakness that is so prevalent in the human population. These mice display reduced grip strength, running speeds, motor coordination, and swimming speeds (14, 15). Thus, the Ts65Dn mouse model provides a suitable platform to identify potential mechanisms for DS-associated muscle dysfunction.

The purpose of this experiment was to directly assess skeletal muscle contractile properties of Ts65Dn mice and to examine potential mechanisms that may be responsible for muscle weakness. We tested the overarching hypothesis that
skeletal muscle from Ts65Dn mice would display force deficits in the nonfatigued state and impaired resistance to fatigue that would coincide with increased markers of oxidative stress and decreased indices of mitochondrial oxidative capacity.

**MATERIALS AND METHODS**

**Ethical Approval and Experimental Animals**

The Syracuse University Institutional Animal Care and Use Committee approved the use of animals for these experiments, which complied with the Guide for the Care and Use of Laboratory Animals. Male B6Eic3Sn a/A-Ts1(171)65Dn (Ts65Dn) mice and wild-type (WT) colony controls were purchased from the Jackson Laboratory (Bar Harbor, ME) (total n = 30; 14 Ts65Dn and 16 WT). Mice were housed in groups of 3–4 by genotype and maintained on a 12:12-h light-dark cycle with food and water provided ad libitum. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (120 mg/kg) with supplemental dosages provided as needed to maintain a surgical plane of anesthesia. Following a procedure to surgically remove the hindlimb musculature, the animals were euthanized by decapitation.

**In Vitro Muscle Contractile Function**

The soleus muscle was excised and immediately placed in a dissecting dish containing Krebs-Henseleit buffer equilibrated with 95% O2-5% CO2 at room temperature. The proximal and distal dissecting dish containing Krebs-Henseleit buffer equilibrated with 95% O2-5% CO2 at room temperature. The proximal and distal dissection was performed with a surgical knife. Following a procedure to surgically remove the hindlimb musculature, the animals were euthanized by decapitation.

**In Vitro Muscle Contractile Function**

The soleus muscle was excised and immediately placed in a dissecting dish containing Krebs-Henseleit buffer equilibrated with 95% O2-5% CO2 at room temperature. The proximal and distal tendons of the muscle were clipped with light-weight Plexiglas clamps (Harvard Apparatus, Holliston, MA), and the muscle was suspended vertically in a water-jacketed organ bath equilibrated with 95% O2-5% CO2 gas at 25°C containing Krebs-Henseleit buffer and 25 μM D-tubocurarine. The clamp attached to the proximal end of the muscle was secured to a force transducer (301C; Aurora Scientific, Aurora, ON) and the clamp attached to the distal end of the muscle was secured to a fixed Plexiglas rod; this positioned the muscle directly between platinum wire stimulating electrodes. Following a 15-min equilibration, optimal contractile length (LO) was determined by stimulating the muscle with supramaximal voltage and adjusting muscle length in ~0.05-g increments until maximum force was achieved. Force output was continuously monitored using a computerized data acquisition system (Aurora Scientific). Following the determination of LO, the bath temperature was increased to 37°C, and the muscle was allowed to equilibrate for an additional 30 min. The force-frequency relationship was determined using contractions evoked at stimulus frequencies of 1, 15, 30, 50, 80, 120, 160, 250, and 300 Hz with train duration of 500 ms with 2 min of recovery between contractions (Grass S48 stimulator; West Warwick, RI). Two minutes following the end of the force-frequency protocol, the muscles underwent a fatigue protocol (40 Hz, 0.5 trains/s, 500 ms trains) for 5 min, followed by a recovery period (40 Hz, 0.5 trains/s, 500-ms trains) with force measurements obtained at 30 s, and 1, 2, 5, 10, and 15 min. At the end of the contractile protocol the muscle was then removed from the bath, trimmed of excess tendon, blotted to remove excess buffer, and weighed. Muscle cross-sectional area was calculated by dividing muscle mass by the product of fiber length and muscle density (12). Fiber length was calculated by multiplying muscle length by the fiber length coefficient of 0.71 (5). A value of 1.06 g/cm^3 was used for muscle density. Force was expressed in absolute values, relative [% of peak tetanic tension (P0)], and/or stress, which was calculated by normalizing to muscle cross-sectional area.

**Western Blot Analysis**

Soleus muscles were homogenized (1:25, wt/vol) in phosphate buffer containing EDTA, 1% Triton-X, 5 mM DTT, 0.1% SDS, and protease inhibitor (Sigma, St. Louis, MO) using a microtube pestle with a conical tip (Research Products International, Mt. Prospect, IL). Following complete disruption of the muscle, the homogenate was centrifuged at 10,000 g for 10 min at 4°C. The protein concentration of the soluble fraction was assessed using the Bradford assay (Sigma-Aldrich, St. Louis, MO). The samples were diluted in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) to yield a final protein concentration. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with Poncous-S to ensure optimal protein loading and transfer. Membranes were then blocked in 1–5% skim milk protein in PBS containing 0.05% Tween 20 (PBS-T), and subsequently incubated with a primary antibody specific for SOD1 (Novus Biologicals, Littleton, CO), manganese SOD (SOD2; Cayman Chemical, Ann Arbor, MI), cathepsin (CAT) (Calbiochem, Darmstadt, Germany), glutathione peroxidase 1 (GPX1; Abcam, Cambridge, MA), 4-hydroxynonenal (4-HNE; Santa Cruz Biotechnology, Santa Cruz, CA), cytochrome-c oxidase subunit II (COX2; Santa Cruz Biotechnology), citrate synthase (CS; GeneTex, Irvine, CA), or GAPDH (Sigma). Membranes were subsequently washed with PBS-T and incubated with a horseradish peroxidase-antibody conjugate (1:1,000–5,000) diluted in blocking buffer provided against the primary antibody for 1 h. Membranes were then washed, treated with chemiluminescent reagents (Thermo Scientific, Rockford, IL), and exposed to light-sensitive film, which was scanned and subsequently analyzed using ImageJ software (42). Bands were quantified and expressed in arbitrary units relative to the GAPDH loading control band. 4-HNE bands were quantified individually and summed to obtain a total value. The 4-HNE membranes were stained with Coomassie G250 (Bio-Rad Laboratories), scanned, and protein per lane was quantified using ImageJ software (42). The 4-HNE bands were expressed relative to total protein per lane.

**Protein Carbonyls**

Protein carbonyls were measured as an index of protein oxidation using the commercially available OxyBlot assay kit (Millipore, Billerica, MA), according to the manufacturer’s instructions. Soluble proteins (15 μg) were reacted with 2,4-dinitrophenyldihydrazine for 15 min. Following the reaction process, 10 μg of 2,4-dinitrophenylhydrazone-derivitized protein was loaded onto 12% polyacrylamide gels, followed by electrophoresis, transfer of protein to nitrocellulose membrane, and visualization of protein according to the procedures described above. Protein bands were quantified using the same procedure as described for the 4-HNE blot.

**Muscle Fiber-Type Distribution**

Soleus muscles were embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) and frozen in either liquid nitrogen chilled isopentane or on dry ice. Sections from frozen muscles were cut at 10–20 μm using a cryostat, mounted on slides, and stored at ~80°C until processing. Muscle sections were air dried for 10 min followed by incubation in PBS containing 0.5% Triton X-100. Sections were simultaneously incubated with prediluted dystrophin (RB-9024-R7; Thermo Fisher Scientific, Fremont, CA), myosin heavy chain type 1 (1:20) (A4.840; DSHB, IA) and myosin heavy chain type IIa (1:1,000) (SC-71; DSHB, IA) antibodies in a dark, humid chamber at room temperature for 1 h. Sections were washed in PBS three times and simultaneously incubated with TRIT-C goat anti-rabbit IgG (1:50) (Invitrogen), Alexa Fluor 350 goat anti-mouse IgM (1:50) (Invitrogen), and FITC goat anti-mouse IgG1 (1:500) (Invitrogen) antibodies diluted in 10% normal goat serum in PBS in a dark humid chamber at room temperature for 1 h. Sections were washed three times in PBS and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were obtained at ×10 magnification using a Zeiss Axiolmager wide-field fluorescence microscope equipped with an AxioCam MRc digital camera and analyzed with AxioVision software (version 4; Carl Zeiss, Germany). For the composite image, dystrophin was labeled red, type
RNAs were isolated from muscle tissue as described above. Muscle fibers were labeled blue, and type IIa fibers were labeled green. The proportions of type I and type IIa myosin heavy-chain isoforms were determined by counting all visible fibers. Fibers that were unlabeled by type I or type IIa antibody were also counted and assumed to be either type IX or type Iib (i.e., IX/b).

RNA Isolation and Microarray Hybridization

The isolation of RNA, microarray hybridization, and initial data processing were performed at the State University of New York Upstate Medical University microarray core facility. Muscle tissue was disrupted by passage through a 22-gauge needle and homogenized with a QIAshredder column (Qiagen, Valencia, CA). RNA was extracted with the RNeasy Mini Kit (Qiagen). Quality and quantification of RNA were performed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). For microarray processing, 9 ng of total RNA was used for each sample. The samples were processed with the WT-Ovation Pico RNA Amplification System (NuGEN Technologies, San Carlos, CA) to produce cDNA, which was then processed with the WT-Ovation Exon Module (NuGEN Technologies) to produce cDNA of the appropriate strand for the Affymetrix Gene ST arrays. The cDNA was then fragmented and hybridized to the FL-Ovation CDNA Biotin Module V2 (NuGEN Technologies). A hybridization mix (5 µg of the labeled cDNA, 50 µM Control Oligo B2, 1× eukaryotic hybridization controls, 1× hybridization buffer and 10% DMSO) was prepared using Affymetrix GeneChip Hybridization Controls and the Affymetrix GeneChip Hybridization Wash and Stain Kit (Affymetrix, Santa Clara, CA). The hybridization mix was incubated at 99°C for 2 min, then 45°C for 5 min, and each sample was added to a GeneChip Mouse Gene 1.0 ST array (Affymetrix). This array contains probes for an estimated 28,853 genes and transcript clusters. Arrays were incubated for 18 h in a GeneChip Hybridization Oven 640 at 45°C with rotation at 60 rpm (Affymetrix). After 18 h, the arrays were washed and stained on the Affymetrix Fluidic Station 450. Arrays were scanned with an Affymetrix GeneChip Scanner 7G Plus. This experiment contained a total of eight arrays (4 animals/group). The microarray data were submitted to the Gene Expression Omnibus of NCBI (accession no. GSE39159).

Microarray Gene Expression Data Analysis

For between-group comparison of the gene expression data, the significance analysis of microarrays (SAM) method (two-class, unpaired) was used with the MultiExperiment Viewer in the TM4 suite of software tools (45, 50). An initial SAM analysis was performed with 1,000 permutations to view the SAM plot. The plot was interactive and allowed the user to select the delta value and examine the effect on the number of genes called significant, but also the false-positive rate. After viewing the test statistic distribution (i.e., observed vs. the expected d-value) a delta (=1.626) was selected that strictly controlled type I error that greatly limited the number of genes called significant by chance alone. We then performed two additional SAM analyses with 1,000 permutations each using the delta established from the initial analysis. The final list of differentially expressed genes comprised those that appeared in all three SAM analyses. We performed this process because the test statistic distribution is different each time the analysis is performed, which provides a slightly different list of genes called significant. For our set of analyses there were 16 genes called significant in at least one but not all three analyses. The final gene list contained 161 genes with a false discovery rate of 0.84%. On the basis of the false discovery rate and the number of genes in our list, there were ~1.3 false positive genes. Given our false discovery rate is far below what is considered acceptable (~5%), we feel this gives us a high degree of confidence that the type I error was adequately controlled. Functional categorization was performed using the National Center for Biotechnology Information and Gene Ontology databases (3).

Microarray Pathway Analysis

Pathway analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (25). The gene list for DAVID pathway analysis was generated by controlling 95% confidence intervals of the fold change of all genes on the array. Genes outside of the 95% confidence intervals were considered genes that were the most differentially expressed (i.e., most interesting). An additional criteria was also applied to this list of genes; the final list of genes included those with a $P < 0.01$ using an unpaired $t$-test. This gene list, which included 803 genes, was imported into DAVID to perform functional annotation clustering. The premise of this analysis is that similar terms are clustered into groups, which can be used to explore their relationship in a network format rather than examining singular terms (25). Clusters with an enrichment score of 1.3 were considered because they are biologically relevant (25). Terms associated with each cluster were considered if they reached statistical significance ($P < 0.05$) with a false discovery rate <5.0%.

Quantitative Real-Time PCR

Microarray data were validated by quantitative real-time PCR from the same RNA sample used for microarray analysis. RNA reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s recommendation. PCR was performed using IQ Supermix (Bio-Rad Laboratories) and the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories) using recommended cycling parameters. Predesigned TaqMan primers and probes were purchased from Applied Biosystems (Foster City, CA), which are designed to have 100% amplification efficiency. The standard curve method was used to quantify the expression of SOD1, amyloid precursor protein (App), ring finger protein 160 (Rnf160), and GAPDH, which was included to normalize gene expression data.

Statistical Analyses

The Shapiro-Wilk test for normality was used to test whether the data were normally distributed for parametric statistical analysis. If data violated the assumption of normality, a Box-Cox transformation was used so that the assumption of normality was met. All data were analyzed using independent sample $t$-tests and presented in the form mean ± SE unless otherwise stated. Data are visually portrayed using box plots, which depict the interquartile range and 10th and 90th percentiles with the mean represented by a plus sign (+). Area under the curve was calculated using the trapezoidal integral method for force-frequency, fatigue, and recovery data. The alpha was set a priori at $P < 0.05$. Stata 10.1 and Prism 5 (La Jolla, CA) statistical package was used for data analysis.

RESULTS

Animal and Soleus Muscle Mass Characteristics

Age (WT: 5.6 ± 0.2 mo; Ts65Dn: 5.6 ± 0.2 mo; $P = 0.90$) and body mass (WT: 34.5 ± 1.2 g; Ts65Dn: 32.6 ± 1.3 g; $P = 0.28$) of the animals were not significantly different between groups. Soleus muscle mass (WT: 10.2 ± 0.4 mg; Ts65Dn: 10.2 ± 0.4 mg; $P = 0.97$) and muscle mass: body weight ratio (WT: 0.30 ± 0.01; Ts65Dn: 0.32 ± 0.02; $P = 0.36$) were also not significantly different between groups.

Soleus Contractility

DS is associated with a number of phenotypic alterations that include hypotonia and low muscle strength, which directly and negatively impacts these individual’s ability to perform activities of daily living (8, 16, 34, 39, 40). The Ts65Dn mouse...
shows evidence of muscle weakness in vivo (14, 15); thus we sought to directly assess skeletal muscle force generation. In vitro twitch and tetanus contractile properties were similar between WT ($n_{11}$) and Ts65Dn ($n_{12}$) muscles: Peak twitch tension (WT: 46.2 $\pm$ 1.8 mN; Ts65Dn: 44.5 $\pm$ 2.7 mN; $P = 0.61$), specific twitch tension (WT: 4.3 $\pm$ 0.2 N/cm$^2$; Ts65Dn: 4.0 $\pm$ 0.2 N/cm$^2$; $P = 0.19$), time-to-peak twitch tension (WT: 19.1 $\pm$ 1.2 ms; Ts65Dn: 19.3 $\pm$ 1.0 ms; $P = 0.92$), half-relaxation time (WT: 22.6 $\pm$ 6.3 ms; Ts65Dn: 24.8 $\pm$ 4.1 ms; $P = 0.34$), peak isometric tetanic tension (WT: 230.7 $\pm$ 10.3 mN; Ts65Dn: 239.7 $\pm$ 11.1 mN; $P = 0.57$), specific tetanic tension (WT: 21.2 $\pm$ 0.6 N/cm$^2$; Ts65Dn: 21.6 $\pm$ 0.6 N/cm$^2$; $P = 0.60$), and twitch-tetanus ratio (WT: 0.20 $\pm$ 0.01; Ts65Dn: 0.19 $\pm$ 0.01; $P = 0.15$). The force frequency response (stress; N/cm$^2$) is presented in Fig. 1. The area under the curve for absolute force ($P = 0.58$), relative force (% of $P_o$) ($P = 0.45$), and stress ($P = 0.71$) were not different between groups.

The response of the soleus muscle to repeated activation and recovery is presented in Fig. 2. Fatigue progressively developed in the muscles as evidenced by the progressive drop in the active force generated during the stimulation protocol (Fig. 2, A and B). The resting force of the muscles gradually increased for both groups, indicating the inability of the muscles to fully relax during the protocol (Fig. 2, C and D). Area under the curve during fatigue for the absolute force ($P = 0.70$; Fig. 2A),

![Figure 1](image1.png)  
Fig. 1. Force-frequency relationship of soleus muscle (stress; N/cm$^2$) determined in vitro. $n_{11}$ for wild-type (•), and $n_{12}$ for Ts65Dn (○).

![Figure 2](image2.png)  
Fig. 2. Fatigue and recovery response of soleus muscle determined in vitro. A: absolute active force during fatigue and recovery. B: fatigue and recovery response expressed as a percentage of initial stress. C: absolute resting force during fatigue and recovery. D: fatigue and recovery response expressed as a percentage of initial resting stress. $n_{11}$ for wild-type (•) and $n_{9}$ for Ts65Dn (○). *Significantly different from wild-type ($P < 0.05$).
percent of initial stress ($P = 0.58$; Fig. 2B), resting absolute force ($P = 0.49$; Fig. 2C), and percent of initial resting stress ($P = 0.79$; Fig. 2D) was not significantly different between groups. Following the fatigue protocol, the muscles gradually recovered as indicated by the increase in active force, as well as gradual decrease in the resting force. The area under the curve during recovery for absolute force was not different between groups ($P = 0.49$; Fig. 2C), but the area under the curve for resting absolute force ($P = 0.02$; Fig. 2C) and percent of initial resting stress ($P = 0.01$; Fig. 2D) was higher in Ts65Dn than WT soleus muscles.

**Muscle Fiber-Type Distribution**

Myosin heavy chain content is known to have a significant impact on skeletal muscle function (47). Immunofluorescence analysis was performed on soleus muscle cross sections (Fig. 3, top) to assess muscle fiber type distribution (Fig. 3, bottom). No significant difference in fiber type distribution was detected between groups.

**Antioxidant Protein Expression and Markers of Oxidative Stress**

SOD1 overexpression is strongly implicated as one of the possible causes of oxidative stress in DS (27, 48). As anticipated, SOD1 protein expression was higher in Ts65Dn soleus by 25% ($P = 0.01$; Fig. 4); however, CAT ($P = 0.53$), GPX1 ($P = 0.55$), and SOD2 ($P = 0.96$) protein expression was not different between groups (data not shown). Oxidative stress is associated with increased production of oxidants and elevated markers of oxidative injury (41). We assessed two well-known markers of oxidative injury (4-HNE and protein carbonyls); protein carbonyls were 20% higher in Ts65Dn soleus ($P = 0.02$; Fig. 5), whereas 4-HNE was not significantly different between groups ($P = 0.39$; data not shown).

**Cytochrome-c oxidase and citrate synthase protein expression**

We hypothesized that the development of postfatigue weakness in Ts65Dn muscle could be attributable, at least in part, to a limitation in mitochondrial function. Thus, we determined the protein expression levels of COX2 and CS as indices of...
mitochondrial oxidative capacity. The rationale for selecting COX2 as a marker of oxidative capacity is based on two principal findings: 1) COX2 protein expression responds to changes in training status and aerobic capacity; and 2) protein expression of COX2 are directly correlated with its enzymatic activity (46). Citrate synthase is also a widely accepted marker of mitochondrial oxidative capacity (23). We found that soleus of Ts65Dn mice displayed a 22% decrease in COX2 expression ($P = 0.03$; Fig. 6), but expression of CS did not differ between groups ($P = 0.55$; data not shown).

**Microarray Analyses and Interpretation**

*Effects of trisomy on skeletal muscle gene expression.* The Ts65Dn mouse is segmental trisomic for mouse chromosome 16, which corresponds to roughly 132 out of the 230 genes in the human condition (21). There were a total of 94 out of the 132 trisomic genes on the array. At nominal $P < 0.05$, 51 genes were differentially expressed compared with WT. Thirty-five genes were up-regulated with a mean fold change of 1.76 and 16 genes were downregulated with a mean change of 1.33 (Supplemental Table S1).

*Gene expression analysis.* The global effects of trisomy on skeletal muscle gene expression were determined using microarray analysis. On the basis of the criteria that we employed to greatly minimize type I error, a final gene list was generated using SAM analysis that contained 159 genes. Of these genes, 106 were downregulated with a mean fold change of 1.6 (range: 1.35–1.85), and 53 were up-regulated with a mean fold change of 2.1 (range: 1.41–4.58). Functional categorization of these genes was performed using the National Center for Biotechnology Information and Gene Ontology databases (3). Ninety-seven of these genes had a known function and are presented along with their annotation and fold change in Supplemental Table S2. The genes with no known function are provided in Supplemental Table S3. The analysis identified genes with roles in metabolism, neuromuscular transmission, inflammation, muscle differentiation, proteolysis and damage...
stimulus, skeletal muscle structure and function, and iron metabolism. A brief summary of these changes is provided in Table 1.

Microarray results were validated using quantitative real-time PCR for 4 genes (SOD1, Rnf160, App, and GAPDH used as the normalizer). In agreement with the array data, GAPDH was not significantly different between groups (P = 0.27). Fold change of SOD1 for Ts65Dn relative to WT was +1.1 and +1.3 for microarray and PCR (P > 0.05 for both), respec-

### Table 1. Summary of gene expression analysis

<table>
<thead>
<tr>
<th>Gene Description (Gene Symbol)</th>
<th>Direction (Ts65Dn/WT)</th>
<th>Fold Change (Ts65Dn/WT)</th>
<th>Potential Effect on Ts65Dn Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes With a Role in Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBC1 domain family, member 1 (Tbc1d1)</td>
<td>Upregulated</td>
<td>1.93</td>
<td>Stimulates glucose uptake</td>
</tr>
<tr>
<td>MLX interacting protein (Mlxip)</td>
<td>Upregulated</td>
<td>1.76</td>
<td>Stimulates glycolysis but suppresses glucose uptake</td>
</tr>
<tr>
<td>Adrenergic receptor, alpha 1a (Adra1a)</td>
<td>Downregulated</td>
<td>1.45</td>
<td>Suppresses glucose uptake</td>
</tr>
<tr>
<td>Trans-acting transcription factor 5 (Sp5)</td>
<td>Downregulated</td>
<td>1.59</td>
<td>Stimulates cytochrome-c oxidase expression</td>
</tr>
<tr>
<td>Sterol regulatory element binding Transcription factor 1 (Srebf1)</td>
<td>Downregulated</td>
<td>1.44</td>
<td>Suppresses lipogenesis</td>
</tr>
<tr>
<td>Tankyrase, TRF1-interacting ankyrin-related</td>
<td>Downregulated</td>
<td>1.54</td>
<td>Regulates mitochondrial mobility in axons</td>
</tr>
<tr>
<td>ADP-ribose polymerase (Tnks)</td>
<td>Upregulated</td>
<td>1.66</td>
<td>Suppresses lipid metabolism</td>
</tr>
<tr>
<td>Melanin-concentrating hormone receptor 1 (Mchr1)</td>
<td>Downregulated</td>
<td>1.53</td>
<td>Increased energy expenditure</td>
</tr>
<tr>
<td><strong>Genes With a Role in Neuromuscular Transmission</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solute carrier family 18 (vesicular monoamine), member 3 (Slc18a3)</td>
<td>Downregulated</td>
<td>1.61</td>
<td>Suppresses synaptic vesicle formation</td>
</tr>
<tr>
<td>Intersectin 1 (SH3 domain protein 1A) (Itsn1)</td>
<td>Upregulated</td>
<td>1.83</td>
<td>Regulates synaptic vesicle cycling</td>
</tr>
<tr>
<td>Amyloid beta (A4) precursor protein (App)</td>
<td>Upregulated</td>
<td>2.41</td>
<td>Necessary for neuromuscular junction integrity</td>
</tr>
<tr>
<td>Syntaphilin (Synph)</td>
<td>Downregulated</td>
<td>1.54</td>
<td>Regulates mitochondria mobility in axons</td>
</tr>
<tr>
<td><strong>Genes With a Role in Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD276 antigen precursor (Cd276)</td>
<td>Downregulated</td>
<td>1.52</td>
<td>Decreased suppression of inflammation</td>
</tr>
<tr>
<td>Tumor necrosis factor-alpha-induced protein 8-like 2 (Tnfaip8l2)</td>
<td>Downregulated</td>
<td>1.60</td>
<td>Decreased suppression of inflammation</td>
</tr>
<tr>
<td>Complement component 3a receptor 1 (C3ar1)</td>
<td>Upregulated</td>
<td>2.37</td>
<td>Stimulates inflammation</td>
</tr>
<tr>
<td><strong>Genes With a Role in Muscle Differentiation/Regeneration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Numb gene homolog (Numb)</td>
<td>Downregulated</td>
<td>1.40</td>
<td>Suppresses muscle differentiation</td>
</tr>
<tr>
<td>Rb1cc1 RB1-inducible coiled-coil 1 (Rb1cc1)</td>
<td>Downregulated</td>
<td>1.41</td>
<td>Suppresses muscle differentiation</td>
</tr>
<tr>
<td>Nephros homolog (Nphs1)</td>
<td>Upregulated</td>
<td>1.58</td>
<td>Suppresses muscle differentiation</td>
</tr>
<tr>
<td>Nima (never in mitosis gene a)-related expressed kinase 1 (Nek1)</td>
<td>Upregulated</td>
<td>1.52</td>
<td>Stimulates muscle differentiation and regeneration</td>
</tr>
<tr>
<td>Avian reticulendotheliosis viral (v-rel) oncogene related B (Relb)</td>
<td>Downregulated</td>
<td>1.53</td>
<td>Stimulates muscle differentiation and regeneration</td>
</tr>
<tr>
<td>Kinase insert domain protein receptor (Kdr)</td>
<td>Upregulated</td>
<td>1.54</td>
<td>Stimulates muscle regeneration</td>
</tr>
<tr>
<td><strong>Genes with a Role in Proteolysis and Damage Stimulus</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase 7 (Enpp7)</td>
<td>Downregulated</td>
<td>1.54</td>
<td>Decreases oxidant production</td>
</tr>
<tr>
<td>AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00312.2012 • <a href="http://www.ajpregu.org">www.ajpregu.org</a></td>
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</tr>
<tr>
<td>c-Fos-like antigen 1 (Fosl1)</td>
<td>Downregulated</td>
<td>1.43</td>
<td>Stimulates antioxidant gene expression</td>
</tr>
<tr>
<td>Transmembrane BAX inhibitor motif containing 6 (Tnbin6)</td>
<td>Upregulated</td>
<td>1.55</td>
<td>Suppresses apoptosis</td>
</tr>
<tr>
<td><strong>Genes With a Role in Skeletal Muscle Structure and Function</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tropomodulin 1 (Tmod1)</td>
<td>Upregulated</td>
<td>1.89</td>
<td>Enhances muscle force production</td>
</tr>
<tr>
<td>Annexin A6 (Anxa6)</td>
<td>Upregulated</td>
<td>1.64</td>
<td>Enhances calcium regulation</td>
</tr>
<tr>
<td>Secretogranin V (Sgcv)</td>
<td>Downregulated</td>
<td>1.45</td>
<td>Stimulates Ryanodine-sensitive calcium release</td>
</tr>
<tr>
<td>Tescalcin (Tesc)</td>
<td>Downregulated</td>
<td>1.53</td>
<td>Stimulates maintenance of muscle pH</td>
</tr>
<tr>
<td>Phosphoglucomutase 5 (Pgm5)</td>
<td>Upregulated</td>
<td>1.65</td>
<td>Enhances structural stability of muscle plasmalemma</td>
</tr>
<tr>
<td>Mesoin (Mios)</td>
<td>Upregulated</td>
<td>1.63</td>
<td>Enhances cytoskeletal stability</td>
</tr>
<tr>
<td>Leprecan-like 1 (Leprel1)</td>
<td>Downregulated</td>
<td>1.55</td>
<td>Depressed collagen IV production for muscle basement membrane</td>
</tr>
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<td>Laminin, gamma 1 (Lamc1)</td>
<td>Upregulated</td>
<td>2.01</td>
<td>Enhances sarcolemma stability</td>
</tr>
<tr>
<td>Myosin, light polypeptide 6B (Myl6b)</td>
<td>Upregulated</td>
<td>2.78</td>
<td>Enhances cytoskeletal stability</td>
</tr>
<tr>
<td><strong>Genes With a Role in Iron Metabolism</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Transmembrane serine protease 6 (Tmprss6)</td>
<td>Downregulated</td>
<td>1.62</td>
<td>Stimulates iron uptake</td>
</tr>
</tbody>
</table>

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sively. Fold change for *App* in Ts65Dn relative to WT was +2.4 and +2.5 by microarray and PCR (*P* < 0.05 for both), respectively. Fold change for *Rgrp160* in Ts65Dn relative to WT was +3.7 and +2.2 for microarray and PCR (*P* < 0.05 for both), respectively. These results provide evidence that the microarray data accurately reflect the gene expression changes in Ts65Dn soleus.

**Pathway analysis.** Pathway analysis was performed using DAVID with an expanded list of the most differentially expressed genes. This list included a total of 803 genes, 68 of which were down-regulated and 735 were up-regulated. The DAVID database identified 745 of the 803 genes, and these were used to perform functional annotation clustering to identify overrepresented pathways. Results of the analysis are shown in Supplemental Table S4.1.

**DISCUSSION**

**Overview of Principle Findings**

Ts65Dn mice show evidence of muscle weakness in vivo (14, 15), but the contribution of skeletal muscle to these force deficits was previously unknown. The purpose of the current experiment was to directly examine skeletal muscle force generation in the Ts65Dn mouse. Moreover, we assessed indices of oxidative stress, mitochondrial oxidative capacity, and global mRNA expression to gain insight into potential mechanisms of DS-associated muscle dysfunction. Several key findings emerged from this experiment that include 1) soleus from Ts65Dn mice demonstrated normal in vitro force generation, but failed to recover from fatiguing contractions to the same degree as muscle from WT mice; 2) SOD1 protein was overexpressed in Ts65Dn skeletal muscle; 3) protein oxidation was greater in Ts65Dn muscle, but lipid peroxidation and expression of CAT, SOD2, and GPX1 were unchanged; 4) COX2 protein was reduced in Ts65Dn soleus but expression of CS was similar between groups; and 5) global gene expression patterns revealed alterations in numerous cell signaling pathways in Ts65Dn muscle. A discussion of these key findings is presented in the following sections.

**Nonfatigued Soleus From Ts65Dn Mice Exhibits Normal Force Production In Vitro**

Reduced grip strength was reported in persons with DS and the Ts65Dn mouse (14, 15, 20). We hypothesized that Ts65Dn muscle would exhibit deficits in force-generating capacity in the nonfatigued state. In contrast to our hypothesis, isometric contractile properties of Ts65Dn muscle were not significantly different from WT controls. The current data reveal that the strength limitations observed in Ts65Dn mice in vivo are not due to differences in the inherent functional properties of the muscle. This finding is consistent with the similarities observed in soleus muscle mass and myosin heavy chain composition between Ts65Dn and WT mice. It should be noted that the in vitro muscle preparation eliminated factors related to neuro-muscular transmission and central activation that are required for muscle force development in vivo. Although microarray analysis identified a number of differentially regulated genes involved in neuromuscular transmission (Table 1) in Ts65Dn muscle, no aspects of neural contributions were assessed in this experiment. The lack of a functional deficit in nonfatigued Ts65Dn muscle is suggestive that neural properties may play a larger role in mediating strength deficits in vivo, and this warrants further investigation.

**Ts65Dn Soleus Demonstrates Postfatigue Muscle Weakness In Vitro**

Ts65Dn muscle did not exhibit greater fatigability as hypothesized but showed evidence of postfatigue muscle weakness. Analysis of resting force values (measured between contractions) during the recovery period revealed that the Ts65Dn soleus did not exhibit similar decreases in resting force compared with WT. As a result, Ts65Dn soleus demonstrated a very modest reduction in active force generation during the recovery period. However, the force deficits observed in vitro may not be applicable to conditions in vivo. The presence of neural deficits would be expected to reduce the degree of muscle activation compared with stimulation conditions in vitro and potentially negate the development of postfatigue muscle weakness. Therefore, the observed functional limitations of the muscle following fatiguing contractions in vitro may play a minor role in mediating DS-associated muscle weakness in vivo.

We postulated that a mitochondrial limitation may underlie the failure of Ts65Dn soleus to recover to the extent of WT muscle following fatiguing contractions. Ts65Dn mice reportedly have a lower basal VO$_2$ (9), and mitochondrial dysfunction has been reported in brain tissue from persons with DS (6) and the Ts16 mouse model of DS (4). The microarray analysis identified alterations in pathways involved in glucose and fat metabolism, as well as ATP biosynthesis that could indicate an underlying mitochondrial limitation (Table 1). We hypothesized that Ts65Dn muscle would demonstrate reduced expression of proteins that are indicative of mitochondrial oxidative capacity. Despite observed reductions in COX2 protein expression, CS protein levels were similar between groups. Unaltered CS expression could signify normal mitochondrial content, but additional mechanisms of mitochondrial regulation may be altered in Ts65Dn muscle. Further experiments are required to test these hypotheses and to obtain definitive evidence of a mitochondrial limitation.

**Soleus Muscle from Ts65Dn Mice Overexpresses SOD1 But Does not Show Evidence of Oxidative Stress**

SOD1 is located in the cytosol and intermembrane space of the mitochondria and functions to dismutate superoxide radicals to hydrogen peroxide (41). Persons with DS, and the Ts65Dn mouse, could overexpress SOD1 because the gene is present in triplicate (21, 22). At the cellular level, overexpression of SOD1 may lead to increased production of hydrogen peroxide. Thus, we hypothesized that skeletal muscle of Ts65Dn mice would overexpress SOD1 and demonstrate oxidative stress. As anticipated, SOD1 expression was higher in Ts65Dn soleus but below what is predicted by gene dosage (i.e., 1.5-fold). There was no change in the expression levels of CAT, GPX1, or SOD2. This finding is consistent with results showing transgenic overexpression of SOD1 does not affect CAT or SOD2 expression in the mouse diaphragm and that

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1 The online version of this article contains supplemental material.
overexpression of a human SOD1 transgene in myoblasts does not affect GPX or CAT activities (31, 52).

Oxidative stress in Ts65Dn muscle is unlikely considering the similar expression levels of key antioxidant proteins in addition to 4-HNE, which is a marker of lipid peroxidation. Furthermore, microarray analyses did not reveal gene expression patterns that would indicate the presence of oxidative stress. The lack of oxidative stress in muscle is surprising, given that other cell types isolated from DS and Ts65Dn tissue demonstrate increased oxidative production and markers of oxidative stress (7, 30). One possibility for this discrepancy might be that SOD1 was not elevated in Ts65Dn muscle to a sufficient degree to elevate oxidant production (52).

A possible explanation for the selective accumulation of protein carbonyls is twofold. First, in spite of the fact that mRNA of proteolytic pathways was upregulated (See Supplementary Table S4), the efficiency of these processes may be compromised in DS tissue. For example, the chymotrypsin-like proteolytic activity of the proteasome is reduced in the cerebellum of Ts65Dn mice (35). Thus, the signal for protein degradation is active in Ts65Dn muscle, but the efficiency of the process may be compromised. The net effect would be the accumulation of oxidized proteins since the proteasome is primarily responsible for degrading oxidized proteins (26). Therefore, we surmise that oxidative stress was not present in Ts65Dn muscle and that elevated levels of protein oxidation could have been the result of decreased rate of oxidized protein degradation.

Perspectives and Significance

There is an urgent need to understand why persons with DS exhibit such dramatic muscle weakness, so researchers can identify potential avenues for treatment. Similar to the human condition, the Ts65Dn mouse displays muscle weakness in vivo, but no mechanisms were previously identified to explain these findings. Our results reveal that Ts65Dn muscle shows normal contractile function in the fatigued state and postfatigue muscle weakness in vitro. Although muscle from Ts65Dn animals lacked evidence of oxidative stress, clear evidence of a mitochondrial limitation in mediating postfatigue muscle weakness requires further investigation. Nonetheless, the postfatigue muscle weakness of Ts65Dn muscle observed in vitro is likely of minor importance to the muscle weakness that is observed in vivo. These findings suggest a greater role of neural activation in mediating in vivo muscle force deficits in Ts65Dn mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


