

Endurance capacity in maturing mdx mice is markedly enhanced by combined voluntary wheel running and green tea extract

Jarrold A. Call,¹ Kevin A. Voelker,¹ Andrew V. Wolff,^{1,2} Ryan P. McMillan,¹ Nick P. Evans,¹ Matthew W. Hulver,¹ Robert J. Talmadge,³ and Robert W. Grange¹

Departments of ¹Human Nutrition, Foods and Exercise, and ²Mechanical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia; and ³Biological Sciences Department, California State Polytechnic University, Pomona, California

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Call JA, Voelker KA, Wolff AV, McMillan RP, Evans NP, Hulver MW, Talmadge RJ, Grange RW. Endurance capacity in maturing mdx mice is markedly enhanced by combined voluntary wheel running and green tea extract. *J Appl Physiol* 105: 923–932, 2008. First published June 26, 2008; doi:10.1152/jappphysiol.00028.2008.—Duchenne muscular dystrophy is characterized by the absence of dystrophin from muscle cells. Dystrophic muscle cells are susceptible to oxidative stress. We tested the hypothesis that 3 wk of endurance exercise starting at age 21 days in young male mdx mice would blunt oxidative stress and improve dystrophic skeletal muscle function, and these effects would be enhanced by the antioxidant green tea extract (GTE). In mice fed normal diet, average daily running distance increased 300% from *week 1* to *week 3*, and total distance over 3 wk was improved by 128% in mice fed GTE. Running, independent of diet, increased serum antioxidant capacity, extensor digitorum longus tetanic stress, and total contractile protein content, heart citrate synthase, and heart and quadriceps β -hydroxyacyl-CoA dehydrogenase activities. GTE, independent of running, decreased serum creatine kinase and heart and gastrocnemius lipid peroxidation and increased gastrocnemius citrate synthase activity. These data suggest that both endurance exercise and GTE may be beneficial as therapeutic strategies to improve muscle function in mdx mice.

antioxidant capacity; muscular dystrophy; oxidative stress

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a severe muscle wasting disease that affects 1 in every 3,500 boys (12). The pervasive and progressive skeletal muscle atrophy and weakness leaves patients wheelchair bound by age 12 yr, and they often die due to respiratory or cardiac failure by the mid 20s (49). DMD results from the absence of dystrophin, a 427-kDa protein normally localized to the inner surface of the muscle fiber sarcolemma (48) and associated with a multimolecular network of integral and subsarcolemmal proteins known as the dystrophin glycoprotein complex [DGC (13)]. Dystrophin and the DGC together form a physical link between the intracellular cytoskeleton and the extracellular matrix. In the absence of dystrophin, the DGC fails to properly aggregate at the sarcolemma (13) and the link is compromised. Two potential outcomes leading to disease onset and progression are 1) the sarcolemmal membrane is mechanically weaker and/or 2) cell signaling via the DGC is disrupted. At present, the precise mechanisms of DMD pathophysiology remain elusive.

Because DMD results in severe muscle atrophy and progressive muscle weakness, appropriate exercise, which is known to

improve muscle strength and endurance, may be a suitable therapy (15). However, it is not presently known if exercise will blunt or exacerbate disease progression in DMD and, at present, exercise parameters (duration, intensity, frequency) have not yet been defined (15). The greatest risk is that exercise may induce or accelerate muscle fiber damage. Because of this risk, we suggested that exercise training be first systematically tested in dystrophic mice (e.g., mdx), and then in dystrophic dogs as a bridge to human studies (15).

On the basis of studies *in vitro*, muscles of older mdx mice are susceptible to contraction-induced injury [age ~100 days (34)], but muscles of maturing mdx mice appear less susceptible [e.g., age 9–12 days (16)]. Young mdx mice [e.g., age 4 wk (4)] also appear to tolerate endurance training on a running wheel better than that of older mice [e.g., age 6 mo (4)], perhaps suggesting that the muscles can better adapt to the stresses of physical activity when young. Thus, to potentially maximize the benefit of endurance training, we considered a reasonable age to begin was weaning age (21 days).

Another advantage of regular endurance exercise for both humans and mice is the upregulation of antioxidant proteins to balance increased reactive oxygen species [ROS (31, 35)]. Oxidative stress appears to precede disease onset in the mdx mouse (10), thus increased antioxidant capacity associated with regular exercise during early maturation may provide relief from oxidative stress in dystrophic muscle. In addition, we considered that exercise combined with a diet supplemented with green tea extract (GTE) may further reduce dystrophic oxidative stress.

Green and black teas are derived from the leaves of the plant *Camellia sinensis*, which contain catechins, polyphenolic plant metabolites that are antioxidants. Green tea, made from unfermented leaves, is rich in catechins; whereas, black tea, made from fermented leaves, is rich in theaflavins, the oxidized form of catechins. It is not yet clear if catechins and theaflavins have equivalent antioxidant capacity (21, 22). However, GTE, the hot water-soluble fraction of unfermented leaves, is a superior ROS scavenger compared with vitamins C and E (52). For example, GTE has the same protective effects as 10-fold greater concentrations of vitamin C (41). Each of these molecules can donate electrons from hydroxyl (OH) groups, but the antioxidant capacity of the catechins in GTE is greater likely because they have more OH groups (5–8 OH groups) compared with either vitamins C (4 OH groups) or E (1 OH group).

Address for reprint requests and other correspondence: J. A. Call, Rehabilitation Sciences Program, MMC 388, 420 Delaware St. SE, Minneapolis, MN 55455 (e-mail: callx017@umn.edu).

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Additionally, GTE catechins have a more stable molecular structure after the electrons are donated (46, 47). Mdx mice supplemented with 0.05% and 0.25% GTE for periods of 1 to 5 wk starting at weaning age showed delayed disease onset and adaptations toward stronger and more fatigue-resistant skeletal muscle fibers (2, 11), effects also associated with endurance exercise (6). Although the precise mechanism(s) by which GTE exerts its positive effects is(are) still not clear, these results suggest it blunts dystrophic disease progression at least in part because of its antioxidant properties.

Endurance exercise and GTE separately are both reported to increase endurance and antioxidant capacities (25, 35, 37), and each alone or in combination may alleviate the effects of the dystrophic process during early maturation. Therefore, in this study, we tested three hypotheses in male mdx mice: 1) 3 wk of endurance exercise initiated immediately postweaning (age 21 days) will improve skeletal muscle function and serum antioxidant capacity and decrease muscle lipid peroxidation relative to nonrunning mice; 2) GTE supplementation without running will demonstrate similar beneficial effects; and 3) GTE supplementation in combination with endurance exercise will be more beneficial than a diet of GTE or endurance exercise alone.

METHODS

Mice

This study was performed on male mdx mice. Breeders were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and from a colony thereafter maintained at Virginia Tech. Mice were housed in plastic microisolator cages on a 12:12-h light/dark cycle and were provided food and water ad libitum. All procedures performed in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Diet Groups

Sunphenon 90DCF, decaffeinated green tea extract [polyphenols >80%, catechins >80%, (-)-epigallocatechin-3-gallate (EGCG) >45%, caffeine <1%] was a kind gift from Taiyo International (Minneapolis, MN). Two commercial diets were supplemented with GTE at a 0.5% concentration by weight by Harlan Teklad (Madison, WI): #7004 for breeders, composed of 16.9% protein, 50.3% carbohydrate, and 11.4% fat, and #2018 for weaned pups, composed of 18.1% protein, 47.7% carbohydrate, and 5.8% fat. Male and female mdx breeder mice were paired at age ~35 days and randomly assigned and then maintained on normal diet (#7004) with or without 0.5% GTE. Pups were weaned at age 21 days and were provided normal diet (#2018) with or without 0.5% GTE. The normal and GTE diets were maintained for the duration of the study.

Body mass and food consumption were measured weekly. An initial body mass at age 21 days was obtained and subsequent measurements were made at ages 28, 35, and 42 days. Each mouse was provided with an initial food mass of 120 g for the 3-wk study. Food consumption was determined from the difference between the beginning and ending food mass each week (e.g., 120 g – 95 g = 25 g consumed). Weekly food consumption was then normalized by body mass to grams of food consumed per gram of body mass per week ($\text{g} \cdot \text{g}^{-1} \cdot \text{wk}^{-1}$).

Treatment Groups

Mdx mice weaned from dams consuming normal chow + 0.5% GTE were randomly separated into sedentary or run groups [treatment groups: Sedentary, GTE diet (SedGTE; $n = 8$) and Run, GTE diet

(RunGTE; $n = 10$)]. Mdx mice weaned from dams consuming normal chow were randomly separated into sedentary or run groups [treatment groups: Sedentary, Normal diet (SedNorm; $n = 8$) and Run, Normal diet (RunNorm; $n = 8$) (Fig. 1)].

Voluntary Wheel Running

At age 21 day, “Sedentary” mice were placed two to a cage (11 in. \times 7 in.) and “Run” mice were placed in individual 11 in. \times 9 in. plastic cages with a running wheel (0.16 m diameter; Silent Spinner) mounted to the side of the cage. Mice were allowed free access to the wheel 24 h/day. A metal tab attached to the rear of the wheel was used to interrupt a light signal to a photoelectric gate. Each signal interrupt was recorded on a laptop computer at a sampling rate of 1 Hz using a digital data-acquisition card (National Instruments USB-6501, Part #779205-01) and a custom Labview program. The running distance and average running speed were saved to a data file every 120 s. At age 42 days, after 3 wk of voluntary run training or normal cage activity, mice were killed via CO_2 inhalation. Voluntary running is an endurance exercise that involves multiple limb skeletal muscles and increased activity of the cardiovascular system. We therefore assessed adaptations in several skeletal muscles involved in running, as well as the heart and serum (Table 1). For practical reasons (i.e., insufficient muscle mass), not all tissues could be assayed for all variables.

Isometric Contractile and Mechanical Properties

The fast-twitch extensor digitorum longus (EDL) muscles were excised. EDL muscles were incubated at 30°C in an oxygenated (95% O_2 -5% CO_2) physiological salt solution (PSS) as previously described (51). Nonabsorbable braided silk suture (4-0) was tied to the distal and proximal tendons at the myotendinous junctions. EDL muscles were then fixed between a clamp and arm of a dual-mode servomotor system (300B, Aurora Scientific) at a resting tension (L_0) of 1.0 g. EDL muscles were maintained at L_0 by a stepper motor (51). The servomotor arm and stepper motor were controlled by Dynamic Muscle Control software (DMC Version 4.1.6, Aurora Scientific) to obtain the position and force output data.

The stimulated muscle protocol consisted of 5 steps: 1) a pretwitch and tetanus; 2) a single passive stress relaxation; 3) a single active stretch; 4) a fatigue protocol; and 5) a post tetanus. The first and fifth steps were performed to determine the effects of steps 2–4 on contractile capability (51). In step 1, the stimulated muscle was subjected to three isometric twitches and tetani (150 Hz) spaced 1 min apart. In step 2, the muscle was stretched instantaneously to 1.05 L_0 , held for 7.0 s, and then returned to 1.00 L_0 to determine passive parallel elastic stiffness (51). After 5 min at L_0 , in step 3 the muscle was stimulated at 80 Hz for 700 ms, and then during the final 200 ms, the muscle was stretched at 0.5 L_0 /s to a total strain of 0.1 L_0 (i.e., an

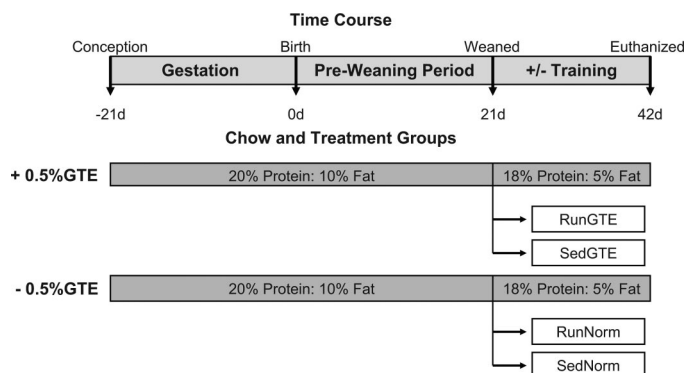


Fig. 1. Schematic for study of time course and treatment groups. Mice for all groups were killed at age 42 days.

Table 1. *Tissues collected postmortem, assays performed, and tissue function*

Tissue	Assay	Function
Extensor digitorum longus	In vitro contractile and mechanical properties Total protein content (BCA) Contractile protein content (SDS-PAGE) MyHC isoform distribution (SDS-PAGE)	Toe extension and foot dorsiflexion
Quadriceps	Metabolic activity (citrate synthase) Metabolic activity (β -HAD)	Knee extension
Soleus	Metabolic activity (citrate synthase)	Foot plantar flexion
Gastrocnemius	Lipid peroxidation (MDA assay)	Foot plantar flexion
Heart	Lipid peroxidation (MDA assay) Metabolic activity (citrate synthase) Metabolic activity (β -HAD)	Blood circulation
Blood	Antioxidant capacity (Trolox assay) Creatine kinase activity	O ₂ /CO ₂ , nutrient, and metabolite transport, etc.

MyHC, myosin heavy chain; β -HAD, β -hydroxy acyl-CoA dehydrogenase.

eccentric contraction) to determine active stiffness. In *step 4*, the muscle was subjected to a 1 s submaximal tetanus (60 Hz) at a rate of 12 tetani/min for 5 min to assess fatigue. At 1-min intervals, beginning with the pre-fatigue measurement, the muscle force output was recorded. After the fatigue protocol, the muscle was quiescent for 20 min at L₀, and then in *step 5* was subjected to a final tetanus (150 Hz). At the conclusion of the protocol, each muscle was weighed to the nearest 0.1 mg using an A-200D electronic analytical balance (Denver Instruments, Denver, CO) and snap frozen in liquid nitrogen. Additional muscles were also excised, snap frozen in liquid nitrogen, weighed to the nearest 0.1 mg using an A-200D electronic analytical balance (Denver Instruments, Denver, Colorado), and assayed as noted below.

Force and position output profiles were analyzed using Dynamic Muscle Analysis software (DMA Version 3.2, Aurora Scientific). For each twitch, the peak force, the time to peak stress (TPS), and half-relaxation time (HRT) were determined. Muscle cross-sectional area (CSA) was determined as previously described (16). Twitch and tetanic forces were normalized to muscle CSA to obtain twitch and tetanic stress. Passive parallel elastic stiffness (g/mm²) for *step 2* (51), and active stiffness for *step 3* were determined as previously described (16).

Contractile Protein Contents and Myosin Heavy Chain Isoform Distribution

For these analyses, we first determined total protein content in each EDL sample and then conducted two additional but separate assays. In the first, the total content of the actin and myosin in the sample was determined. In the second, we determined the myosin heavy chain (MyHC; i.e., myosin isoform) distribution for each sample.

Contractile protein content. Individual EDL muscles were homogenized in 10 mM phosphate buffer (pH 7.0), assayed in triplicate using the BCA protein assay (Pierce), and then subjected to SDS-PAGE to determine total sample MyHC and actin contents (24). Briefly, homogenates were diluted with an equal volume of Laemmli sample buffer containing 5% β -mercaptoethanol and boiled for 3 min. Ten micrograms total protein from each sample was separated on a 4% stacking, 7.5% separating Tris SDS-PAGE gel run at 150 V for 93 min. Four MyHC standards (2, 3, 4, and 5 μ g) and four actin standards (1, 2, 3, and 4 μ g) and 11 samples were run on each gel. Purified rabbit MyHC was a kind gift of Dawn Lowe (University of Minnesota) and actin was purchased from Sigma (A2522). Gels were stained for 1 h with 0.1% Coomassie Blue R-250, 30% methanol, 10% glacial acetic acid, and then destained overnight in 20% methanol and 10% glacial acetic acid. Stained gels were scanned using an EPSON (Expression 1680) Twain imaging densitometer and analyzed using SynGene. Linear regressions of the optical density for MyHC and

actin protein standards were used to determine the contents of MyHC and actin proteins for each sample.

MyHC. We determined the proportional content of MyHC isoforms as described (45). Briefly, frozen EDL muscles obtained from SedNorm, RunNorm, SedGTE, and RunGTE mice aged 42 days were thawed on ice in microcentrifuge tubes, homogenized, assayed for total protein, and then diluted in sample buffer to a final concentration of 0.04 mg/ml. The samples were then heated to 100°C for 10 min. Ten microliters of each sample was subjected to SDS-PAGE using 4% stacking and 8% separating gels. Samples were run on a mini-gel electrophoresis unit (BioRad) for 24 h at 80 V (constant voltage). Gels were silver stained and scanned using a FluorChem image analysis system (Alpha Innotech). Band densities for each MyHC isoform were expressed as a percentage of total MyHC band density.

Serum Creatine Kinase Activity and Antioxidant Capacity

Serum creatine kinase (CK) activity and antioxidant capacity were determined only at age 42 days for mice in all treatment groups. This age represented the end of the 3 wk of voluntary wheel running (RunGTE, RunNorm groups) or 3 wk of normal cage activity (SedGTE, SedNorm groups).

Serum collection. Following death of the mice, blood from cardiac puncture was collected (~1 ml) directly into Microtainer serum separator tubes (Becton Dickinson) and refrigerated (4°C) for 30 min to clot blood. The tubes were then centrifuged (Brinkmann Instruments, 5417 R) at 10,000 rpm for 10 min at 4°C to separate serum, which was then stored at -80°C until analyzed.

Serum CK. Serum CK concentration was determined by the Clinical Pathology Laboratory at Virginia Tech, using an Olympus AU400 chemistry analyzer at a wavelength of 670 nm and is expressed in units per liter (Olympus America, Center Valley, PA).

Serum antioxidant capacity. The capacity of each serum sample to inhibit ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) oxidation by metmyoglobin was determined with an antioxidant assay kit (Cayman Chemical). Briefly, each serum sample was diluted 1:20 with assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose). A Trolox standard range (0–0.660 mM) was obtained by diluting a 1 M Trolox stock solution with the same assay buffer. Trolox is a water-soluble tocopherol analog. Reactions in duplicate were performed in a 90-well plate. To each well was added: 10 μ l metmyoglobin, 150 μ l chromogen, 10 μ l of either standard or sample, and 40 μ l of 441 μ M H₂O₂ to initiate the reaction. The plate was covered, placed on a shaker at low speed for 5 min, and then read at 750 nm (Bio-Tek Instruments, μ Quant). Linear regressions of the absorption vs. Trolox standard concentrations were used to determine the antioxidant capacity of the samples.

Lipid peroxidation. Lipid peroxidation was determined in the gastrocnemius and heart. Individual gastrocnemius and heart muscles were carefully excised, homogenized in 10 mM phosphate buffer (pH 7.0), and sonicated (550 Sonic Dismembrator, Fisher Scientific) at 40 V over ice for 15 s. Homogenates were assayed in duplicate using the TBARS Assay Kit (Cayman Chemical) to determine the content of malondialdehyde (MDA). Homogenates were boiled for 1 h, incubated on ice for 10 min, and centrifuged at 1,600 *g* at 4°C for 10 min. Samples were then loaded with standards onto a 96-well plate and read at 530 nm. Linear regressions of the absorption for MDA standards were used to determine the amount of MDA in each sample. Standards ranging from 0 to 50 μ M MDA were prepared by diluting a 125 μ M MDA stock solution with HPLC-grade water.

Citrate synthase activity. Citrate synthase (CS) activity was determined in the soleus, quadriceps, and heart. CS catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloacetate. Because CoASH reduces DTNB, each sample was assayed in duplicate from the reduction of DTNB over time. Briefly, individual muscles were homogenized and protein concentration determined in triplicate using the BCA protein assay (Pierce) (42). Then 10 μ l of a 1:5 diluted muscle homogenate [previously diluted 1:20 (1 mg/ μ l in 10 mM phosphate buffer)] were added to 170 μ l of a solution containing Tris buffer (0.1 M, pH 8.3), DNTB (1 mM, in 0.1 M in Tris buffer) and oxaloacetate (0.01 M, in 0.1 M Tris buffer). Following a 2-min background reading, 30 μ l of 3 mM acetyl CoA were added to initiate the reaction. Absorbance was measured on a spectrophotometer (SPECTRAMax ME, Molecular Devices, Sunnyvale CA) at 405 nm every 12 s for 7 min at 37°C. Maximum CS activity was calculated and reported as micromoles per milligram protein per minute (43).

β -Hydroxy acyl-CoA dehydrogenase activity. β -Hydroxy acyl-CoA dehydrogenase activity (β -HAD) activity was determined in the quadriceps and heart. The third reaction of the β -oxidation cycle is the oxidation of the hydroxyl group at the β -position to produce a β -ketoacyl-CoA derivative. The second oxidation reaction is catalyzed by L-hydroxyacyl-CoA dehydrogenase, an enzyme that requires NAD⁺ as a coenzyme. Each NADH produced in mitochondria by this reaction drives the synthesis of 2.5 molecules of ATP in the electron transport pathway. For the determination of β -HAD, oxidation of NADH to NAD was measured in triplicate. Briefly, 35 μ l of whole muscle homogenate [1:20 wt/vol (mg/ μ l)] was added to 190 μ l of buffer containing 0.1 M triethanolamine, 5 mM EDTA, 0.45 mM NADH, and 15 μ l of 2 mM acetoacetyl CoA to initiate the reaction. Absorbance was measured on a spectrophotometer (SPECTRAMax PLUS 384, Molecular Devices) at 340 nm every 12 s for 6 min at

37°C. Maximum β -HAD activity was calculated and reported as micromoles per milligram protein per minute (7).

Statistics

Results are expressed as means \pm SE for figures and tables. Percentages (%) are expressed as either increases or decreases, not relative to initial values (e.g., a 100% increase means the original value has doubled). Daily wheel running data and weekly body mass and food intake data were analyzed by a two-way repeated-measures ANOVA. All other data were analyzed by two-way ANOVA (diet \times activity) with Tukey's honest significant difference post hoc analysis when necessary ($P < 0.05$). In this report, when no interactions were detected, we describe a main effect of running as "independent of diet" (i.e., RunNorm and RunGTE data combined compared with SedNorm and SedGTE data combined), and a main effect of diet as "independent of running" (i.e., SedNorm and RunNorm data combined compared with SedGTE and RunGTE data combined).

RESULTS

Voluntary Wheel Running

There was a significant interaction between diet groups (RunNorm vs. RunGTE) and time ($P = 0.0003$), indicating wheel running distance over time was dependent on diet. Within the RunNorm mice, average daily distance run each week was increased (i.e., *week 1* < *week 2* < *week 3*, $P < 0.05$). Within the RunGTE mice, average daily distance run was different between *week 3* and *weeks 1* and *2* [(*week 1* = *week 2*) < *week 3*, $P < 0.05$]. During the second week of running, RunGTE had a 94% greater average daily distance than RunNorm mice (2.3 vs. 1.2 km/day, $P < 0.05$). During the third week of running, RunGTE mice had a 170% greater average daily distance compared with RunNorm mice and had an average daily run distance similar to C57Bl/10 mice (Fig. 2A). RunGTE mice ran a total distance 128% greater than RunNorm mice over the 3-wk period (61.2 vs. 26.8 km, $P < 0.0001$) and RunGTE total distance was statistically equivalent to C57Bl/10 total distance (Fig. 2B).

Body mass and muscle masses normalized to body mass are reported in Fig. 3, A and B. There was no significant interaction between treatment groups and time for body mass. Independent

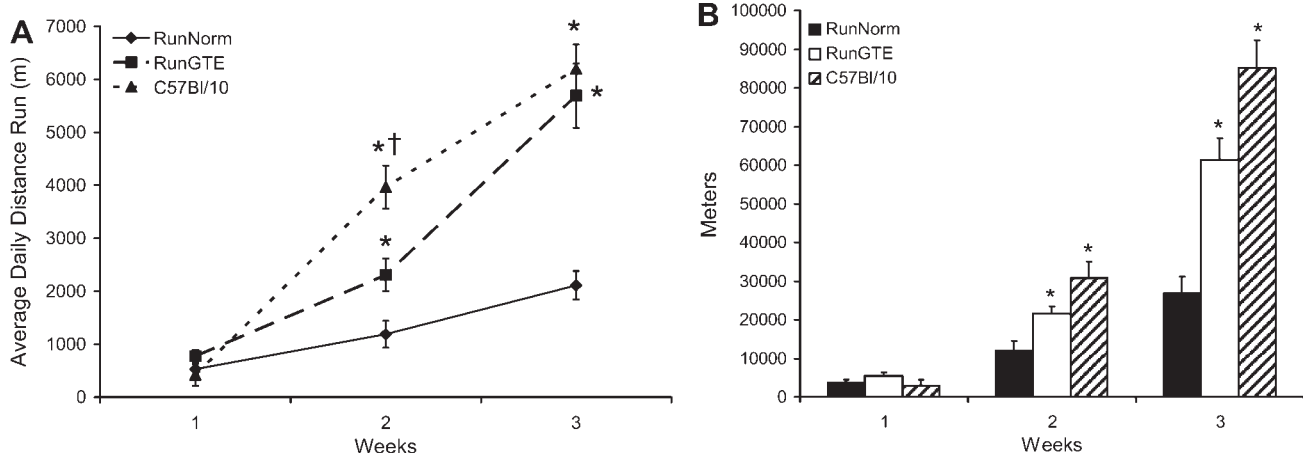


Fig. 2. Green tea extract increased running distance in mdx mice. A: average daily distance run by week increased with green tea extract (GTE). B: after 2 and 3 wk of training, Run GTE diet-fed (RunGTE) mice ran a greater total distance compared with Run, normal diet-fed (RunNorm) mice. Values are means \pm SE. *C57Bl/10 and RunGTE are greater than RunNorm ($P < 0.05$). †C57Bl/10 greater than RunGTE ($P < 0.05$).

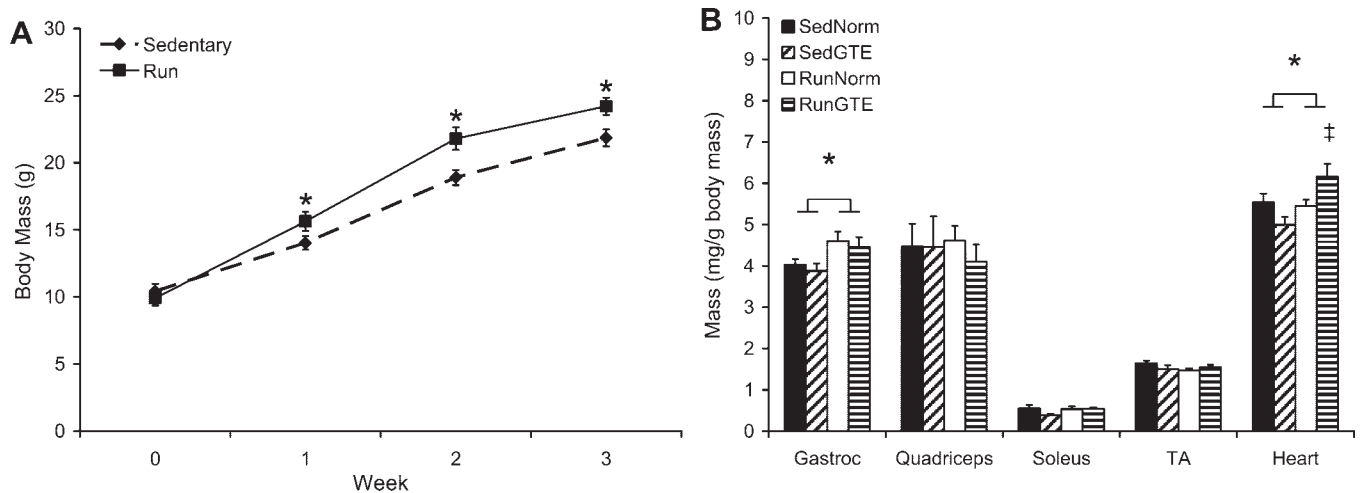


Fig. 3. Body mass and normalized muscle masses were increased with running. *A*: body mass increased with running. *B*: running elicited hypertrophy in both the gastrocnemius and heart. Values are mean \pm SE. *Greater than Sedentary mice ($P < 0.05$). †Greater than Sedentary normal diet fed (SedNorm), SedGTE, and RunNorm ($P < 0.05$).

of diet, Run compared with Sedentary mice had greater body masses at weeks 1, 2, and 3 ($P < 0.05$) of the treatment period (Fig. 3A), with Run mice gaining 4.6 g/wk compared with 3.6 g/wk for Sedentary mice. The masses of Run compared with Sedentary EDL, gastrocnemius, and heart muscles were increased when expressed relative to body mass (14%, 15%, and 11%, respectively, $P < 0.05$; EDL, Table 2; gastrocnemius and heart, Fig. 3B). These results suggest the increased body mass of runners was in part due to greater muscle mass. RunGTE mice had greater normalized heart masses compared with hearts of SedNorm, RunNorm, and SedGTE mice (+11%, +13%, and +23%, respectively; $P < 0.05$).

There was no significant interaction between treatment groups and time for food consumption. Independent of running, the GTE compared with Normal diet mice consumed more grams of chow per gram of body mass per week ($1.51 \text{ g} \cdot \text{g} \text{ bm}^{-1} \cdot \text{wk}^{-1}$ vs. $1.34 \text{ g} \cdot \text{g} \text{ bm}^{-1} \cdot \text{wk}^{-1}$, $P < 0.05$; data not shown).

Contractile and Mechanical Properties

Morphological, contractile, and mechanical data for the EDL muscles are shown in Table 2. Independent of diet, Sedentary mice had a slower time to peak stress than Run mice (13.1 vs.

11.5 ms, $P < 0.001$). Independent of diet, Run mice produced a greater tetanic stress compared with Sedentary mice (24.0 vs. 20.5 g/mm^2 , $P < 0.0434$; Table 2). There were no main effects or interactions for fatigue resistance for the four treatment groups (data not shown).

Mechanical Properties

Passive and active stiffness values for EDL muscles from SedNorm, RunNorm, SedGTE, and RunGTE mice are shown in Table 2. Active stiffness was 16% greater in Run compared with Sedentary mice, independent of diet ($P < 0.0121$) and 42% greater in RunNorm mice compared with SedNorm mice ($P < 0.0171$). Passive stiffness was increased 64% ($P < 0.0149$) in SedGTE mice compared with RunGTE mice.

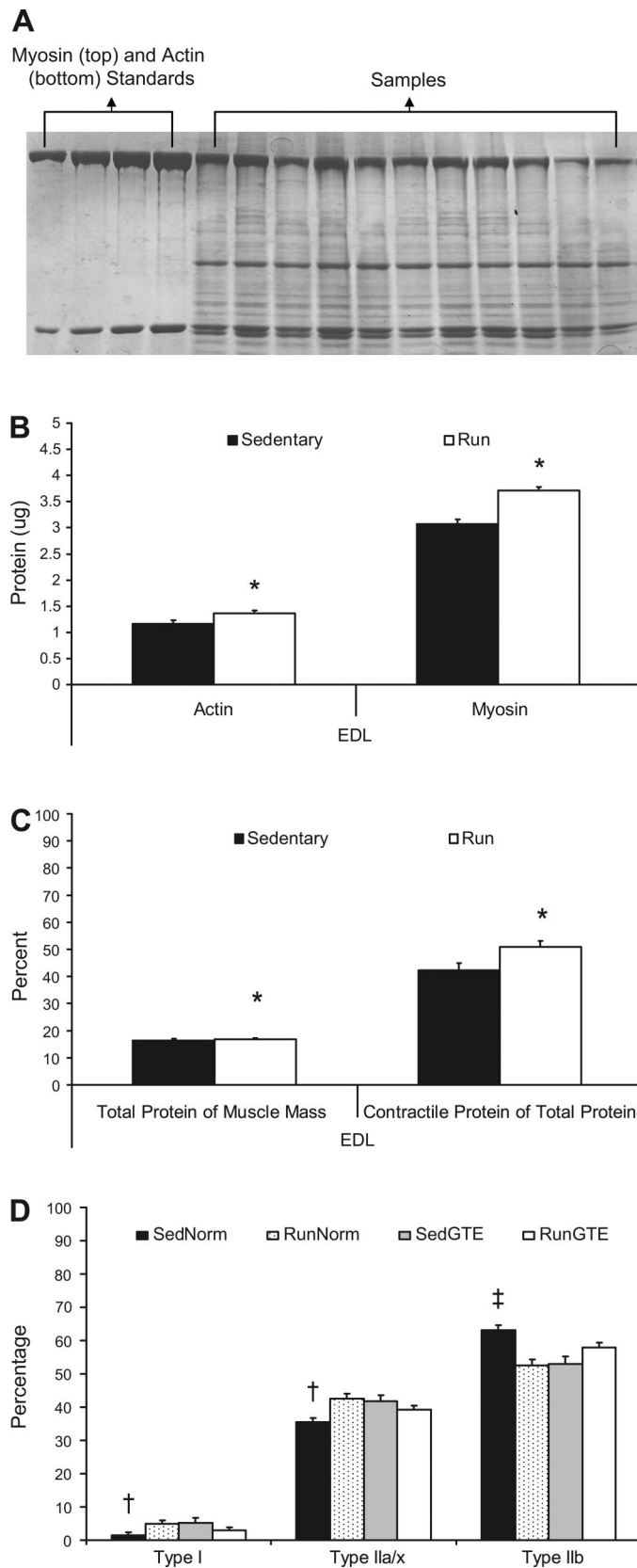
Contractile Protein Content and Fiber Type Distribution

Independent of diet, absolute myosin content (Fig. 4B), total muscle protein relative to muscle mass (Fig. 4C), total contractile protein relative to total muscle protein (Fig. 4C) were all increased in Run compared with Sedentary mice for the EDL (+21%, +2%, +20%, respectively; $P < 0.05$). We considered a shift in fiber type might be responsible for the increased endurance

Table 2. Morphological, contractile, and mechanical data following 3 wk of diet and activity interventions

	<i>n</i>	EDL Length, Mm	EDL Mass, mg	CSA, mm ²	TPS, ms	HRT, ms	P _t , g/mm ²	P _o , g/mm ²	PS, MPa	AS, MPa
Diet										
Normal	16	10.0 (0.2)	8.1 (0.2)	0.78 (0.02)	11.9 (0.3)	25.7 (1.5)	4.4 (0.2)	21.3 (1.2)	1.23 (0.11)	1.64 (0.08)
GTE	18	10.0 (0.1)	8.3 (0.2)	0.78 (0.02)	12.6 (0.3)	25.1 (1.4)	4.1 (0.2)	23.5 (1.2)	1.39 (0.11)	1.70 (0.07)
Activity										
Sedentary	16	9.6 (0.2)	7.7 (0.2)	0.76 (0.02)	13.1 (0.3)	26.7 (1.5)	4.0 (0.2)	20.5 (1.2)	1.44 (0.11)	1.52 (0.08)
Run	18	10.4 (0.1)*	8.7 (0.2)*	0.80 (0.02)	11.5 (0.3)*	24.2 (1.4)	4.5 (0.2)	24.0 (1.2)*	1.18 (0.11)	1.82 (0.07)*
Treatment groups										
SedNorm	8	9.4 (0.2)	8.0 (0.3) ^b	0.80 (0.03)	12.2 (0.4) ^b	25.2 (2.1)	4.1 (0.3)	19.6 (1.7)	1.16 (0.16)	1.36 (0.12) ^b
RunNorm	8	10.5 (0.2)	8.3 (0.3)	0.75 (0.03)	11.7 (0.4) ^b	26.3 (2.1)	4.6 (0.3)	22.9 (1.7)	1.30 (0.16)	1.93 (0.11) ^a
SedGTE	8	9.7 (0.2)	7.4 (0.3) ^b	0.72 (0.03) ^b	14.0 (0.4) ^a	28.1 (2.1)	3.9 (0.3)	21.3 (1.7)	1.72 (0.16) ^a	1.69 (0.11)
RunGTE	10	10.3 (0.2)	9.1 (0.3) ^a	0.84 (0.03) ^a	11.3 (0.4) ^b	22.2 (1.9)	4.4 (0.2)	25.2 (1.5)	1.05 (0.14) ^b	1.71 (0.1)

Values are means (SE). CSA, cross sectional area; TPS, time to peak stress; HRT, half relaxation time; P_t, twitch stress; P_o, tetanic stress; PS, passive parallel elastic stiffness; AS, active stiffness determined from stretch protocol. *Different from Sedentary ($P < 0.05$). Treatment means not followed by the same letter are significantly different ($P < 0.05$).



capacity and so we also assessed the MyHC isoform composition of the EDL. We found type I and IIA fibers were increased and type IIB fibers were decreased in the EDLs of RunNorm and SedGTE mice compared with SedNorm mice (Fig. 4D), suggesting a shift toward more fatigue-resistant fibers. Surprisingly, these fiber type changes were blunted in the RunGTE EDLs.

Serum Antioxidant and Membrane Integrity Markers

Antioxidant capacity was determined from the serum's ability to inhibit oxidation of ABTS by metmyoglobin. Independent of diet, Run compared with Sedentary mice had a 22% greater antioxidant capacity (Fig. 5A; 156 vs. 128 mM Trolox, $P < 0.0017$). Lipid peroxidation, a marker of oxidative stress, was determined from MDA content in gastrocnemius and heart homogenates. Independent of running, GTE compared with Normal diet mice demonstrated 64% less lipid peroxidation (Fig. 5B; 0.103 vs. 0.281 $\mu\text{mol MDA/mg protein}$, $P < 0.0001$) in the gastrocnemius, and 29% less lipid peroxidation (0.311 vs. 0.22 $\mu\text{mol MDA/mg protein}$, $P < 0.05$) in the heart.

SedGTE mice had CK levels 57% less than SedNorm mice (Fig. 5C; 3,375 vs. 5,949 U/l, $P < 0.0308$). Independent of running, GTE mice demonstrated lower serum CK (2,981 vs. 4,659 U/l, $P < 0.0314$). Independent of diet, RunNorm compared with SedNorm mice demonstrated decreased but not significant serum CK levels (3,369 vs. 5,949 U/l; Fig. 5C).

Markers of Oxidative Metabolism

CS activity was determined in quadriceps, soleus, and heart muscles, all of which would be active to support endurance activity. Independent of running, GTE compared with Normal diet mice had 95% greater CS activity in quadriceps muscles (Fig. 6A; 448 vs. 285 $\mu\text{mol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, $P < 0.003$). Additionally, CS activity was 35% greater in heart muscle in Run mice compared with Sedentary mice, independent of diet (Fig. 6A; 998 vs. 742 $\mu\text{mol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, $P < 0.0099$), but there were no differences in soleus muscle CS activity for any of the treatment groups.

β -HAD activity was determined in quadriceps and heart muscles. Independent of diet, Run compared with Sedentary mice had β -oxidation activity that was 36% greater (242 vs. 178 $\mu\text{mol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, $P < 0.0028$) in the quadriceps and 35% greater (200 vs. 148 $\mu\text{mol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, $P < 0.0099$) in heart muscle (Fig. 6B).

DISCUSSION

The major findings were 1) mdx males who began voluntary wheel running at age 21 days demonstrated a significant increase in weekly distance over the 3-wk period, and 2) running distance was increased by $\sim 128\%$ by 0.5% GTE in the diet. For each condition alone, there were beneficial, but no obvious dele-

Fig. 4. Running resulted in greater contents of total protein, contractile protein, and myosin, and a shift to fatigue-resistant fibers in EDL relative to the sedentary condition. A: representative gel used to determine actin and myosin content. B: actin and myosin contents expressed in $\mu\text{g}/10 \mu\text{g}$ total protein. C: total protein is expressed as a percent of tibialis anterior (TA) or extensor digitorum longus (EDL) whole wet muscle mass, and contractile protein is expressed as a percent of total protein. D: fiber type distribution. Values are mean \pm SE. *Greater than Sedentary ($P < 0.05$). †Less than RunNorm and SedGTE ($P < 0.05$). ‡Greater than RunNorm and SedGTE ($P < 0.05$).

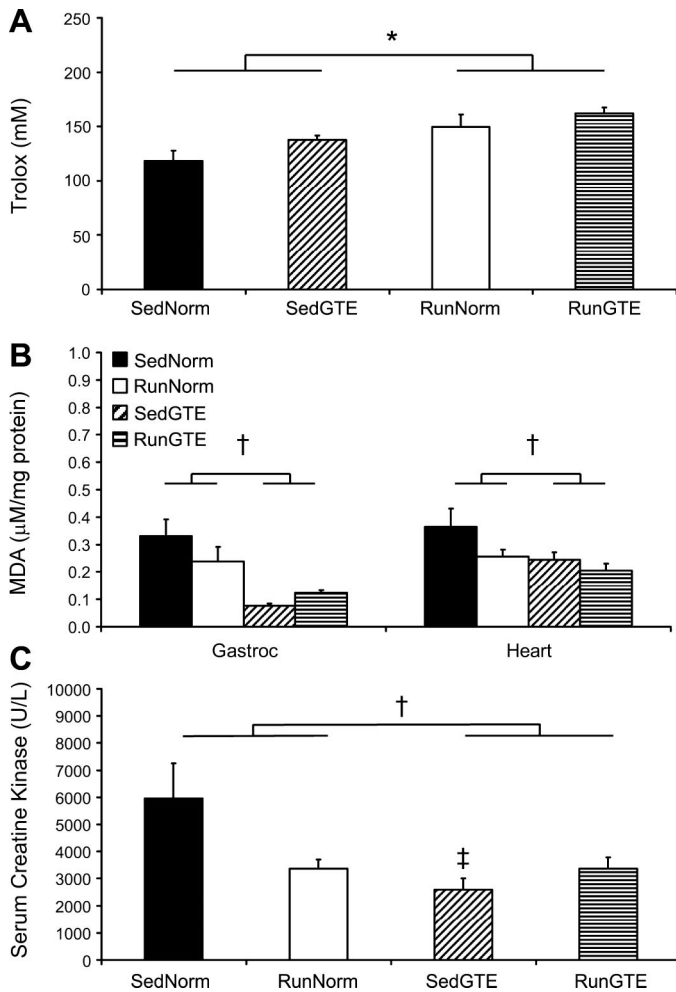


Fig. 5. Running and GTE both protect the sarcolemma from oxidative damage. *A*: Running, independently of diet, increased serum antioxidant capacity. *B*: GTE, independently of running, attenuated lipid peroxidation in the gastrocnemius and heart. *C*: GTE, independently of running, reduced serum CK. Values are means \pm SE. *Runners greater than Sedentary mice ($P < 0.05$). †GTE diet mice less than Normal diet mice ($P < 0.05$). ‡SedGTE greater than SedNorm.

rious, outcomes. Synergistic effects between running and GTE included improved skeletal and cardiac muscle aerobic metabolism, serum antioxidant capacity, decreased lipid peroxidation, and cardiac hypertrophy. These data suggest that combined endurance training and GTE improves skeletal muscle function in young mdx mice.

In this study, we assessed various adaptations in several locomotor skeletal muscles, the heart, and serum in mdx mice in response to GTE and voluntary running. For practical reasons, not all assays were conducted in each of the different muscle samples. Nevertheless, we believe it is instructive to integrate results from discrete tissues to understand potential global adaptations in the dystrophic mice.

Effects of Running

Running alone. Male mdx mice began running at age 21 days because young mdx mice do not experience the same membrane damage as older mdx mice (16, 51). Furthermore, young (age 4 wk) compared with older (age 6 mo) mdx mice

can better tolerate running (4, 17). After 3 wk, male mdx mice increased the average daily distance run from the first to the last week by $\sim 300\%$ (0.5 vs. 2.1 km/day, $P < 0.05$), indicating they could positively adapt with minimal detrimental effects. Dystrophic muscle can positively respond to both short (herein)- and long (17)-term exercise training, particularly if initiated at a young age.

Surprisingly, running alone (i.e., RunNorm different than SedNorm) elicited only one significant effect in EDL muscles. Active stiffness was increased in RunNorm mice by 42% compared with SedNorm mice. This increase could reflect improved actin-myosin interaction, (40) and improved transmission of force between the contractile element and the tendons.

Running independent of diet. Body mass of runners was greater than that of sedentary mice (Fig. 3A) due in part to increased mass for the EDL, gastrocnemius, and heart muscles (Table 2; Fig. 3B). Although wheel running is endurance training, EDL tetanic stress output was increased $\sim 17\%$ (Table 2). This increase could be due to at least three possible mechanisms: 1) increased expression and stability of contractile proteins (i.e., myosin and actin); 2) a shift in fiber type; and 3) improved membrane integrity.

First, a previous report revealed that stress output of fast-twitch skeletal muscle was closely related to total myosin content [$r = 0.81$, $P < 0.05$ (3)]. Total contractile protein and myosin content were increased in the EDL after 3 wk of wheel running, independent of diet (Fig. 4, B and C). MyHC is susceptible to oxidative stress, which could decrease contractile stress output (8). This decrement in MyHC function could

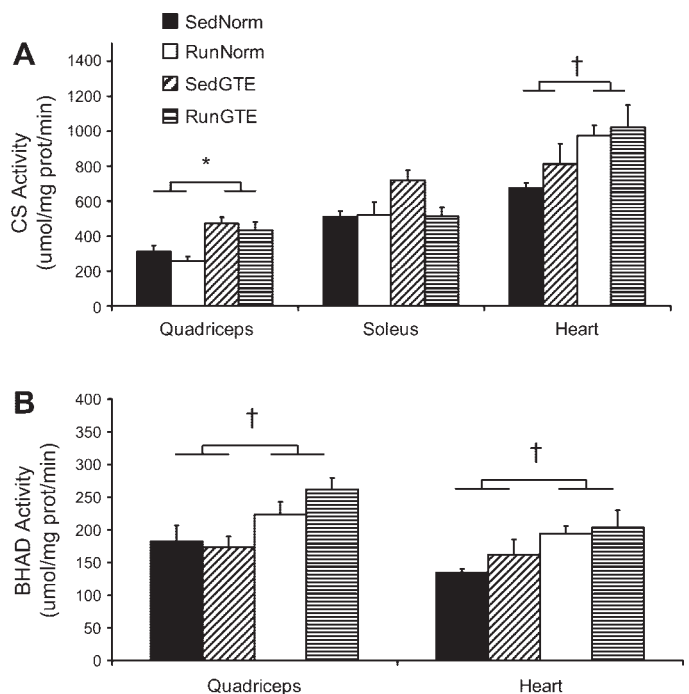


Fig. 6. GTE and Running increased metabolic activity differentially. *A*: citrate synthase activity was increased with GTE in the quadriceps and with Running in the heart. *B*: Running increased β -oxidation in the quadriceps and heart. Note, β -hydroxy acyl-CoA dehydrogenase activity (β -HAD) was not determined in the soleus because of insufficient sample for the assay. Values are means \pm SE. *GTE diet greater than Normal diet mice ($P < 0.05$). †Runners greater than sedentary mice ($P < 0.05$).

be alleviated by increased antioxidant capacity (30). Serum antioxidant capacity was increased ~22% with running (Fig. 5C). EDL active stiffness was also increased (Table 2), suggesting an increased proportion of cross bridges in the strong binding state (40). We did not assess intracellular antioxidant capacity; however, Kaczor et al. (20) reported an ~30% decrease in MDA content and 40% decrease in protein carbonyls in white gastrocnemius muscles from mdx mice subjected to 8 wk of light treadmill run training (~9 m/min). This outcome suggests that intracellular antioxidant capacity may have also been enhanced by wheel running for 3 wk. To confirm this possibility, intracellular antioxidant capacity should be assessed as a potential mechanism by which MyHC are protected following run training in mdx mice.

Second, a modest increase in stress output coupled with improved endurance capacity could reflect increased fast glycolytic-oxidative fibers (e.g., Type IIA). The amount of voluntary wheel running required to elicit fiber type shifts in skeletal muscle is not clear (1, 33). Allen et al. (1) reported a transition toward more fatigue-resistant MyHC IIA fibers in tibialis anterior (TA) of male C57Bl/6 mice after 1 wk, whereas Pellegrino et al. (33) reported no changes in TA of male C57Bl/6 mice after 8 wk. Our EDL MyHC isoform analysis indicated a shift to fatigue-resistant fibers in mdx mice (I and IIA; Fig. 4D) after 3 wk of wheel running that likely contributed to improved endurance capacity.

Third, the dystrophic process is thought to render the sarcolemma susceptible to mechanically induced injury, which contributes to depressed whole muscle stress output (34). This effect may be more evident in old compared with young mdx muscles (16, 51). EDL muscle tetanic stress output was increased ~17% coincident with a modest but not significant decrease in serum CK with running, both of which could be considered indexes of muscle fiber integrity. Thus our data suggest voluntary wheel running, independent of diet, improves resistance to contractile stress-generating capability, and does not exacerbate sarcolemmal membrane leakiness, but may actually reduce it.

Metabolic properties. To account for the improved endurance capacity, we determined metabolic adaptations in 1) CS and 2) β -HAD activities for muscles that contribute to running. CS activity was determined in the soleus, quadriceps, and heart, and β -HAD in quadriceps and heart. Running on a Normal or on a GTE diet alone did not increase either CS or β -HAD activities above those of the respective sedentary diet groups (SedNorm and SedGTE; Fig. 6, A and B). Runners, independent of diet, demonstrated increased CS activity, an index of mitochondrial content, in cardiac muscle but not in the gastrocnemius or soleus muscles. β -HAD activity was increased by 37% in Run compared with Sedentary mice independent of diet in the gastrocnemius and 35% in cardiac muscle. These results are similar to the 20% increase in β -HAD reported for 8-wk treadmill trained wild-type FVB mice (6). Our data suggest some (gastrocnemius) but not all (soleus) dystrophic skeletal muscles adapt to endurance training, while the dystrophic cardiac muscle showed the greatest adaptability (improved CS and β -HAD activities). The reasons for the differential metabolic adaptations in dystrophic skeletal muscle are not clear, but suggest the dystrophic process may disrupt the "normal" mechanisms of adaptation. Nevertheless,

those adaptations that did occur contributed to improved running performance.

Effects of GTE

GTE alone. GTE had several beneficial outcomes on sedentary dystrophic muscle, likely through its antioxidant and signaling properties. Oxidative stress is believed to contribute substantially to dystrophic muscle pathology (36, 50). GTE is considered an amphiphilic antioxidant because it can act at the lipid bilayer, and intracellularly as a ROS scavenger (32). Mdx mice treated with either 0.05% GTE, 0.25% GTE, or 0.1% EGCG (epigallocatechin gallate, the major catechin in GTE) for 1–5 wk after weaning had increased antioxidant capacity, improved contractile properties, and decreased muscle pathology (11). In agreement with these findings, our data indicate one of GTE's primary beneficial antioxidant effects could be to enhance membrane integrity, possibly by decreasing oxidative stress of the lipid bilayer. We observed increases in passive (+48%) stiffness in EDL muscles (Table 2), coincident with decreased serum creatine kinase (–52%, Fig. 5C). Because membrane fragility and increased permeability are thought to contribute to dystrophic fiber degeneration, our findings support the idea that fiber necrosis and regenerating surface area may have been reduced by GTE (2, 11).

It is also possible that GTE could act via signaling to improve membrane integrity. For example, EGCG can bind a 67-kDa laminin receptor (44) to modulate actin remodeling and inhibit degranulation of human basophilic KU812 cells (14). The increased passive parallel elastic stiffness we observed with GTE alone may represent similar cytoskeletal remodeling, possibly through GTE binding to α 7 β 1 integrin. The α 7 β 1 integrin is a laminin receptor upregulated as a compensatory mechanism to possibly stabilize the costameric lattice in human DMD patients and mdx mice (18). Additionally, GTE could initiate signaling cascades that regulate cell viability and the immune/inflammatory response in dystrophic muscle (23). However, the precise mechanisms of GTE action at the cellular level remain unclear (23). Additional studies to demonstrate a mechanistic role for GTE could be invaluable to support its use in the treatment of DMD.

GTE independent of running. The most intriguing findings were the significant decreases in lipid peroxidation, an index of membrane degradation, and subsequent decreases in serum CK, a benchmark index of muscle fiber damage (Fig. 5, C and B). Polyunsaturated fatty acids comprise the plasmalemma (i.e., the lipid bilayer) and are highly susceptible to oxidative damage (28). GTE protected C2C12 myotubes from induced oxidative stress (2). Our results suggest that similar protection may be elicited *in vivo*, as we observed a strong correlation ($r = 0.82$; data not shown) between lipid peroxidation and serum CK levels.

Metabolic properties. When GTE was considered independent of running, we observed increased CS activity in quadriceps (+59%) but not in soleus. In soleus, running blunted the enhanced CS activity induced by GTE. However, in both muscles, the data suggest that mitochondrial capacity could be improved with GTE supplementation, even when physical exercise may not be possible. This may be especially important for DMD patients who could benefit from improved fatigue resistance. GTE alone has been shown to reduce obesity by

suppressing fatty acid synthesis (39) and increasing liver β -oxidation (27), but we observed no increase in skeletal muscle β -oxidation either in SedGTE vs. SedNorm, nor in RunGTE vs. RunNorm mice.

Effects of Running and GTE Combined

Endurance capacity was markedly enhanced so that the average daily distances covered per day during the 3rd wk of training were approaching those of C57BL/10 male runners [herein (4, 17)]. Although running and GTE both independently appear to elicit beneficial outcomes in dystrophic muscle, a major synergistic effect of combined running and GTE was an \sim 128% increase in endurance running capacity. This result is consistent with the increased endurance capacity (i.e., duration) reported for mice fed GTE who performed either swim or treadmill run training (25, 26). In these studies, increased β -oxidation, increased nonesterified fatty acid content in the gastrocnemius muscles, and decreased serum glucose suggested GTE coupled with activity promoted fatty acids as a primary energy source and preserved intramuscular glycogen levels (26). We did not see a clear distinction in β -HAD activity between running with and without GTE, but running independent of diet increased β -HAD activity. We suggest the dramatically improved endurance capacity likely resulted because GTE improved CS activity and running improved β -HAD activity in the gastrocnemius, while running improved both CS and β -HAD activities in the heart. Furthermore, other deleterious effects such as lipid peroxidation were depressed. Running activates a signal cascade in skeletal muscle that favors gene and protein expression to support adaptation to endurance exercise, including activation of AMPK (29); GTE also activates AMPK (9). EGCG has also been shown to activate the MAP kinase family of intracellular signal cascades, including JNK, p38 (5), and ERK (5, 19), which are thought to regulate cell signaling in response to exercise such as treadmill running (38). Thus there are multiple potential pathways by which EGCG could exert intracellular effects.

Surprisingly, rather than additive positive adaptations when running and GTE were combined, several of the beneficial effects of GTE alone were attenuated by running. These included passive parallel elastic stiffness, active stiffness, serum CK, lipid peroxidation, and CS activity. However, although these effects were attenuated, values for each did not return to levels seen in Sedentary normal fed mice (e.g., serum creatine kinase).

Conclusion

In summary, male mdx mice on a Normal diet tolerated voluntary wheel running starting at age 21 days and improved their daily distance over a 3-wk period. In none of the parameters assessed (e.g., tetanic stress, serum creatine kinase) was running detrimental. GTE supplementation appeared to be a beneficial supplement with or without exercise, but markedly increased endurance running distance in those mice provided running wheels. Our findings suggest the beneficial effects of endurance exercise and GTE result from adaptations in both skeletal and cardiac muscle. Future studies should explore the mechanistic reasons for these adaptations, as combined GTE and running could hold great promise as a potential therapy for DMD.

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