Nitric oxide and voluntary exercise together promote quadriceps hypertrophy and increase vascular density in female 18-mo-old mice

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Leiter JRS, Upadhaya R, Anderson JE. Nitric oxide and voluntary exercise together promote quadriceps hypertrophy and increase vascular density in female 18-mo-old mice. Am J Physiol Cell Physiol 302: C1306–C1315, 2012. First published February 8, 2012; doi:10.1152/ajpcell.00305.2011.—Age-related sarcopenia reduces the size, strength, and function of muscle, and the diameter of muscle fibers. It also disrupts the dystrophin-glycoprotein complex, dislocating nitric oxide synthase 1 (NOS-1) and reducing sarcolemmal integrity. This study of quadriceps muscle in 18-mo-old mice showed that NO-donor treatment with isosorbide dinitrate (I) for 6 wk, in combination with voluntary exercise for 3 wk, increased muscle mass by 25% and stimulated cell proliferation. The resulting fiber hypertrophy was accompanied by a lower ratio of protein:DNA, consistent with myogenic-cell hyperplasia. Treatment enhanced the ratio of NOS-1:β-dystroglycan in correlation with fiber diameter, improved sarcolemmal integrity, and increased vascular density after an increase in vascular endothelial growth factor protein at 3 wk. Results demonstrate that age-related muscle refractoriness to exercise can be overcome with NO-donor treatment. Since activation of muscle stem cells and vascular perfusion are limiting factors in the maintenance, regeneration, and growth of aged muscle, results suggest the feasibility of using NO-donor drugs to combat atrophy and muscle ischemia. Improved function and quality of life from the NO-amplified effects of exercise may be useful in aging and other conditions such as disuse, insulin resistance, or microgravity.

sarcopenia; satellite cells; nitric oxide donor; beta-dystroglycan; VEGF

AGE-RELATED MUSCLE ATROPHY in humans is arguably inevitable, since 100% of older individuals experience some degree of sarcopenia (32). Muscle wasting results from atrophy of individual fibers, loss and expansion of motor units (24, 37, 79) that becomes increasingly severe from late-middle age to senescence in both fast- and slow-twitch muscles (63) and ultimately weakness limits function and quality of life. Current treatments, such as exercise and growth-promoting drugs, are not completely effective, in part as we do not fully understand the many facets of age-related atrophy.

Progressive refractoriness of satellite cells (SC) to stimuli that promote growth in young mice, such as stretch (43) and exercise (36, 44, 45), is a major contributor to age-related atrophy. Muscle SC are required to maintain mass and repair muscle and are involved in growth via proliferation (4). Deficient activation of these cells by stretching in aged muscle (65, 72, 84) reduces or inhibits the potential for regeneration and hypertrophy (15, 17, 18). Since the normal process of SC activation depends on nitric oxide (NO) and signaling via hepatocyte growth factor and the c-met receptor (3, 73, 83), drugs that increase the NO concentration have potential to promote growth in old muscle. Research using a whole muscle culture system showed that the age-related reductions in muscle-specific neuronal nitric oxide synthase (NOS-1) concentration and activity are reversed by treatment with a NO-donor drug, one that releases NO without activity of NOS (43). Similarly, in old rats, NOS protein levels in muscle decrease with aging and increase after exercise training (68). In culture, the effectiveness of stretch to activate SC increases when coupled with NO-donor treatment (43), but application of NO-donor compounds with exercise to treat age-related atrophy in vivo is not yet tested.

The dystrophin-glycoprotein complex (DGC) connects sarcoplasm across the sarcolemma to the extracellular matrix (66). It has a critical role in sensing and responding to mechanical forces imposed on the sarcolemma (55). In normal muscle, NOS-1 is anchored in the DGC in an optimal position for communication with satellite cells (3). Diffusion of NO from the fiber either maintains SC quiescence or activates SCs, depending on the amount of NO produced (81, 82). Aged and dystrophic muscle have an abnormal response to loading compared with young, healthy muscle (53, 58). Defects in the DGC (62) and subsequent dislocation of NOS-1 (71) at least partly explain deficits in mechanochemo signaling that normally induce SC activation. During aging, muscle also displays reduced perfusion (76) while exercise stimulates angiogenesis via increases in vascular endothelial growth factor (VEGF), which in turn simulates NO production by NOS-3 (42, 77). Interestingly, angiogenesis is related to both VEGF and NO signaling, and is marked by production of VEGF (40) by endothelial and satellite cells (61). Both types of cells respond to NO and make NOS-1 and NOS-3 proteins (in different relative amounts), and endothelial cells produce endothelin-1, which inhibits NO-dependent vasodilation (74). As well, the vascular proliferative response to increased VEGF in electrically stimulated muscle contraction is NO dependent through NOS-1 in muscle fibers and NOS-3 in endothelial cells (51). Changes in perfusion and the DGC with increasing age would be exacerbated by changes in capillary density and SC responsiveness to NO and mechanical signals, and ultimately reduce the capacity for maintenance, growth, and repair of muscle as age increases.

We investigated the effects of a NO-donor drug, isosorbide dinitrate (ISDN), and exercise, separately and in combination, on muscle mass and fiber size in sarcopenic muscle by studying cell proliferation, NOS-1 and β-dystroglycan (βDG) in the DGC, fiber integrity, vascular density, and the level of VEGF protein. The hypothesis was that NO-donor treatment com-
bined with exercise would promote growth of muscle in 18-
mo-old female mice in vivo.

MATERIALS AND METHODS

Animals and experimental design. Female C57BL/6 mice [bred or
maintained from Jackson Laboratory stock (Bar Harbor, MA)] were
housed at the Animal Holding Facility until 18 mo of age (mo) on a
12:12-h light:dark cycle, as approved by the Protocol Management
and Review Committee at our institution in accordance with the
Canadian Council on Animal Care. Between 18 and 19.5 mo, sar
copenia is established (Leiter and Anderson, unpublished observa-
tions) and comorbidity is much less pronounced for this strain than
they would be in senescent mice at 24–37 mo. Mice were randomly
assigned to one of four groups with or without treatment with isosorbide
mitrate (I) and/or exercise, as follows. Daily oral treat-
ment with I (66 mg/kg in canola oil, po) started at the beginning of the
experiment and continued for 6 wk. Half the mice had access to a
voluntary running wheel for voluntary exercise (Ex) for the first 3 wk
of the treatment period, as reported (5). A previous study showed
quadriceps from 18-mo mice were refractory to hypertrophy follow-
ing 3 wk of voluntary exercise, whereas younger 8-mo mice were
responsive (44); this experiment tested whether NO-donor treatment
could change the responsiveness to exercise and induce muscle
growth in the 18-mo group. Groups were control (C, oil only, n = 6),
Ex (n = 8), I (n = 6), and I + Ex (n = 3). At the end of 6 wk, mice
were injected with tritiated thymidine (3H-tdr: 1
Ci/g body wt) and
euthanized 2 h later by cervical dislocation under isoflurane
anesthesia (Baxter, Mississauga, ON).

Tissues. The quadriceps, gastrocnemius (GAST), and tibialis ante-
rior (TA) muscles were dissected and weighed; mass was normalized to
body mass. Muscles were cut in half across the muscle belly, and
halves were either placed in Eppendorf tubes (Westbury, NY) and
snap frozen in liquid nitrogen and stored at −80°C until extraction of
DNA or protein, or embedded in optimal cutting medium, frozen in an
isopentane slurry, and cryosectioned.

Fiber diameter. Sections (7 µm, 50 µm apart) were stained with
hematoxylin and eosin (H&E, Fisher, NJ) and photographed using an
Olympus BH-2 microscope (at ×100). Using NIH-Image J software,
fiber diameter was measured along the shortest diameter (>600 fibers
per group). Mean (and SE) and frequency distribution of diameter
were compared among groups.

Cell proliferation. Frozen muscle was weighed, minced over dry
ice, placed in lysing matrix-A tubes (MP Biomedicals, Solon, OH),
and homogenized in DNA extraction solution (1 M NH4OH; 0.2%
 Triton X-100) at a speed of 4.0 m/s for 40 s using a FastPrep-24 (MP
Biomedicals, Solon, OH). After 10 min incubation at 37°C, 600 µl of
homogenate was mixed with 300 µl of 2 M acetic acid, mixed gently
by inversion, and centrifuged at 14,000 relative centrifugal force (rcf)
at 4°C for 5 s. Supernatant was transferred to new vials; one aliquot
was used to determine the DNA concentration with a Hoeschst dye
assay using a Victor microplate reader (Perkin Elmer, Waltham MA).
A second aliquot was transferred to scintillation vials containing 5 ml
of scintillation fluid (Fisher Scientific, Ottawa, ON) to count disintegrations
per minute (dpm) over a 15-min period using an LS 6500 scintillation
counter (Beckman-Coulter, Mississauga, ON). Cell proliferation was
represented as disintegrations per minute per microgram DNA
(dpm/µg DNA) as previously reported (4, 5).

Western blotting. Muscle was weighed, minced over dry ice, placed
in lysing matrix-D tubes (MP Biomedicals, Solon, OH) and
homogenized in lysis buffer (63 mM Tris, 2% SDS, 10% sucrose) at 4.0 m/s
for 40 s using a FastPrep-24, followed by 1 h incubation on ice.
Homogenate was centrifuged at 14,000 rcf at 4°C for 5 min, and
supernatant transferred to new tubes. Total protein concentration was
measured using BCA Protein Assay Reagent (Pierce Protein Research
Products, Thermo Fisher Scientific, Mississauga, ON) standardized
against bovine serum albumin. Quadriceps from 6-wk-old normal
C57BL/6 mice was included as a “young muscle” baseline for comparison
to muscle experimental groups at 19.5 mo.

Samples were handled on ice and were diluted with 2 × sample
buffer [100 mM dithiothreitol, 4.0% SDS, 0.16 M Tris-HCl (pH 6.8),
43% glycerol, 0.2% bromophenol blue] and lysed buffer to a final
protein concentration of 1.3 mg/ml. Samples were heated at 97°C for
3 min, loaded into 9% polyacrylamide gels (20 µg/well) along with
dual-color Precision Plus protein standards (Bio-Rad Laboratories),
electrophoresed, and blotted onto nitrocellulose membranes. Mem-
branes were blocked with 5% wt/vol skim milk in phosphate-buffered
saline with 0.5% Tween 20 for 1 h and incubated overnight at 4°C
with one of the following primary antibodies: rabbit polyclonal
anti-NOS-1 (sc-1025, Santa Cruz Biotechnology, Santa Cruz, CA,
1:200); anti-β-dystroglycan [NCL-43DAG, Novocastra (Leica Micro-
systems, Richmond Hill, ON), 1:200]; mouse monoclonal anti-Pax7
(PAX7, Developmental Studies Hybridoma Bank, 1:1,000); goat
anti-VEGF-P-20 (sc-1836, Santa Cruz Biotechnology, 1:500); and
anti-actin (MAB1501, Millipore, Bedford, MA, 1:200,000). Mem-
branes were washed and incubated with the appropriate secondary
antibody [donkey anti-rabbit IgG (NA9340V, GE Healthcare, Little
Chalfont Buckinghamshire, UK), goat anti-mouse IgG (A2304,
Sigma Aldrich, St. Louis, Missouri) or bovine anti-goat IgG (sc-2350,
Santa Cruz Biotechnology, Santa Cruz, CA)] at 1:5,000 dilution for
1 h at room temperature. Bands were visualized using chemilumines-
cence according to the manufacturer (Santa Cruz Biotechnology, sc-2048).
The optical density of bands (OD minus background) was measured using
NIH-Image J software and normalized to actin.

Immunostaining. Double immunostaining for NOS-1 and βDG was
performed using the IHC World protocol, which is a parallel approach
(31) that employs multiple blocking steps including normal goat
serum, the unconjugated Fab fragment of goat anti-rabbit or anti-
mouse IgG, and avidin and biotin as reported (52). After blocking,
sections were incubated in a mixture of two primary antibodies, rabbit
polyclonal anti-NOS1 (sc-1025, Santa Cruz Biotechnology, 1:50) and
mouse monoclonal anti-β-dystroglycan (NCL-43DAG, Novocastra,
1:100) overnight at 4°C. After washing in Tris-buffered saline with
TWEEN (TBST, 0.5 M Tris base and 9% NaCl in water with 0.5%
Tween 20) sections were incubated with a mixture of two secondary
antibodies (AMCA-conjugated Fab fragment goat anti-rabbit, and
DYLight 649-conjugated Fab fragment goat anti-mouse, Jackson
ImmunoResearch Laboratories, West Grove, PA, 1:200) for 1 h. After
washing in TBST, sections were mounted under coverslips using
Vectashield (Vector Laboratories, Burlingame, CA). Omission of
primary antibody served as a negative control. Sections were viewed
without knowledge of source group.

Sarcosomal integrity. Since βDG forms an essential core of the
DGC that links the internal cytoskeleton across the membrane to the
extracellular matrix (27), we examined the age-related reduction in
βDG in relation to functional changes that increase fiber permeability
to serum proteins such as IgG. Immunostaining for mouse IgG was
performed as follows. Briefly, sections were incubated for 1 h in
blocking solution (10% goat serum in phosphate-buffered saline and
2 M glycine, 20:1), rinsed, and incubated with goat antibody to mouse
IgG (DYLight 649-conjugated Fab fragment, Jackson Immuno-
Research Laboratories, 1:200) for 1 h. After washing in TBST,
sections were mounted under coverslips using
Vestashield (Vector Laboratories, Burlington, CA). All imaging parameters were kept
constant between samples, viewed without knowledge of source group.

Histochemistry. Nonspecific alkaline phosphatase (AP) histochem-
istry was used to visualize vascular endothelial cells in quadriceps
muscle cryosections. The reaction provided substrate (5-Br-4-Cl-3-
indolyl phosphate, Sigma) dissolved in a solution of dimethylforma-
mide and tetranitroblue tetrazolium salt (Sigma) in 1 ml of 0.1 M Tris
HCl, pH 9.4, according to a standard method (8). Vascular density was
assessed using the Weibel-Gomez method, viewing three random

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fields of transversely cut fibers in each section (avoiding areas of muscle-tendon junctions) through a 10 × 10 ocular grid at 200× magnification (29, 70, 78). The mean (number per field) of AP-stained vessel profiles intersecting grid lines was counted on slides coded to minimize observer bias. Counts were reproducible with <8% variation among five repeat counts on a test section.

Statistical analysis. Values are expressed as means ± SE. Groups were compared by analysis of variance (ANOVA) followed by post hoc Tukey’s HSD means-comparison or Chi-squared tests (for frequency distribution), as appropriate using Excel 2007 and SAS (JMP). A probability of $P \leq 0.05$ indicated statistical significance.

RESULTS

Muscle mass and fiber diameter. Quadriceps mass increased ~25% after treatment with I + Ex compared with the control group (Table 1, $P < 0.01$). The mass of tibialis anterior and gastrocnemius muscles did not change with treatment (Table 1). Fiber diameter in the quadriceps also increased in the I + Ex group ($P < 0.05$) and was reduced by exercise alone ($P = 0.05$) compared with C (Fig. 1). The distribution of fiber diameter was shifted to larger fibers in the I + Ex group compared with both C and Ex groups ($P < 0.01$, Chi-squared). There was no difference in distance run over a 24-h period between the two exercised groups (distance ranged from 2,649 ± 911 m/24 h to 4,230 ± 1,512 m/24 h) during the 3-wk course of exercise.

Cell proliferation and protein:DNA ratio. Cell proliferation, assessed by incorporation of $^3$H-trd into DNA, was lower after Ex compared with all other groups ($P < 0.05$, Fig. 2). Proliferation increased after I compared with the control group ($P < 0.01$). The I + Ex group showed greater proliferation in quadriceps than C and Ex groups ($P < 0.01$) and was ~30% higher than in the I group. The overall ratio of total protein to total DNA in muscle homogenates was less in groups receiving ISDN (I and I + Ex) compared with those without (C and Ex) ($P < 0.01$, Fig. 3). Although the cell types responsible for proliferation were not identified, there was no apparent infiltration by immune cells or change in the level of inflammation apparent among muscles of the different groups.

Proteins. NOS-1 concentration (relative to actin) was higher in muscle from all old groups (19.5 mo) compared with quadriceps from 6-wk-old young mice ($P < 0.01$, Fig. 4A). NOS-1 in old mice did not change after any treatment, although NOS-1 tended toward an increase after I treatment compared with the C group ($P = 0.08$). The concentration of βDG (relative to actin) was also unchanged with treatment (Fig. 4B). However, the ratio of NOS-1:βDG was increased by I in the I group compared with all other groups ($P < 0.05$). The NOS-1:βDG ratio was also higher at 19.5 mo (all 4 groups) than at 6 wk ($P < 0.05$, Fig. 4C). The NOS-1:βDG ratio was significantly correlated to fiber diameter ($P < 0.01$, $R^2 = 0.26$, Fig. 4E). The levels of Pax7 and MyoD proteins did not change with treatment. However, the NOS-1:βDG ratio was correlated to the level of Pax7 protein ($P < 0.001$, $R^2 = 0.57$, Fig. 4F).

Immunostaining of NOS-1 and β-dystroglycan. Immunostaining studies of NOS-1 and βDG localization in quadriceps

![Fig. 1. Fiber diameter in quadriceps muscle. Groups: Ex, exercise only; I, isosorbide dinitrate (ISDN) only; Ex + I, exercise + ISDN; control (C), without exercise or ISDN. A: mean (±SE) fiber diameter. Bars identified by with one or more different letters were significantly different, as follows. I + Ex treatment increased fiber diameter in 19.5-mo-old mice compared with the Ex group ($P < 0.01$). Exercise decreased fiber diameter compared with the C group ($P = 0.05$). B: frequency distribution of fiber diameter in the four groups shows significant shift toward larger fibers after I + Ex treatment vs. C and shift toward smaller fibers in the Ex group.](http://ajpcell.physiology.org/)
fibers (Fig. 5) showed striking changes between 19.5 mo groups compared with muscle from young mice. In 6 wk mice, immunoreactivity for NOS-1 and βDG was a uniformly smooth outline around fibers. In quadriceps from older C mice, staining for anti-βDG staining was discontinuous, almost punctate, around fibers and in some locations was completely absent. In the same sections, NOS-1 staining was more generalized throughout the cytoplasm compared with the peripheral outline around fibers in muscle from young mice. After I treatment, muscle fibers displayed NOS-1 and βDG proteins that were similar to the profile in young muscle. Staining was more evenly distributed around fibers compared with staining in C muscle, although some fibers had discontinuous segments of βDG, resembling “beads.” Immunostaining was absent in negative-control slides.

Sarcolemmal integrity evaluated by IgG staining. Immunoreactivity for serum IgG protein was not observed in the cytoplasm of quadriceps fibers from young mice (Fig. 6A). By contrast, in quadriceps from 19.5-mo controls, many fibers showed cytoplasm with intense labeling for IgG. Fibers with the internal profile of cytoplasmic IgG immunostaining were much less frequent in the I + Ex group compared with the C group. Quadriceps fibers in the Ex group showed the same staining pattern as in the C group.

Vascular density and VEGF. Vascular endothelium was visualized using AP histochemistry and vessel profiles quantified by counting intersections per field on a Weibel grid. Vessel profiles were observed more frequently in quadriceps sections from I + Ex-treated animals (Fig. 7A) and vascular density was nearly twofold higher in I + Ex than C or Ex groups (P < 0.01) (Fig. 7B). Although the level of 41-kDa VEGF protein (relative to actin) did not differ among groups after 6 wk treatment (Fig. 7, C and D), quadriceps muscle from a subset of I and I + Ex groups (n = 3–4) euthanized after 3-wk treatment (i.e., tissues collected at the end of the exercise period) showed a threefold increase in VEGF compared with all four groups after 6-wk treatment (P < 0.001).

DISCUSSION

This study in old mice examined whether treatment with a NO-donor drug, ISDN, for 6 wk would improve the impact of 3 wk of voluntary exercise starting at 18 mo. NO-donor...
treatment stimulated exercise-induced muscle growth by a functionally important 25% increase in mass. Hypertrophy was accompanied by modifications in the DGC, particularly the ratio and localization of NOS-1 and βDG ratio. As well, the same muscles showed increased vascular density and a reduced influx of serum IgG into fibers. Muscle mass, fiber diameter, and cell proliferation in quadriceps muscle increased after combined treatment with I/Ex compared with age-matched controls. The response was muscle-specific and restricted to quadriceps, as noted in an earlier study in 8-mo-old mice. Exercise alone in younger mice induced an increase in fiber cross-sectional area and NOS-1 protein, and reduced the growth inhibitor, myostatin (38, 44, 49, 50, 67). By contrast, in older mice in the present study, I + Ex also partially restored the normally close association of NOS-1 and βDG in the DGC, and increased the prevalence of NOS-1 at the fiber membrane. Notably, the correlation of fiber diameter and the NOS-1:βDG ratio accompanied by a decrease in anti-IgG staining in fibers suggested that cytoskeletal integrity was stabilized by NO-donor treatment, which should be investigated as underpinning the growth response to exercise. The increase in vascular density after 6 wk I + Ex treatment followed an earlier increase in VEGF protein after 3 wk. While not conclusive as to mechanism, collectively, findings illustrate NO acts as a potent adjuvant to exercise in inducing fiber growth. NO-donor treatment during a relatively short intervention of voluntary exercise was efficacious in producing hypertrophy by sarcopenic muscle in old mice, muscle that typically resists exercise-induced growth (44, 68). Results have strong potential for application to improve quality of life through prevention and treatment of age-related atrophy, since strength and muscle mass are so strongly correlated (12, 30). Notably, only longer-term voluntary exercise was previously noted to induce hypertrophy in old mice (10, 80).

Exercise alone in 18-mo female mice decreased quadriceps fiber diameter (44). Since only combined I + Ex treatment induced fiber growth by effects on proliferation, the cytoskeleton, and vascularity, muscle hypertrophy was likely dependent on a combination of effects on sarcomemmal stability, SC activation, the mechanobiology of exercise, and perfusion. SC

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**Fig. 5.** Immunostaining for NOS-1 and β-dystroglycan (β-DG) demonstrated that Ex and ISDN treatment partly restored the integrity of NOS-1 and β-dystroglycan protein around the sarcolemma of muscle fibers. Arrows in the middle column indicate that the contour of staining for β-dystroglycan is smooth and continuous surrounding fibers in young muscle (top row), discontinuous, almost punctuate, around fibers in old control muscle (middle row) and more continuous and evenly localized around the large fibers in quadriceps muscle of the I + Ex group (bottom row). Bar = 20 μm.
activation by stretching is typically perturbed beyond 6 mo; this leads to SC becoming increasingly refractory to mechanical stimuli (stretch, exercise, strain) with increasing age. This was demonstrated by experiments using cultures of single fibers (7) and whole muscles (43), and by in vivo experiments (44). Although the measure of cell proliferation in this study included SC and other cell types in the assay for [³²P]thymidine uptake, the notable hypertrophy in context of the decreased protein:DNA ratio in quadriceps after I treatment (± exercise) indicates myogenic cells contributed to the proliferating population that supported fiber growth. Similar contribution from myonuclear addition during hypertrophy was required for muscle growth following exercise or endurance training (6, 45, 57). NO-donor treatment in vivo and l-arginine treatment in whole muscle cultures from old mice (43) both show that exogenous NO can overcome the age-related disruption of NO-dependent SC activation by exercise. In the present study, that bypass of SC refractoriness produced fiber hypertrophy in a powerful fast-twitch muscle. We therefore propose that combining exercise with an exogenous source of NO has potential as a method to combat age-related muscle atrophy. NO donors improved regeneration in mdx mouse diaphragm during prednisone treatment (52) and effects of nonsteroidal anti-inflammatory drugs on mdx mouse dystrophy and alpha-sarcoglycanopathy (11, 64). By extension, therefore, since SC activation is a critical step in muscle regeneration, NO-based interventions very likely have strong potential to rescue the regenerative capacity of stem cells in aged muscle.

The impact of exercise and ISDN treatment to induce hypertrophy was restricted to the quadriceps muscle in this study. Although fiber type-independent responses are known (14), the specific features of exercise that impact particular muscles and fiber types are important aspects of adaptive muscle function in relation to training and levels of NOS (68), physiology of recruitment and loading (69), dislocation response to unloading from suspension (71), and muscle loading in voluntary exercise (41, 77). Additional differences in muscle architecture, the configuration of fiber insertion, developmental fiber-type patterns, and the functional angle of applied tension in lengthening and shortening contractions also impact the range of phenotypic responses resulting from aging and interventions in vivo (44). Indeed, the gait cycle of voluntary exercise, including alternating plantar flexion/hip extension with hip flexion/knee extension, would have distinctive functional demands on particular muscles, depending on changes in pennation and fiber shortening with age, and including need for stabilization by co-contraction around the knee. Identifying the source of muscle-specific effects of exercise is thus limited by lack of metabolic information such as exercise effects on maximal oxygen consumption (V⁰₂max), cytochrome oxidase, citrate synthase or other components of the Krebs cycle in muscles that increased after 16 wk wheel running in oxidative muscles of young female mice (19). Therefore, muscle-specific changes, such as hypertrophy from wheel running, were not unexpected. However, it is interesting that responses to exercise were most apparent in the quadriceps in this study and in dystrophinopathy (5).

The level of NOS-1 protein is generally accepted to be stable during aging in adult muscle, and we previously reported that quadriceps muscle NOS-1 did not change from 8 to 18 mo in mice (44), consistent with a study in rat plantaris between 2 and 22 mo of age (7). However, the current study found NOS-1 relative to actin in quadriceps increased between 6 wk and 19.5 mo, and increased in response to exercise and/or NO-donor treatment. A similar response to exercise was reported for gastrocnemius and soleus muscles of 24-mo rats (68). Variations may relate to muscle or species-specific changes during aging and/or use of different loading controls [actin (here and Ref. 7) vs. GAPDH (44, 68)]. Supposedly stable “housekeeping” genes are noted to vary in relation to activity (13, 33) or
fiber type (46), since metabolism, the cell cycle, contractility, vascular perfusion, and disease state all impact the plastic interactions of function on structural compartments. Notably, the GAPDH promoter has 3 insulin-response elements in its promoter and responds to metabolic changes (2, 54). Levels of GAPDH protein also decrease as a result of transcriptional modification in fast-glycolytic muscles of senescent rats compared with slow-twitch muscles (46). Given that muscle fiber diameter was increased by combined I\(\times\)H11001 Ex treatment vs. I alone, reporting changes in protein concentration relative to actin would actually be a conservative estimate of those changes, since the level of actin protein itself in blots did not change across treatments (arbitrary OD units, means ± SD: C 9,485 ± 3,308; Ex 8,303 ± 2,016; I 7,050 ± 3,384; I + Ex 9,364 ± 1,975) whereas levels of GAPDH protein did change (means ± SD: C 17,941 ± 1,894; Ex 18,342 ± 3,051; I 8,335 ± 4,111; I + Ex 14,440 ± 6,930). A recent review eloquently demonstrated site-specific production and effects of NO in various tissues in the body, including skeletal muscle, lung, central nervous system, and liver (75). The altered level of NOS-I in old muscle may be related to the compartmentalized environment within a particular muscle, and the location of the protein in fibers (internalized in the sarcoplasm vs. localized with the DGC).

There were intriguing relationships between the NOS-1:βDG ratio, the pattern of localization of the two proteins, and
sarcolemmal integrity with fiber diameter. The NOS-1:βDG ratio in quadriceps at 19.5 mo increased after ISDN treatment, with or without exercise. Here, study of the ratio of two proteins (probed on two halves of the same blot), both typically localized within the dystroglycan complex, obviates the need for an additional loading control. Immunostaining studies showed that the age-related disruption of NOS-1 and βDG localization was restored by I + Ex toward a more even pattern of distribution at the fiber periphery, as observed in young-adult muscle. Secondary age-related changes in transmembrane signaling (60) by MAPK phosphorylation (62) and NO release from NOS-1 (71) would each be counteracted by normalizing the localization of the two DGC proteins. A physiological improvement in sarcolemmal integrity was shown by the decrease in IgG that had penetrated fibers after I + Ex treatment compared with old controls. Such sarcolemmal disruption was observed previously in 36-mo rat muscle, and increased further under tension (62), a feature that is reminiscent of DGC disruption and membrane leakiness (47) of muscle in old animals and in muscular dystrophies (58). As well, during hindlimb suspension, there is displacement of NOS-1 into the sarcoplasm that is reported to induce atrophy via apoptosis (71). Together the present results on DGC proteins and sarcolemmal integrity strongly suggest that an increase in the NOS-1:β-DG ratio produced after I + Ex treatment may be important in preventing fiber damage in aged muscle by reducing membrane leakiness, secondarily reducing low-grade inflammation in aged muscle (56), and reducing the activity of apoptotic pathways leading to atrophy. Notably, DGC integrity and NOS-1 localization are essential in the normal regulation of satellite cell activation during regeneration and muscle growth (3, 59), and loss of membrane integrity raises susceptibility to contraction-induced injury and reduces regeneration in aged muscle (1, 9, 21, 23, 47, 48). Complex interactions between aging, DGC proteins, fiber integrity, muscle atrophy, and muscle growth and regeneration likely underlie the remarkable hypertrophy of quadriceps muscle that was observed after treatment with voluntary exercise plus the NO donor drug, ISDN. The significant correlations between the NOS-1:βDG ratio, relative Pax7 levels and muscle fiber size, and changes in the protein:DNA ratio provide suggestive evidence of these interactions, although changes in DGC proteins may be unrelated to the hypertrophic response to exercise as neither the protein levels or the NOS-1:βDG ratio tended to normalize after treatment toward the levels seen in muscle from younger 6 wk mice. However, results do indicate that the reduced capacity of aged muscle to grow in response to exercise can be significantly reversed through NO-donor treatment, and that the treatment response included partial restoration of sarcolemmal integrity and changes in the DGC.

Exercise induces angiogenesis and vascular remodeling through a complex regulatory cascade involving VEGF (including VEGF alternative splicing, dimerization, and receptor interactions), NO-related vascular permeability and proangiogenesis, matrix metalloproteinases, and Wnt, Notch, and fibroblast growth factor signals (16, 20, 22, 25, 39). Notably, capillary density declines with age (26). In 12-mo-old rats, L-arginine treatment alone increased blood vessel density with only a marginal increase in soleus mass relative to body weight after 6 wk of endurance training (fiber size was not measured) (70). Interestingly, capillary density (8) and exercise-induced angio-

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

version of manuscript; R.U. and J.E.A. conception and design of research; R.U. and J.E.A. prepared figures; J.E.A. analyzed data; J.E.A. drafted manuscript.

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