Regulation of neuregulin/ErbB signaling by contractile activity in skeletal muscle

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The proliferative and metabolic responses of skeletal muscle to contractile activity involve the complex integration of both intra- and intercellular signaling pathways. The activation of diverse intracellular kinase cascades has been demonstrated in skeletal muscle, and these have been associated with fiber hypertrophy and alterations in metabolism. The extracellular stimuli and cell-cell interactions that trigger the activation of these pathways during and after exercise, however, have not been elucidated. In multiple cell types, growth factors relay key extracellular signals to trigger diverse cellular events via receptor tyrosine kinases (RTKs) (9). One such candidate system in the regulation of exercise signaling that has pleiotropic effects in skeletal muscle is neuregulin (NRG) and its cognate RTKs ErbB2, ErbB3, and ErbB4.

The NRGs (also known as heregulin, neu differentiation factor, ACh receptor-inducing activity, glial growth factor II, and sensory motor neuron-derived factor) are a complex family of proteins structurally related to the classical polypeptide mitogen-epidermal growth factor (EGF; see Ref. 19). More than 15 distinct soluble and membrane-anchored NRG isoforms result from alternative splicing of mRNA from one of four known NRG genes (21). All NRGs identified to date feature an EGF-like motif that is both necessary and sufficient for biological activity (6, 29). NRG signals are mediated via activation of the type I subfamily RTKs: ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4) (7, 13, 30, 41). Heteromeric complexes of ErbB2, ErbB3, and ErbB4 and homomeric ErbB4 are activated by NRG binding and lead to phosphorylation of cytoplasmic tyrosine residues that initiate a diverse array of downstream signaling events (5, 46).

NRGs activate growth, differentiation, and survival signaling pathways in multiple cell types, including epithelium (44), nerve (23), cardiac (47), and skeletal muscle. Targeted mutations in mice have demonstrated an essential role for NRG and ErbBs in the development of the nervous and cardiovascular systems (11, 18, 23). In skeletal muscle, NRG has been studied extensively in vitro as a regulator of differentiation and development of the neuromuscular junction. Nerve- and muscle-derived members of the NRG family have been observed to stimulate myotube formation and muscle-specific gene expression (10, 16), induce ACh receptor expression (AchR; see Refs. 8, 15, and 22), and regulate formation and maintenance of chemical synapses (34). Generation of a muscle-specific ErbB2 deletion resulted in mice with impaired motor coordination and poor body condition. Isolated ErbB2-deficient myoblasts suffered extensive apoptosis upon differentiation to myofibers (3). Furthermore, NRG appears to play a role in regulating metabolism, in that NRG stimulation of skeletal muscle facilitates glucose uptake in a manner that is additive to the effects of insulin (38).

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To date, the physiological regulation of NRG/ErbB signaling has not been examined. Given the evidence of NRG mediation of skeletal muscle growth and metabolism in vitro, we tested the hypothesis that contractile activity in vivo elicits NRG/ErbB activation. We employed two distinct modes of contractile activity that initiate specific intracellular signaling pathways and induce unique functional and morphological properties in skeletal muscle. We used electrical stimulation as a model of resistance exercise, which is known to enhance muscle strength through fiber hypertrophy and contractile characteristics, and treadmill running as a model of endurance exercise, which is known to enhance muscle oxidative capacity through changes in key metabolic enzymes, mitochondrial density, and capillary supply with no resultant hypertrophy.

MATERIALS AND METHODS

Materials. ErbB2, ErbB4, and extracellular NRG [Hersgenulin Ab1 (clone 7D5)] antibodies were purchased from NeoMarkers (Fremont, CA). ErbB3, intracellular NRG [HRGαC20]: sc-348] specific to the carboxy terminus of the NRG precursor and PY99 phosphotyrosine antibodies, and protein A/G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Molecular mass markers were from Invitrogen Life Technologies (Carlsbad, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-goat, and anti-mouse IgG whole antibodies were also from Santa Cruz Biotechnology, and an enhanced chemiluminescence system was purchased from Pierce Biotechnology (Rockford, IL). Dye reagent for determination of protein concentrations was from Bio-Rad Laboratories (Hercules, CA). Secondary antibodies for immunofluorescence (Alexa Fluor 488 goat anti-rabbit and anti-mouse IgG) and α-bungarotoxin were from Molecular Probes (Eugene, OR). Actin antibody and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Animals. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the Boston University Medical Center and were in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats weighing 180–220 g obtained from Charles River Laboratories (Wilmington, MA) were fed standard laboratory chow and water ad libitum. Rats were fasted overnight (10:00 PM to 8:00 AM) before the experiment.

In vivo muscle contraction. To examine the acute effects of a single bout of an in vivo model of resistance exercise (RX), a protocol was chosen based on its efficacy in eliciting contractile activity and inducing skeletal muscle hypertrophy. In this model, hindlimb muscles of the posterior compartment (gastrocnemius, soleus, and plantaris) perform concentric contractions, and muscles of the anterior compartment [tibialis anterior and extensor digitorum longus (EDL)] undergo high-resistance eccentric contractions facilitating hypertrophy. Briefly, animals (n = 5) were anesthetized using an intraperitoneal injection of a ketamine (75 mg/kg) and xylazine (25 mg/kg) cocktail. The sciatic nerves to both legs were surgically exposed, and two fine-wire platinum electrodes were placed around the nerves. Muscle contractions were produced in one hindlimb using a Grass S48 stimulator (Grass Instruments, Quincy, MA) at a frequency of 100 Hz to recruit both fast- and slow-twitch muscle fibers (6–12 V, 3-s duration, and 10-s delay, for 10 sets of 10 repetitions). A 1-min recovery was given between sets, resulting in a protocol time of 45 min. The contralateral hindlimb served as a sham-operated control.

To analyze the acute effects of a single bout of endurance exercise (EX) in vivo, a 45-min treadmill running protocol was employed (32). Briefly, control (n = 5) and experimental (n = 5) animals were acclimated to a motor-driven treadmill (Quinton Instruments, Seattle, WA) for 3 days (5 min at 21.7 m/min). On the 4th day, experimental animals were subjected to a three-stage exhaustive bout of exercise. The protocol consisted of 15 min at 21.7 m/min and a 15% incline and 15 min at 26.7 m/min and a 20% incline and concluded with 15 min at 31.7 m/min and a 25% incline, or until exhaustion, whichever came first. For the equivalent duration, control animals were placed on an adjacent treadmill, and the belt remained stationary.

After RX and EX, a lethal dose of pentobarbital sodium was administered to experimental and control animals, and gastrocnemius (medial and lateral head), soleus, plantaris, tibialis anterior, and EDL muscles were removed rapidly and quick-frozen in liquid nitrogen. For immunofluorescence studies, a portion of soleus and EDL muscles was oriented with National Institutes of Health guidelines. Male rats (6–12 V, 3-s contraction, 15-min recovery) were used in accordance with National Institutes of Health guidelines. Male rats (6–12 V, 3-s contraction, 15-min recovery) were used in accordance with National Institutes of Health guidelines.

Analysis of NRG/ErbB expression and activation. To characterize the expression of NRG and ErbB receptor subtypes in whole muscle lysates, aliquots (50 μg) of muscle protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were blocked in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 (TBST) and 5% BSA for 1 h at room temperature and then probed with NRG antibodies for intracellular- and extracellular-specific sequences (1:1,000) or ErbB receptor subtypes with antibodies specific to ErbB2, ErbB3, or ErbB4 (1:1,000) in 2% BSA overnight at 4°C. To determine if ErbB receptors were activated in response to contractile activity, aliquots (500 μg) of muscle protein were incubated with antibodies specific to ErbB2, ErbB3, or ErbB4 (1:100) overnight at 4°C and precipitated with protein A/G plus agarose. Immunoprecipitates were collected and released by boiling in Laemmli buffer. Samples were fractionated by SDS-PAGE, transferred to PVDF membranes, and blocked in TBST and 2% BSA. Membranes were probed with a PY99 anti-phosphotyrosine antibody (1:1,000) in TBST and 5% BSA for 1 h at room temperature.

Bound antibodies were detected with anti-mouse hors eradish peroxidase-linked whole antibody (1:5,000). Protein immunoblots were visualized by enhanced chemiluminescence, and bands were quantified with scanning densitometry using Molecular Analyst Software (Bio-Rad Laboratories). The sizes of the antibody-bound proteins were verified using standard molecular mass markers.

Immunofluorescence studies. To determine the localization of NRG and its receptors, control soleus and EDL muscle serial tissue cross sections (8 μm) were cut and mounted on
glass slides using a cryostat microtome (Leica Microsystems) and fixed in cold acetone at −20°C. After rehydration in PBS (pH = 7.4), samples were blocked in 10% BSA for 1 h at room temperature and then incubated overnight at 4°C in a humidified chamber with anti-ErbB2 (1:100), -ErbB3 (1:100), -ErbB4 (1:100), or -NRG (EGF-like domain; 1:300) in PBS with 3% BSA. To identify neuromuscular junctions (NMJ), sections were coincubated in Texas red-conjugated a-bungarotoxin (1:1,000). Control sections were incubated in 3% BSA. Sections were washed in PBS and then incubated for 1 h at room temperature in FITC-labeled secondary antibody (1:300) diluted in PBS with 3% BSA. Sections were rinsed in PBS, mounted in Vectashield medium (Vector Laboratories, Burlingame, CA), and examined using a Nikon Eclipse E400 (Melville, NY) and either ×40 or ×100 magnification. Images were captured using the Bioquant Nova image analysis system (Bioquant Image Analysis, Nashville, TN). Overlay images were processed in Adobe Photoshop.

Statistics. Data are expressed as means ± SE. Statistical analysis was undertaken using a paired Student’s t-test and one-way ANOVA. When ANOVA revealed significant differences, further analysis was performed using Tukey’s post hoc test for multiple comparisons. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

Expression and localization of ErbB2, ErbB3, and ErbB4 in skeletal muscle. To quantify the relative expression of ErbB receptors in adult skeletal muscle, we probed lysates from multiple hindlimb muscle types using specific ErbB2, ErbB3, and ErbB4 antibodies. As shown in Fig. 1A, ErbB2 and ErbB4 are abundant in soleus and EDL muscles. Interestingly, ErbB3 expression appears to be decreased in the soleus relative to the EDL. Immunofluorescence analysis of muscle cross sections showed that ErbB2, ErbB3, and ErbB4 were localized clearly to the myocyte, and ErbB3 immunoreactivity was decreased in the soleus compared with the EDL (Fig. 1B). All three receptors colocalized to the NMJ (Fig. 2), although there was staining for each receptor on the myocyte membrane remote from the NMJ.

Multiple NRG isoforms are expressed in skeletal muscle. In multiple cell types, ErbB2, ErbB3, and ErbB4 receptor interaction and phosphorylation is triggered by the ligand NRG (7, 17, 30, 31, 37, 41). We examined NRG expression in skeletal muscle using antibodies specific to intracellular vs. extracellular domains of NRG. Under basal conditions, several bands were detected in whole skeletal muscle with both NRG antibodies (Fig. 3, A and B, representative blots), consistent with the expression of multiple NRG splice variants. The molecular size of several of these isoforms corresponds to known masses of recombinant NRG proteins (1, 24, 35, 43), including the 115-kDa species that is immunoreactive to both the intracellular and extracellular domain antibodies. The 183-kDa band (Fig. 3A) is also detectable with the extracellular domain antibody with prolonged exposure (data not shown) and may represent “mature” type III NRG (43). The reduced sensitivity compared with its detection using the intracellular antibody may represent epitope modification during protein maturation. As shown in Fig. 2, examination of muscle cross sections revealed that the distribution of NRG was enriched but not confined to the NMJ.

Contractile activity stimulates NRG processing in vivo. Alternatively spliced transcripts of NRG generate both secreted and membrane-anchored isoforms. Cleavage of transmembrane isoforms and the release of their extracellular EGF-like domain may be critical for NRG activation (21, 45). To test the hypothesis that contractile activity initiates NRG processing, we used intracellular and extracellular NRG antibodies to detect a shift in the expression profile of NRG. In response to both forms of exercise (RX and EX), the relative distribution of NRG changed markedly in all muscles examined. Most notably, we observed the disappearance of the 183-kDa band (Fig. 3C, top, representative blot) and the concurrent increase in the 64- and 48-kDa bands (Fig. 3C, middle) in exercised vs. control muscles. These changes arguably represent acute proteolytic processing of mature NRG transmembrane protein to soluble ligand (24, 35, 43).

Skeletal muscle contractile activity stimulates ErbB2, ErbB3, and ErbB4 phosphorylation. To test the hypothesis that NRG/ErbB signaling is activated in response to contractile activity, we stimulated contractile activity either by sciatic nerve stimulation (RX) or treadmill running (EX) and rapidly isolated hindlimb skeletal muscles. ErbB immunoprecipitates from lysates were probed with an anti-phosphotyrosine antibody as an indicator of receptor activation. Both RX and EX resulted in significant activation of skeletal muscle ErbB2, ErbB3, and ErbB4. Activation of ErbB receptors was comparable among muscles despite decreased ErbB3 expression in the soleus. Compared with sham-operated muscles, RX resulted in an approximately fourfold increases in ErbB2, ErbB3, and ErbB4 phosphorylation for all muscle groups examined (Fig. 4, A and C). Similarly, compared with nonexercised control muscles, EX resulted in more than twofold increases in ErbB2, ErbB3, and ErbB4 phosphorylation (Fig. 4, B and C). Moreover, RX significantly increased ErbB2 (P < 0.02) and ErbB4 (P < 0.04) receptor phosphorylation compared with EX; however, differences in ErbB3 phosphorylation were not significant (P < 0.08).

DISCUSSION

NRG and its receptors have been implicated in multiple aspects of skeletal muscle development (10, 16) and in the regulation of skeletal muscle metabolism (38). In the present study, we have shown that ErbB2, ErbB3, and ErbB4 receptors and multiple NRG isoforms are expressed at high levels in adult skeletal muscle and are activated in response to contractile activity. Two distinct modes of exercise activated proteolytic processing of NRG with concomitant stimulation of ErbB2, ErbB3, and ErbB4 signaling in vivo. To our knowledge, this is the first demonstration of physiological regulation of NRG/ErbB signaling in any organ and implicates this pathway in the metabolic and proliferative responses of skeletal muscle to exercise.
Consistent with other investigators, we have demonstrated the expression and localization of ErbB2, ErbB3, and ErbB4 proteins in skeletal muscle and the enrichment of these receptors at the NMJ (2, 14, 15, 25, 40, 48). A novel finding in our study is the increased expression of ErbB3 in the EDL, a predominantly fast-twitch, glycolytic muscle compared with the soleus, a predominantly slow-twitch, oxidative muscle. Whether

Fig. 1. ErbB2, ErbB3, and ErbB4 receptors are expressed in adult skeletal muscle. A: soleus (SOL) and extensor digitorum longus (EDL) muscle lysates were resolved on SDS-PAGE and immunoblotted with specific antibodies for ErbB2, ErbB3, and ErbB4 (representative of 6 experiments). B: immunofluorescent staining of ErbB2, ErbB3, and ErbB4 in soleus and extensor digitorum longus muscle cross sections (sections representative of 5 animals, ×40 magnification).

Fig. 2. ErbB receptors and neuregulin (NRG) are enriched at the neuromuscular junction (NMJ) in adult skeletal muscle. Soleus muscle cross sections were labeled with specific ErbB2, ErbB3, ErbB4, and NRG antibodies. Sections were counterstained using α-bungarotoxin (αBTX) to label the postsynaptic membrane (representative of 5 animals, ×100 magnification).
this differential ErbB expression confers distinct metabolic and/or morphological properties in muscle fibers warrants further investigation. It is interesting that ErbB3 is not expressed in adult cardiac myocytes (47), which have a similar phenotype to slow-twitch skeletal muscle.

There appear to be multiple products of the NRG-1 gene expressed in skeletal muscle. The use of NRG antibodies to extracellular and intracellular domains reveals that there are multiple NRG isoforms in whole muscle lysates that likely represent both precursor pro-NRG and mature NRG (13, 44). Immunohistochemistry suggests NRG expression in skeletal muscle and motor neurons. Currently available tools have not permitted us to resolve potential differences in nerve- and muscle-derived NRG isoform expression. Previous reports have also shown that NRG mRNA is highly expressed in motor neurons (8, 21) and is detectable in muscle cells (NK LeBrasseur, unpublished observation, and Refs. 25 and 27). These studies provide a framework for both autocrine and paracrine NRG signaling. Cleavage of transmembrane NRG isoforms may be essential for function given that the ErbB receptors could be located a significant distance from the ligand. We observed a marked shift in NRG isoform distribution in response to contractile activity, consistent with proteolytic cleavage. After RX and EX, we detected a significant increase in the relative amounts of 64- and 48-kDa NRG isoforms, which are consistent with the predicted molecular sizes of the soluble and excreted species (8, 13, 28, 42). The lower molecular mass bands seen may represent truncated NRG fragments (8). Definitive identification of these awaits improved antibody reagent and/or mass spectrometric methods. The proteolytic mechanisms responsible for the solubilization of membrane-anchored NRGs are not known, although prior studies of NRG and other transmembrane growth factors suggest a role for the ADAM (a disintegrin and metalloprotease) family of proteases (24, 35). Our data suggest that, with exercise, there is rapid activation of these enzymes as a proximal step in the activation of NRG/ErbB signaling.

In muscle and various other cells, ErbB3 and ErbB4 bind NRG ligand and dimerize with ErbB2 to initiate Fig. 3. Multiple NRG isoforms are expressed in skeletal muscle. Contractile activity initiates NRG processing. Muscle lysates (representative blot of soleus) were subjected to Western blotting and probed for NRG isoforms using intracellular (A) and extracellular (B; EGF) specific NRG antibodies (representative of 10 experiments). C: to identify alterations in NRG after an acute bout of treadmill running (EX), control (C), EX soleus, and extensor digitorum longus muscle lysates were resolved on SDS-PAGE and immunoblotted with the specific intracellular (top) and extracellular (middle) NRG antibodies. Membranes were eluted and probed for actin to control for protein loading (representative of 5 experiments).

Fig. 4. Contractile activity stimulates ErbB receptor phosphorylation. After an acute bout of sciatic nerve stimulation (RX) or treadmill running (EX), muscle lysates were immunoprecipitated (IP) with anti-ErbB2, -ErbB3, or -ErbB4 antibody and blotted with anti-phosphotyrosine (pY) antibody. Representative blots of receptor phosphorylation in sham control and RX gastrocnemius muscle (A; n = 5) and control (C) and EX (B; n = 5) gastrocnemius muscle. C: mean activation of ErbB2, ErbB3, and ErbB4 in multiple hindlimb muscles after RX (filled bars) and EX (hatched bars) relative to controls (gray bars). *P < 0.01 vs. control. †P < 0.05 vs. EX.
signaling by phosphorylation of intrinsic tyrosine residues (7, 17, 30, 31, 37, 41). In this study, we observed significant phosphorylation of ErbB2, ErbB3, and ErbB4 in response to two distinct modes of exercise (RX and EX). The relatively similar degree of ErbB2, ErbB3, and ErbB4 expression and activation supports the possibility of multiple receptor interactions (i.e., ErbB3/ErbB2 or ErbB4/ErbB2 heterodimers and ErbB4/ErbB4 homodimers) and underscores the likelihood of this system playing multiple biological roles in skeletal muscle.

In vitro studies have demonstrated multiple effects of recombinant NRG on myoblasts, myotubes, and isolated skeletal muscle (10, 16, 38). Development of the NMJ remains the most extensively studied target of NRG action. NRG-stimulated homo- and heteromeric ErbB complexes activate the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K) signaling pathways and subsequently upregulate transcription of various AchR genes in cultured cells (36, 39). More recently, evidence for a metabolic role of NRG has been recognized. In muscle cells and tissue, NRG stimulates glucose transport by PI-3K- and/or Akt/protein kinase B-dependent translocation of GLUT4, GLUT1, and GLUT3 transporters to the cell membrane (38). The effect of NRG on glucose uptake was comparable with and additive to insulin. Furthermore, in vivo studies support an essential role for NRG/ErbB signaling in skeletal muscle. Muscle-specific ErbB2 deletion in mice resulted in impaired motor coordination and poor body condition, whereas isolated myoblasts lacking ErbB2 suffered extensive apoptosis upon differentiation to myofibers (3). It is now well established that specific intracellular signaling pathways (e.g., MAPK and PI3-K) are activated by contractile activity and orchestrate alterations in muscle growth and metabolism that occur after exercise (for review, see Ref. 33). Our finding that exercise is a potent activator of NRG/ErbB signaling suggests that NRG may in part mediate some of these biological effects of exercise on skeletal muscle.

In summary, physical exercise activates NRG/ErbB signaling in skeletal muscle. To our knowledge, this is the first demonstration of physiological regulation of NRG/ErbB signaling in adult tissue. On the basis of our work and in vitro work by other investigators, activation of NRG/ErbB signaling may mediate one or more adaptive growth and metabolic responses of skeletal muscle to exercise. To better understand this complex system and its contributions to skeletal muscle physiology, future work is needed in characterizing the signaling events, identifying age- or disease-associated alterations, and examining the consequences of manipulating the NRG/ErbB system.

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REFERENCES

22. Marchionni MA, Goodearl AD, Chen MS, Bermingham-McDonogh O, Kirk C, Hendricks M, Danehy F, Misumi D,


