Neuregulin-dependent protein synthesis in C2C12 myotubes and rat diaphragm muscle

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Hellyer, Nathan J., Carlos B. Mantilla, Eunice W. Park, Wen-Zhi Zhan, and Gary C. Sieck. Neuregulin-dependent protein synthesis in C2C12 myotubes and rat diaphragm muscle. Am J Physiol Cell Physiol 291: C1056–C1061, 2006. First published June 21, 2006; doi:10.1152/ajpcell.00625.2005.—The nerve-derived trophic factor neuregulin (NRG) is a prime candidate molecule for modulating muscle fiber growth. NRG regulates signal transduction in skeletal muscle through activation of ErbB receptors present at the neuromuscular junction. In this study, we hypothesize that NRG increases protein synthesis in maturing muscle via a phosphatidylinositol 3-kinase (PI3K)-dependent mechanism. NRG signal transduction and its ability to stimulate protein synthesis (measured by incorporation of [3H]phenylalanine into the protein pool) were investigated in differentiated C2C12 myotubes and rat diaphragm muscle (DIAm). In C2C12 myotubes, NRG dose dependently increased phosphorylation of ErbB3 and recruitment of the p85 subunit of PI3K. NRG also increased phosphorylation of Akt, a downstream effector of PI3K. NRG treatment increased total protein synthesis by 35% compared with untreated control myotubes. This NRG-induced increase in Akt phosphorylation and protein synthesis was completely blocked by wortmannin, an inhibitor of PI3K but was unaffected by PD-98059, an inhibitor of MEK. In DIAm obtained from 3-day-old rat pups, Akt phosphorylation increased ~30-fold with NRG treatment (vs. untreated DIAm). NRG treatment also significantly increased protein synthesis in the DIAm by 29% after 3 h of incubation with [3H]phenylalanine (vs. untreated DIAm). Pretreatment with wortmannin abolished the NRG-induced increase in protein synthesis, suggesting a critical role for PI3K in this response. The results of the present study support the hypothesis that nerve-derived NRG contributes to the regulation of skeletal muscle mass by increasing protein synthesis via activation of PI3K.

Akt; ErbB; heregulin; protein biosynthesis; skeletal muscle

NRG activates receptor tyrosine kinases of the ErbB family expressed in motoneurons, Schwann cells, and muscle fibers (40, 44). NRG signal transduction in muscle requires the phosphorylation of ErbB receptors and subsequent activation of the phosphatidylinositol 3-kinase (PI3K) and/or Akt and Ras/Raf/ERK intracellular pathways (8). Interestingly, PI3K/Akt activation appears to be a critical regulator of muscle protein synthesis and hypertrophy via the downstream regulation of translational initiators (4, 20, 26). All four members of the ErbB receptor family can recruit the PI3K enzyme (9, 17, 29). In particular, ErbB3 contains six consensus binding motifs, which, when phosphorylated by the coreceptor ErbB2, are able to recruit and activate PI3K (15). Therefore, the ErbB2-ErbB3 coreceptor complex may be particularly well adapted for PI3K signaling, although it is possible that NRG can utilize a variety of ErbB receptor combinations to increase muscle protein synthesis via PI3K/Akt activation (25). To our knowledge, the ability of NRG to modulate global protein synthesis in mature skeletal muscle has not been reported. Therefore, we hypothesized that NRG-dependent activation of PI3K and/or Akt increases protein synthesis in differentiated C2C12 myotubes and in the rat diaphragm muscle (DIAm).

MATERIALS AND METHODS

Cell culture. C2C12 murine myoblasts (ATCC) were cultured at 37°C in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) with 5% CO2 until 80–90% confluent growth was reached. Medium was then changed to DMEM supplemented with 10% horse serum to induce differentiation. After ~2 days, myoblasts began to fuse. After an additional 5 days, distinct, multinucleated myotubes were evident. Myotubes were then serum starved 24 h before growth factor treatment.

Ex vivo DIAm preparations. All experimental procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee and were in agreement with the American Physiological Society’s “Guiding Principles in the Care and Use of Animals.” The costal DIAm was quickly excised from 3-day-old Sprague-Dawley rat pups, washed in 0.9% NaCl, and immediately pinned on a silicone rubber (Sylgard; Dow Corning, Midland, MI)-coated dish at approximately resting length. For Akt analysis and protein synthesis assays, DIAm were incubated in DMEM at 37°C with 5% CO2 for 30 min and then treated with growth factor. Experiments were terminated by quickly rinsing the DIAm in PBS and snap freezing the DIAm in liquid nitrogen.

Growth factor stimulation. Recombinant heregulin β177–244 and insulin-like growth factor (IGF)-1 (both from Upstate Biotechnology, Waltham, MA) were used in experiments examining PI3K signaling and protein synthesis. The heregulin β177–244 fragment was used to
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NRG induces protein synthesis in skeletal muscle. Western analysis of NRG effects because it contains the EGF domain necessary for ErbB activation (14). IGF-1 activates PI3K and increases protein synthesis in muscle (4), and thus its effects were compared with those of NRG.

Western analyes. Differentiated C2C12 myotubes were treated with 50 ng/ml of NRG (~10 nM) or IGF-1 (~7 nM), and DIAM was treated with 200 ng/ml NRG (~40 nM) for 30 min. C2C12 myotubes were immediately lysed in RIPA 1640 lysis buffer. Ex vivo DIAM was quickly rinsed in PBS and snap frozen before they were placed in a modified RIPA 1640 lysis buffer [1% Igepal CA-630, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 5 mM sodium fluoride, 5 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, and complete mini protease inhibitor (Roche, Indianapolis, IN) in Tris-buffered saline (TBS)]. The homogenate was cleared by centrifugation and diluted 2:1 in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). Samples were electrophoretically separated on 4–15% SDS-PAGE mini-gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked in TBS containing 5% milk and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO) before an overnight incubation with primary antibodies for Akt, phosphorylated Akt (serine residue 473; both from Cell Signaling Technology, Beverly, MA), p85 (Upstate), and ErbB3 and phosphorosyline (both from Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies were diluted 1:1,000 in TBS containing 1% BSA and 0.1% Tween 20. After three washes, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Santa Cruz) for 1 h. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL). For immunoprecipitation experiments, homogenates (200 μg of protein) were incubated with 2 μg ErbB3 antibody (Santa Cruz) for 1 h at 4°C. Protein-antibody conjugates were precipitated with protein G-PLUS agarose (Santa Cruz) and washed three times with RIPA 1640 buffer. Precipitate was resuspended in 40 μl of Laemmli sample buffer, electrophoretically separated by SDS-PAGE, and detected by immunoblotting as above. Protein amounts were semi-quantitated by measuring the density of autoradiographs with Metamorph 6.3 software (Molecular Devices, Downingtown, PA). Akt activation is reported as the ratio of phosphorylated to total Akt.

Protein synthesis assay. Assays were performed as previously described, with minor modifications (35). Myotubes were exposed to either 200 ng/ml NRG (~40 nM) or 50 ng/ml IGF-1 (~7 nM) for 30 min. Ex vivo DIAM preparations were treated with 200 ng/ml NRG for 30 min. In other experiments, 5 nM wortmannin (Sigma) or 50 μM PD-98059 (Sigma) was added to the cell or muscle baths 20 min before growth factor treatment. Cells and DIAM preparations were then incubated in the presence of 5 μCi [3H]phenylalanine (Amer sham Biosciences, Piscataway, NJ) diluted in 2 mM unlabeled phenylalanine. The time period for incorporation was 1 h, except for time-course experiments (Fig. 6) in which DIAM preparations were incubated for 1-, 2-, or 3-h periods. Myotubes and DIAM were rinsed in TBS and then placed in 500 μl of 5% TCA. Myotubes were scraped from their wells, whereas DIAM were mechanically homogenized by mortar and pestle. Homogenization chambers were rinsed twice with 250 μl TCA. The homogenate and rinses were pooled and placed in 1.5-ml conical tubes and then incubated on ice for 1 h. Proteins were precipitated by centrifugation at 6,000 g for 10 min and washed three times with TCA, each wash followed by centrifugation at 10,000 g for 10 min. The precipitate was resuspended in 0.1 N NaOH and 0.1% SDS at 60°C for 1 h. Protein concentration was determined with the Bio-Rad DC protein assay according to the manufacturer’s protocol. Incorporation of [3H]H was determined with a Beckman LS5000TD liquid scintillation counter (Beckman Coulter, Fullerton, CA) and is expressed as counts per minute (cpm) per microgram of protein.

Statistical analyses. Statistical analyses were performed with JMP 5.12 Statistical Discovery software (SAS Institute, Cary, NC). The statistical test and significance level are reported for each individual comparison. Phosphorylation of Akt and protein incorporation were analyzed by one-way ANOVA for the comparisons of growth factor effects. The effect of pharmacological inhibitors was assessed by two-way ANOVA, with growth factor and inhibitor treatment conditions as grouping variables. Significant differences between individual treatment conditions were determined post hoc by the Tukey-Kramer honestly significant difference test. For the analysis of protein incorporation across time points in the DIAM, data were analyzed by a two-way ANOVA, with the experimental group and time as grouping variables. Significant differences between individual group and time comparisons were determined post hoc by the Tukey-Kramer honestly significant difference test. In all cases, statistical significance was established at P < 0.05. All values are reported as means and SE, unless otherwise specified.

RESULTS

NRG signal transduction in differentiated C2C12 murine myotubes. NRG dose dependently increased ErbB3 phosphorylation in differentiated C2C12 murine myotubes (Fig. 1). NRG treatment also resulted in a dose-dependent increase in the association of the PI3K regulatory subunit p85 with ErbB3 (Fig. 1), suggesting that critical PI3K signaling motifs were phosphorylated within the ErbB3 COOH-terminal domain by ErbB coreceptors. Accordingly, NRG treatment induced activation of the intracellular protein kinase Akt, a downstream target of PI3K (Figs. 2 and 3). At 50 ng/ml (~10 nM), NRG increased Akt phosphorylation on serine residue 473, a site previously shown to be sufficient for activation of mammalian target of rapamycin-regulated protein translation (5, 30). Akt phosphorylation was maximal after 30 min of NRG treatment. IGF-1, a prototypical growth factor for the induction of Akt activation, increased Akt phosphorylation with a similar time course (Fig. 2). After 30 min of growth factor treatment, both NRG and IGF-1 significantly increased Akt phosphorylation (F2,22 = 16.3, P < 0.001) compared with control (Fig. 3). Akt phosphorylation induced by NRG was prevented by pretreatment with the PI3K inhibitor wortmannin (F2,22 = 18.1, P < 0.001), suggesting that NRG-induced Akt phosphorylation requires PI3K activation (Fig. 3).

NRG-induced protein synthesis in C2C12 myotubes. Incorporation of [3H]phenylalanine per total amount of precipitated...
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NRG induced cellular protein was measured in differentiated C2C12 myotubes after NRG or IGF-1 treatment and after wortmannin or PD-98059 treatment in the presence of NRG (Fig. 4). A significant growth factor treatment effect was evident ($F_{2,70} = 19.1$, $P < 0.001$). Post hoc analyses revealed that NRG and IGF-1 increased protein synthesis significantly compared with untreated control ($P < 0.05$ in each case). In fact, NRG and IGF-1 comparably increased protein synthesis (by 35% and 39%, respectively).

NRG-induced increase in protein synthesis was abolished by inhibition of PI3K activity, whereas inhibition of the MAPK pathway had no effect (Fig. 4; $F_{5,87} = 22.2$, $P < 0.001$). Protein synthesis was 49% less in myotubes treated with NRG in the presence of wortmannin compared with those treated with NRG alone. Additionally, we tested a chemically distinct PI3K inhibitor, LY-294002 (30 $\mu$M). An equivalent reduction in protein synthesis (~33%) was evident in myotubes treated with NRG in the presence of LY-294002 compared with those treated with NRG alone (data not shown). Myotubes treated with wortmannin or LY-294002 showed a comparable decrease in protein synthesis relative to control (31% and 44%, respectively), indicating that PI3K activity is critical for basal protein synthesis and necessary for NRG effect. In contrast, treatment with the MEK inhibitor PD-98059 had no significant effect on basal protein synthesis or the NRG-induced increase in protein synthesis (Fig. 4), indicating that MAPK activation is not necessary for the NRG effect.

To evaluate any chronic, longer term effects of NRG on protein synthesis, $[^{3}H]$phenylalanine incorporation was measured after 24 h of incubation with NRG at two different doses ($n = 10$ in each case) and compared with control, untreated myotubes ($n = 9$). Importantly, the effect of NRG treatment was comparable after 1 h (35% increase over control at 200 ng/ml) and 24 h (37% increase at 200 ng/ml and 21% at 50 ng/ml). Thus the effect of NRG treatment on protein synthesis is sustained.

NRG signal transduction in rat pup DIAm. ErbB2 and ErbB3 were both present in rat pup DIAm (data not shown), consistent with previous results (24). Exogenously applied NRG (200 ng/ml) increased Akt phosphorylation ~30-fold compared with untreated 3-day-old DIAm preparations (Fig. 5; $P < 0.05$), an effect similar to that demonstrated in C2C12 myotubes. Thus this dose of NRG was used in experiments examining protein synthesis in the rat pup DIAm.

NRG-induced protein synthesis in the rat DIAm. NRG produced significant, time-dependent increases in $[^{3}H]$phenylalanine incorporation over the 3-h incubation period (Fig. 6;
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Fig. 5. NRG-stimulated Akt phosphorylation in rat diaphragm muscle (DIAm). DIAm from 3-day-old rat pups were incubated with NRG or an equal volume of PBS. The ratio of phosphorylated to nonphosphorylated Akt was analyzed as described in MATERIALS AND METHODS. The mean and SE of 6 experiments are represented in the bar graph. *Significantly different (P < 0.05) from CTL.

$F_{2,22} = 45.5, P < 0.001$. The rates of $[^{3}H]$phenylalanine incorporation were not significantly different between the control and NRG groups ($F_{2,22} = 2.3, P = 0.12$), but the overall amount of $[^{3}H]$phenylalanine incorporation was significantly different ($F_{1,22} = 10.9, P = 0.003$). In the control group, $[^{3}H]$phenylalanine incorporation was significantly greater at 3 h compared with the 1- and 2-h control groups ($P < 0.05$). Similarly, $[^{3}H]$phenylalanine incorporation was significantly greater at 3 h compared with the 1- and 2-h NRG groups. At the 3-h time point, NRG treatment demonstrated a significant increase in protein synthesis compared with all other groups, including the time-matched control ($P < 0.05$). Thus the 3-h time point was used in further experiments examining the cellular pathways activated by NRG.

Inhibition of PI3K activity with wortmannin (5 nM) abolished any NRG-increased increase in protein synthesis in the rat DIAm (Fig. 7). As expected, a 3-h NRG treatment increased protein synthesis by 29% compared with control ($F_{2,23} = 4.5, P < 0.05$), an effect comparable to that observed with IGF-1 treatment. Preincubation with wortmannin (5 nM) significantly blunted the NRG-induced increase in protein synthesis:

$[^{3}H]$phenylalanine incorporation was 42% less in the combined NRG and wortmannin group compared with NRG alone ($F_{3,29} = 31.5, P < 0.05$). In accordance with the results in C$_2$C$_{12}$ myotubes, wortmannin had a significant effect on basal protein synthesis, decreasing $[^{3}H]$phenylalanine incorporation significantly below control levels ($P < 0.05$). These results demonstrate that PI3K activation is necessary for both basal and the NRG-induced increase in protein synthesis at the rat DIAm.

**DISCUSSION**

The results of the present study support the hypothesis that NRG augments protein synthesis in muscle via a PI3K-dependent mechanism. Although NRG is known to regulate the development of the neuromuscular junction and myogenesis, this study uniquely demonstrates that NRG positively impacts anabolic activity in differentiated skeletal muscle. Indeed, NRG may be an important nerve-derived trophic factor that helps maintain muscle mass.

Both ErbB receptors and NRG are present in mature skeletal muscle fibers and their innervating motoneurons. The receptors ErbB2, ErbB3, and ErbB4 are concentrated at rat and mouse neuromuscular junctions (2, 23, 24, 31, 40, 44), providing a means for a nerve-derived influence on muscle mass. It is presently unknown whether NRG exerts its effects solely at synaptic sites (12). ErbB receptors may also be distributed throughout muscle fibers at nonsynaptic sites (2, 23, 24, 44), and NRG may be released from muscle fibers (19). Regardless of its origin in vivo, the results of the present study indicate that NRG clearly increases protein synthesis in C$_2$C$_{12}$ myotubes and rat DIAm.

Other mediators, e.g., IGF-1, are known to increase protein synthesis and induce muscle hypertrophy (32, 42). IGF-1 and NRG may share similar intracellular signaling pathways, particularly PI3K/Akt. Activation of PI3K and Akt increases protein translation, resulting in muscle hypertrophy in both cultured myotubes and regenerating skeletal muscle (4, 26, 32). Indeed, PI3K activation was necessary for NRG-induced effects on Akt phosphorylation and protein synthesis. In the
present study, the effects of NRG on Akt activity and protein synthesis were compared with those of the known muscle trophic factor IGF-1. In cultured C2C12 myotubes, NRG demonstrated greater maximal Akt activation than IGF-1. In both cultured myotubes and rat DIAm, the NRG-induced increase in protein synthesis was comparable to that induced by IGF-1 treatment, suggesting a possible ceiling level in the protein synthesis response to Akt activation. Indeed, treatment with NRG produced sustained increases in protein synthesis comparable at 1 and 24 h. Together, these results indicate that NRG may be a critical factor in maintaining muscle protein homeostasis. Whether NRG actually regulates muscle mass or hypertrophy in the adult animal has not been explored directly.

Several members of the ErbB family of NRG receptors are present in skeletal muscle (3, 22, 23, 31, 36, 44). Unfortunately, identifying the role of each ErbB receptor family member is complicated by the fact that heterodimerization is common and critically determines receptor signaling. For example, ErbB2 is unable to bind NRG directly and its activation requires heterodimerization with ErbB3 or ErbB4. ErbB3 does not contain a functional tyrosine kinase domain and thus requires heterodimerization with either ErbB2 or ErbB4 for tyrosine phosphorylation of PI3K binding motifs (43). An ErbB2/ErbB3 heterodimer combination is likely the strongest mediator of NRG-dependent PI3K recruitment and activation (27, 29, 37), due to the higher affinity for NRG shown by this coreceptor (34) and the multiple PI3K binding sites present in ErbB3 (14, 28). Both ErbB2 and ErbB3 are expressed in mature skeletal muscle (23, 36), including C2C12 myotubes (18) and rat DIAm (this study) and thus may mediate NRG anabolic effects. Indeed, NRG strongly induced ErbB3 phosphorylation in C2C12 myotubes. Although ErbB4 is not expressed in C2C12 myotubes (18), we cannot rule out the possibility that ErbB4 is involved in NRG signaling in the DIAm (9).

Of note, NRG does not induce Akt activation across all skeletal muscles. For instance, in the rat soleus, NRG treatment did not increase Akt activation (7). Interestingly, the soleus muscle has a lower level of ErbB3 expression compared with the extensor digitorum longus muscle (23). It is provoking to speculate that the relative levels of ErbB receptor expression determine the signaling pathways activated by NRG binding (41, 43). Whether differential levels of ErbB receptor expression or heterodimerization are responsible for differences in ErbB signaling across muscles is presently unknown.

NRG may selectively increase the expression of specific muscle proteins via differing signaling pathways. For instance, NRG increases the expression of the large structural protein utrophin in dystrophic mdx mice (21), an effect presumably mediated through the activation of GABP transcription factors, known downstream targets of MAPK. NRG increases expression of ACh receptor α-subunits via activation of MAPK in chick myoblasts, but activation of PI3K-dependent pathways suppresses this effect (1). Both PI3K and MAPK activation are required for NRG-dependent expression of ACh receptor α-subunits in mouse skeletal muscle cells (38).

Interestingly, PI3K activation was critical, and MAPK activation was not, for the NRG-dependent increase in global protein synthesis observed in the present report. These results are consistent with the pivotal role of PI3K in muscle growth (26, 32) and support a role for NRG in providing trophic support for mature skeletal muscle. Consistent with this role, NRG was shown to increase glucose transport in skeletal muscle (36), as a result of increased glucose uptake, glucose transporter translocation, and transporter expression.

In summary, NRG enhances protein synthesis in mature skeletal muscle, which is, to our knowledge, the first demonstration of such a physiological effect. NRG may be released at the neuromuscular junction in an activity-dependent fashion and thus may regulate muscle hypertrophy in response to increased activity. Both sciatic nerve stimulation and treadmill exercise result in ErbB receptor phosphorylation in hindlimb muscles (23). Future work is needed to elucidate the conditions under which NRG is released at adult skeletal muscles, including the DIAm.

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REFERENCES


