Neuregulins increase mitochondrial oxidative capacity and insulin sensitivity in skeletal muscle cells

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Running Title: Neuregulins increase muscle oxidative capacity

Carles Cantó, PhD, Sara Pich, PhD, José C. Paz, Rosario Sanches, Vicente Martínez, Meritxell Orpinell, PhD, Manuel Palacín, PhD, Antonio Zorzano, PhD, Anna Gumà, PhD

Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona and Institute of Research in Biomedicine, Parc Científic de Barcelona, E-08028 Barcelona, Spain.

$ To whom correspondence should be addressed:
Anna Gumà, e-mail: aguma@ub.edu
Antonio Zorzano, e-mail: azorzano@pcb.ub.es

For editorial communications, please send correspondence to:
Anna Gumà
Dept. Bioquímica i Biologia Molecular
Facultat de Biologia, Universitat de Barcelona
Avda. Diagonal, 645
E-08028-Barcelona, Spain

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ABSTRACT

Objective. Neuregulins are growth factors that are essential for myogenesis which also regulate muscle metabolism. The addition of a recombinant neuregulin-1 isoform, heregulin-β1\textsubscript{177-244} (Hrg), containing the bioactive EGF-like domain, (3 nM) to developing L6E9 myocytes has acute and chronic effects on glucose uptake and enhances myogenesis. Here we studied metabolic adaptation of L6E9 to chronic treatments with Hrg.

Research Design and Methods. L6E9 and C2C12 myocytes were chronically treated with low concentrations of Hrg (3 pM) which do not induce myogenesis. We analyzed the effects of Hrg on cellular oxidative metabolism and insulin sensitivity, and explored the mechanisms of action.

Results. Hrg increased the cell content of GLUT4 without affecting basal glucose uptake. Glucose and palmitate oxidation increased in Hrg-treated cells while lactate release decreased. Hrg increased the abundance of oxidative phosphorylation (OXPHOS) subunits, enhanced mitochondrial membrane potential and induced the expression of PGC-1\textalpha\ and PPARδ. Furthermore, we identified PPARδ as an essential mediator of the stimulatory effects of Hrg on the expression of OXPHOS subunits. The higher oxidative capacity of L6E9 myotubes after neuregulin treatment also paralleled an increase in insulin sensitivity and insulin signaling potency.

Conclusions. These results indicate that neuregulins act as key modulators of oxidative capacity and insulin sensitivity in muscle cells.
Neuregulins are members of the EGF (Epidermal Growth Factor) family, encoded by 4 different genes (neuregulin-1 to -4), that render multiple isoforms by alternative splicing of the transcripts (1). They are key to skeletal muscle development (2; 3) and they modulate muscle metabolism by inducing glucose uptake, independently of insulin (4), and by regulating glucose transporters expression (5). Recent data show that neuregulin receptors are activated during electrically-stimulated contraction and endurance training in skeletal muscle (6; 7) and that anti-neuregulin receptor-blocking antibodies impair contraction-induced glucose uptake (7).

One of the main adaptations of skeletal muscle to endurance training is an increase in oxidative capacity by stimulation of mitochondrial biogenesis (8). This complex process involves cooperation between nuclear and mitochondrial genomes (see (9) for review), which increases cell mitochondrial content. This, in turn, improves endurance performance by increasing the expression of components of the OXPHOS system (8) and of enzymes involved in fatty-acid oxidation (10), among others. The effects of endurance training on mitochondrial biogenesis can be mimicked by electrical stimulation of the motor nerve (11) or by increased cytosolic calcium in L6 cells (12).

Exercise and agents that increase cytosolic calcium induce mitochondrial biogenesis by similar mechanisms. In initial steps, the increase in cytosolic calcium induced by muscle contraction leads to the activation of calcium-dependent kinases, and CAMKII (Calcium/calmodulin-dependent protein kinase II) plays an essential role in calcium-induced mitochondrial biogenesis in L6 cells (12). Other kinases, such as common and novel PKCs (protein kinase C) and MAPKs (Mitogen-activated protein kinases), are also activated by increased intracellular calcium concentrations (see (13) for review), though their role is not fully understood. In subsequent steps, exercise leads to the induction of proteins that control the transcriptional regulation of the OXPHOS system as well as the transcription and replication of mitochondrial DNA. Among these, the transcriptional co-activator PGC-1α (Peroxisome proliferator-activated receptor (PPAR) γ co-activator) drives and coordinates initial steps in the regulation of mitochondrial biogenesis (for review, see (14)). PGC-1α is expressed mostly in tissues that have high energy demands, such as skeletal muscle, heart, liver, brain or brown fat, and is highly induced under conditions of energy requirement (15). Over-expression of PGC-1α stimulates mitochondrial biogenesis in C2C12 myocytes, 3T3 adipocytes (15; 16) and neonatal cardiac myocytes (17). PGC-1α action involves interaction and/or induction of the expression of several transcription factors apart from PPARγ, such as NRF-1 (Nuclear respiratory factor-1), NRF-2, ERRα (Estrogen-related receptor α), MEF2 (Myocyte enhancer factor 2), PPARα and PPARδ. These, in turn, regulate the expression of many enzymes related to lipid oxidation, subunits of the mitochondrial OXPHOS system and GLUT4 glucose transporters, among others (14). Furthermore, expression of PGC-1α under the control of muscle creatine-kinase promoter leads to a conversion of the fiber composition of skeletal muscle, and to type-II fibers acquiring type-I fiber phenotype (18). Similar observations were reported on transgenic mice over-expressing PPARδ (19), which is the predominant PPAR isoform present in skeletal muscle.
In L6 myocytes, a rise in cytosolic calcium leads to an increase in the expression of PGC-1α, mTFA (Mitochondrial transcription factor A), NRF-1 and NRF-2 (12), and induces mitochondrial biogenesis (20) and GLUT4 expression (21). These observations indicate that increases in cytosolic calcium trigger metabolic adaptations induced by exercise and endurance training in skeletal muscle.

Since exercise, muscle contraction or increases in cytosolic calcium trigger the release of neuregulins in rat skeletal muscle, and we have previously shown that Hrg modulates the expression of GLUT4, we examined the chronic effects of Hrg on the metabolic properties of L6E9. Our results indicate that Hrg enhances oxidative metabolism and increases insulin sensitivity. Furthermore, we provide evidence that Hrg increases PGC-1α and PPARδ expression, and that the latter regulates the effects of Hrg on mitochondrial genes.

**RESEARCH DESIGN AND METHODS**

**Cells, reagents and materials**

The L6E9 rat skeletal muscle cell line was provided by Dr. B. Nadal-Ginard (Harvard University, Boston, MA). C2C12 cells overexpressing wild type (WT) or dominant negative (DN) forms of PPARδ were provided by Dr. P.A. Grimaldi (Université de Nice-Sophia Antipolis, Nice, France). Dulbecco’s Modified Eagle Medium (DMEM), glutamine and antibiotics were purchased from BioWhittaker (Walkersville, MD) and Fetal Bovine Serum (FBS) was obtained from Gibco (Invitrogen Ltd.). Purified porcine insulin was a gift from Eli Lilly Co. (Indianapolis, IN). Hrg was donated by Genentech, Inc. (South San Francisco, CA). Commonly used chemicals, along with caffeine, dantrolene, CCCP (carbonyl cyanide m-chlorophenylhydrazone) and anti-β-actin monoclonal antibody (MAb), were purchased from Sigma (St. Louis, MO). Anti-ErbB3 (Ab5) blocking MAb against ErbB3 ligand-binding domain and anti-neuregulin-1 extracellular domain (Ab1) or EGF-domain (Ab2) were purchased from Neomarkers (Fremont, CA). Anticaveolin-3 MAb was from Transduction Laboratories. Anti-PKCζ (C-20), anti-PCG-1α (K-15), anti-ErbB2 (C-18) and anti-ErbB3 (C-17) polyclonal antibodies (PAb) were from Santa Cruz Inc. (Santa Cruz, CA.). Anti-myosin heavy chain (MF-20) and anti-α1(Na+/K+)ATPase (α6F) MAb were from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). Anti-PKB, anti-phospho-Ser473-PKB, anti-phospho-Thr410-PKCζ, anti-insulin receptor (IR) β-chain and anti-IRS-1 (Insulin Receptor Substrate-1) PAbs were from Cell Signaling (Danvers, MA). Anti-phosphotyrosine (4G10) MAb and the anti-p85 subunit of phosphatidylinositol 3-kinase 3-subunit PAb were from Upstate Biotechnology Inc. (Charlottesville, VA). Anti-PPARδ and PPARα PAb were from Affinity Bioreagents (Golden, CO). PAb OSCRX (raised against the 15 C-terminal residues from GLUT4) was produced in our laboratory. Anti-GLUT1 PAb was from Abcam (Cambridge, UK). Anti-porin MAb was from Calbiochem (La Jolla, CA). Anti-cytochrome C MAb was from PharMingen (San Jose, CA). Anti-L-CPT-1 (liver isoform of Carnitine Palmityl Transferase 1, predominant in muscle cell cultures) PAbs were raised against amino acids 317-430 of the rat L-CPT-1, and were a gift from Dr. Carinne Prip-Buus (Université René Descartes, Paris). NAO (Nonyl acridine orange) and JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,1’-tetraethylbenzimidazolylcarbocyanine iodide) probes and antibodies against several subunits of the OXPHOS system were purchased from Molecular Probes (Eugene, OR). Molecular-weight markers were from Bio-Rad (Hercules, CA). BCA
Protein Assay Reagent Kit was from Pierce (Rockford, IL). Immobilon polyvinylidene difluoride (PVDF) membranes, ECL reagents, 2-Deoxy-D-\(^{3}\)H-glucose, D-[U-\(^{14}\)C]-glucose and [U-\(^{14}\)C]-palmitic acid were from Amersham Pharmacia Biotech (Buckinghamshire, UK). All chemicals were of the highest purity available.

**Cell culture**

L6E9 myoblasts were grown and induced to myotube formation, as described elsewhere (5). To analyze the chronic of neuregulins, we added 3 pM Hrg 24 hours after cells were changed to differentiation medium and studies were carried out 48 hours later. L6E9 were serum-starved for 4 hours before insulin treatments, which lasted 15 minutes to obtain total cell lysates or 30 minutes for 2-deoxyglucose uptake assays.

C2C12 were grown in a similar way to L6E9, but we used a differentiation medium consisting of DMEM supplemented with 5% Horse Serum, 1% (v/v) antibiotics (10,000 units/mL penicillin G and 10 mg/ml streptomycin), 2 mM glutamine and 25 mM HEPES (pH 7.4). Stable retrovirally infected C2C12 cells over-expressing WT PPAR\(\delta\) or dominant negative (DN) PPAR\(\delta\) (E411P) (22), were grown in the presence of 0.4 mg/ml geneticin.

**Preparation of extracts from L6E9 and C2C12 myocytes**

Total protein extracts from L6E9 and C2C12 were obtained as described elsewhere (4). Cellular fractions enriched in mitochondria were obtained by homogenizing L6E9 myotubes with a homogenization buffer (HB) (0.25 M sucrose, 1 mM EGTA, 10 mM Hepes pH 7.4 and freshly-added protease inhibitors: 0.2 mM PMSF, 1 \(\mu\)M leupeptin, 1 \(\mu\)M pepstatin and 1 U/ml aprotinin), then further homogenized with a glass homogenizer and a motor pestle. The homogenate was then centrifuged at 4500 rpm. for 10 minutes at 4\(^{\circ}\)C. The pellet was resuspended with HB and centrifuged again. The resulting pellet, the mitochondrial-enriched fraction, was resuspended in HB. Crude plasma membranes (PM) and low-density microsomal membranes (LDM) were obtained as reported elsewhere (5).

**Immunoprecipitation and immunoblotting**

Immunoprecipitation assays for IR, p85 subunit of the PI3K and PKC-\(\zeta\) were performed as described elsewhere (4). Protein samples containing Laemmli sample buffer (LSB) were subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted as described (5). Proteins were detected by the ECL method and quantified by scanning densitometry.

**Metabolic measurements**

2-Deoxyglucose uptake assays were performed as described elsewhere (5). Glucose and palmitate oxidation were measured in cells by incubation in Hank's balanced salt medium (2% FBS, 5 mM glucose) containing 0.32 \(\mu\)Ci D-[U-\(^{14}\)C]-glucose (306 mCi/mmol) for glucose oxidation, or 0.1 mM palmitate with 0.1 \(\mu\)Ci [U-\(^{14}\)C]-palmitic acid (60 mCi/mmol) for palmitate oxidation. Cells were incubated for 3 hours and \(^{14}\)CO\(_2\) was trapped and measured as described (23). A commercially available kit (Sigma-826-UV) was used to measure lactate release in 1 ml of incubation medium from cells incubated in the presence or absence of Hrg for 48 hours. Glycogen synthesis was measured as incorporation of glucose into glycogen, as described elsewhere (24). Protein was measured by the BCA method.

**Measurement of mitochondrial mass**

NAO is a metachromatic dye that specifically binds cardiolipin, an inner mitochondrial membrane lipid, regardless of the energetic state of the cell. Cells
were incubated with FBS-free medium containing NAO (100 ng / ml) for 30 minutes at 37ºC, then washed twice in PBS and finally trypsinized to measure mean cell fluorescence at 580 nm by flow cytometry.

**Measurement of mitochondrial membrane potential (JC-1)**

JC-1 is a cationic fluorescent dye (green as monomer; 539 nm) that accumulates in mitochondria in a potential-dependent manner. Its accumulation in mitochondria leads to the formation of red fluorescent aggregates (J-aggregates) (597 nm). The concentration of JC-1 green monomers in mitochondria increases in proportion to the membrane potential, and J-aggregates are formed when membrane potential exceeds −240 mV. Thus, mitochondrial membrane potential can be analyzed by determining the ratio of red vs. green fluorescence, independently of the number of mitochondria measured.

To this end, cells were incubated with 1 µM of JC-1 diluted in DMEM without FBS for 30 minutes at 37ºC. Cells were then washed three times in PBS before trypsinization. Mean green and red fluorescence was measured by flow cytometry. The accuracy and specificity of the JC-1 potentiometric method was validated using the mitochondrial uncoupler CCCP (10 µM) (not shown).

**RNA extraction and quantitative Real-Time PCR analysis**

Total RNA was isolated using Trizol Reagent, according to the manufacturer’s instructions (Invitrogen, CA). Reverse transcription was performed from 3 µg of total RNA at 42ºC for 90 min using Oligo dT and SuperScript II reverse transcriptase (Invitrogen, CA). First-strand cDNA was subjected to real-time PCR sequence detection. PCR products were quantified by measuring fluorescence from the progressive binding of SYBR green I dye to double-stranded DNA using the ABI Prism 7700 sequence detection system (Applied Biosystems). The relative quantification value of PCR transcripts was calculated by using the manufacturer’s protocol (comparative Ct method) with normalization to ARP (acidic ribosomal phosphoprotein P0) as endogenous control. The sets of primers used were as follows: PGC-1 (5’-AAAGAGGGCCCGGTACAGTGAGT-3’ and 5’-GGCCCTTTCTTGTTGGAGTGG-3’); ARP (5’-GAGCCAGCAGCCACACT-3’ and 5’-GATCAGCCCGAAGGAGAAGG-3’). Real-time PCR was performed with the following cycling parameters: activation at 95ºC for 10 min; PCR cycling, 40 cycles at 95ºC for 15 s (denaturation), and 60ºC for 1 min (annealing/extension).

**Neuregulin detection in incubation medium**

For the detection of released neuregulins in the incubation medium, 3 mL was concentrated 10x by centrifugation using centriicon. The concentrated medium was subjected to immunoprecipitation with an irrelevant antibody (anti-actin MAb) bound to sepharose beads to diminish unspecific binding. The resulting supernatant was incubated with a biotin-labeled MAb against neuregulins extracellular domain (Ab1) for 2 hours and then immunoprecipitated overnight using strepatavidin-agarose beads (Pierce, Rockford, IL). The resulting immunoprecipitate was resuspended in 50 µL of LSB and used for Western blot analysis. Neuregulins were detected using a PAb against their EGF-domain (Ab2).

**Statistical analysis**

Data are shown as mean ± standard error (SE). Significant differences at \( P < 0.05 \) were determined using unpaired Student’s \( t \) tests. For experiments with more than two groups, significant
differences were determined using ANOVA and Dunnet’s post-hoc test.

RESULTS

**Neuregulins increase oxidative metabolism.** To test the metabolic effects of neuregulins without affecting myogenesis, which occurs above 100 pM (recombinant neuregulin-α1) in L6 (3) or above 30 pM (Hrg) in L6E9 (not shown), we treated L6E9 with a lower concentration (3 pM) of Hrg for 48 hours. At this concentration, Hrg-treated cells did not display any change in myotube formation (Online-Only Appendix Fig. 1), in the protein levels of myogenic markers such as myosin heavy chain (MHC) and caveolin-3, or in the expression of neuregulin receptors, ErbB2 and ErbB3 (Fig. 1A and Online-Only Appendix Table 1). Chronic treatment with 3 pM Hrg increased GLUT4 levels, while GLUT1 levels were not significantly different from those of control cells (Fig. 1A and Online-Only Appendix Table 1). The increase in total GLUT4 levels was detected in intracellular LDM membranes, where this transporter is located in basal conditions, while abundance in plasma membrane fraction remained unaffected (Online-Only Appendix Table 1). Consequently, we observed no changes in basal glucose uptake between control and cells treated with 3 pM Hrg (Fig. 1B). However, Hrg treatment increased glucose (85%) and palmitate (89%) oxidation (Figs. 1C and 1D, respectively), while lactate release decreased (Fig. 1E). Under these circumstances, glycogen synthesis was similar in control and Hrg groups (Fig. 1F).

**Chronic neuregulin treatment increases mitochondrial activity.** To test whether the increase in substrate oxidation was due to changes in cellular mitochondrial content in L6E9, we measured the abundance of mitochondrial markers in total cell lysates. Cells treated with Hrg showed increases in total protein levels of porin, an outer-mitochondrial membrane protein, CPT-1, a regulatory step in fatty acids oxidation, and COX-I, a subunit of OXPHOS complex IV that is encoded by mitochondrial DNA (Fig. 2A). NAO staining confirmed the increase in mitochondrial content induced by Hrg (Fig. 2B). Immunofluorescence confocal microscopy using COX-I antibody showed that Hrg treatment did not alter the mitochondrial network architecture of L6E9 (not shown).

The increase in COX-I induced by Hrg was proportionally higher than that of porin or CPT-I. To test whether mitochondria were enriched in OXPHOS complexes in Hrg-treated cells compared to controls, we analyzed the abundance of different OXPHOS complex subunits in mitochondrial fractions from L6E9 incubated with Hrg for 48 hours. Hrg increased the abundance of all OXPHOS complexes in mitochondria, while porin and cytochrome C content per mg of mitochondrial protein was similar (Fig. 3A, Online-Only Appendix Table 2). Furthermore, mitochondrial membrane potential, measured with JC-1 staining, was increased in Hrg-treated cells (Fig. 3B). These data indicate that Hrg increased not only mitochondrial content in muscle cells, but also the oxidative capacity of mitochondria.

**Neuregulins stimulate PGC-1α and PPARδ expression.** To explore how Hrg increased mitochondrial content, we analyzed whether Hrg affected PGC-1α expression, since this protein is a master regulator of mitochondrial biogenesis in skeletal muscle. Chronic treatment with Hrg promoted an 82% increase in PGC-1α protein levels (Fig. 4A) and a 3-fold increase in PGC-1α RNA expression (Fig. 4B). Next, we determined whether Hrg affects the expression of PPARα or PPARδ. These two members of the PPAR family regulate lipid metabolism
and oxidative metabolism in skeletal muscle and are coactivated by PGC-1α (see (25-28) for reviews). As previously reported, no protein expression of PPARα was detected in L6E9 (29; 30) in control or in Hrg-treated cells. In contrast, there was a clear increase in PPARδ protein levels in response to Hrg in L6E9 (Fig. 4C).

**PPARδ mediates neuregulin-induced increases in mitochondrial proteins.** To test the role of PPARδ in the effects of neuregulin on mitochondrial genes, we used stable retrovirally infected WT or DN C2C12 cells (22). Tranfected cells did not show any difference in the levels of late myogenic markers, caveolin 3 and MHC (Online-Only Appendix Fig. 2). As in L6E9, Hrg increased the protein levels of mitochondrial markers, subunits of OXPHOS complexes and PPARδ in control C2C12 cells (Fig. 5). While WT PPARδ only showed a tendency to potentiate Hrg effects on the protein levels of the mitochondrial markers tested, DN PPARδ completely abolished Hrg effects on OXPHOS complexes and porin protein levels (Fig. 5). Moreover, Hrg did not stimulate mitochondrial membrane potential, measured with the JC-1 probe, in the DN PPARδ cells (Online-Only Appendix Fig. 3). These results clearly implicate PPARδ in the effects of neuregulin on mitochondrial activity. In contrast, Hrg did not affect total PPARγ levels in untransfected, WT or DN C2C12 cells, although impairment of PPARδ activity decreased PPARγ levels (Online-Only Appendix Fig. 4). The increase in mitochondrial protein content induced by Hrg in L6E9 or C2C12 cells was not affected by inhibition of PPARγ using the antagonist compound, GW-9662 (1 µM) (Online-Only Appendix Fig. 5).

**Neuregulins increase insulin sensitivity.** Given that the oxidative capacity of skeletal muscle correlates with insulin sensitivity (31; 32), we tested whether chronic treatment with Hrg also increased insulin sensitivity. We examined glucose uptake at a range of insulin concentrations in cells pre-treated or untreated for 48 hours with 3 pM of Hrg. Hrg increased insulin sensitivity by almost one order of magnitude, but, although higher total GLUT4 levels were observed in Hrg-treated cells, maximal insulin response was not altered (Fig. 6A). The increase in insulin sensitivity was also observed in GLUT4 recruitment at the plasma membrane (Fig. 6B). To test whether increase in insulin sensitivity was due to increased activation of insulin signaling effectors, we measured the abundance and response of several insulin mediators of glucose uptake, such as the IR, IRS-1, p85 subunit of PI3K, PKB and PKCζ, at submaximal insulin concentration (100 nM) in L6E9 chronically pre-treated or not with Hrg. Hrg-treated cells increased expression of IR, IRS-1, p85 regulatory subunit of PI3K, PKB and PKCζ (Fig. 6C and Online-Only Appendix Table 3A) and also enhanced insulin-stimulated activation of these proteins (Fig. 6D and Online-Only Appendix Table 3B).

Next, we examined whether the effect of Hrg on insulin sensitivity was dependent on PPARδ, using DN C2C12 cells. Since insulin does not stimulate glucose transport in C2C12 cells due to the low expression of GLUT4, we analyzed Hrg effects on insulin signaling (Online-Only Appendix Fig. 6). Treatment with Hrg in untransfected control cells increased insulin-stimulated IRS-1 tyrosine-phosphorylation, IRS-1 binding to the p85 subunit of the PI3K and Ser473-PKB phosphorylation, at a submaximal insulin concentration (10 nM). These Hrg effects were abrogated in PPARδ DN C2C12 cells, indicating that Hrg required PPARδ to increase insulin sensitivity. In contrast with the observations made in
L6E9, Hrg did not increase total IR protein or insulin-stimulated IR tyrosine-phosphorylation in C2C12 cells. Hrg treatment did not affect the total protein levels of the p85 subunit of PI3K, although Hrg increased p85 binding to IRS-1 in response to insulin (Online-Only Appendix Fig. 6).

Increases in cytosolic calcium regulate PGC-1α levels through neuregulin action. All the effects reported above elicited by chronic Hrg treatment are similar to those observed in skeletal muscle in response to endurance exercise. In L6, increases in cytosolic calcium induced by caffeine mimic the effects of exercise on PGC-1α and mitochondrial biogenesis (12; 20). Interestingly, muscle contraction releases neuregulins through a calcium-regulated pathway (7). Thus, to explore whether neuregulins mediated calcium-induced effects on mitochondrial biogenesis, first we analyzed caffeine effects on neuregulins release and ErbB3 activation in L6E9 myotubes (Fig. 7A). Incubation of L6E9 with caffeine for 3 hours led to neuregulins release and consequent increase in ErbB3 tyrosine-phosphorylation. Blockage by dantrolene of calcium release from the sarcoplasmatic reticulum prevented caffeine action on both effects (Fig. 7A), indicating that, similar to what has been reported for incubated skeletal muscle, increased cytosolic calcium induces neuregulin activity. Next, we tested whether blockage of neuregulins action affected caffeine-induced increases in PGC-1α levels. Caffeine treatment for 16 hours induced PGC-1α protein levels in differentiated myotubes (Fig. 7B), and both dantrolene and ErbB3 blocking antibodies prevented those effects. The effect of Hrg on PGC-1α expression was not blocked by dantrolene, indicating that neuregulins are the main pathway by which increases in cytosolic calcium modulate PGC-1α protein levels. Incubations with dantrolene or anti-ErbB3 receptor-blocking antibodies for periods that would be long enough to reveal changes in mitochondrial proteins were not possible since these treatments compromised cell differentiation and viability. Taken together, these results implicate neuregulins in the metabolic adaptations of the oxidative metabolism that occur in skeletal muscle in response to contraction.

DISCUSSION

Our study demonstrates that the sustained action of Hrg at low concentrations increases oxidative metabolism, mitochondrial cell content and expression of OXPHOS subunits by a mechanism dependent on PPARδ. Moreover, Hrg markedly enhances insulin sensitivity in muscle cells. Based on this global pattern and on the observation that neuregulins are released from muscle during contraction, we conclude that neuregulins are crucial mediators of the adaptive metabolic responses of skeletal muscle to contraction. Hrg effects promoting mitochondrial activity confirm recent data on isolated cardiomyocytes, showing that neuregulins promote reprogramming that leads to increased expression of many OXPHOS- and β-oxidation-related genes (33), and that inhibition of ErbB2 causes mitochondrial dysfunction, involving decreased oxidative capacity (34).

Contractile activity also stimulates mitochondrial biogenesis. This effect could be generated by contractile activity mediators such as increased cytosolic calcium and activated AMPK (AMP-activated protein kinase). In L6 cells there is little evidence for the involvement of AMPK in mitochondrial biogenesis (20), although several experimental in vivo approaches strongly suggest that AMPK is involved in the regulation of mitochondrial biogenesis in skeletal muscle (35; 36). We did not detect any effect of Hrg on total or
phosphorylated AMPK (Online-Only Appendix Fig. 7). However, there is evidence that intermittent increases in cytosolic calcium induce mitochondrial biogenesis in L6 by increased expression of the co-activator PGC-1α (12; 20). Since neuregulins are released in skeletal muscle in response to contractile activity (6; 7) and by increases in cytosolic calcium (7), we hypothesized that these growth factors were involved in calcium-induced metabolic changes in skeletal muscle. Indeed, the stimulation of L6 myotubes with caffeine increases PGC-1α expression (12; 20), but this effect is completely blunted in L6E9 when caffeine-induced ErbB3 activation is blocked.

This is the first study providing evidence that neuregulins induce PGC-1α and PPARδ expression. The latter is necessary for neuregulins to increase cellular mitochondrial content. The study confirms previous reports indicating that PPARδ is a key element in the regulation of oxidative metabolism in skeletal muscle (19; 37-39). However, our results do not rule out the need for other transcription factors in neuregulin action in mitochondrial gene expression such as GABP-α and -β (their human heteromeric binding form is also known as NRF-2), whose transcriptional activity is increased by neuregulins in muscle cells (40).

More than twenty years ago, it was reported that oxidative slow-twitch muscle fibers are more sensitive to insulin (31). One of the effects of exercise training is the modulation of the skeletal muscle phenotype, through the acquisition of “slow-twitch” characteristics, such as an increase in mitochondrial oxidative enzymes and insulin sensitivity (8-10; 41). PGC-1α and PPARδ may be crucial in regulating these transitions, since transgenic mice expressing PGC-1α or PPARδ at physiological levels display putative type-II fibers with type-I phenotype (18; 19). Interestingly, exercise increases the expression of both genes (38; 42), whereas in several insulin-resistant muscle conditions, PGC-1α and other genes involved in OXPHOS are down-regulated (43-45). Our results indicate a parallelism between increased insulin sensitivity and enhanced mitochondrial metabolism as a consequence of chronic exposure to Hrg in skeletal muscle cells, and they are consistent with a role of mitochondrial metabolism in insulin sensitivity in muscle tissue (46; 47). Since impaired mitochondrial activity might contribute to the pathogenesis of insulin resistance in skeletal muscle (see (48) for review), further studies should analyze whether neuregulins can improve muscle metabolic alterations associated with insulin-resistant states.

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REFERENCES

10. Mole PA, Oscai LB, Holloszy JO: Adaptation of muscle to exercise. Increase in levels of palmitoyl CoA synthetase, carnitine palmitoyltransferase, and palmitoyl CoA dehydrogenase, and in the capacity to oxidize fatty acids. *J Clin Invest* 50:2323-2330, 1971


Figure legends.

Fig. 1. Neuregulins increase oxidative metabolism. L6E9 cells were treated with 3 pM Hrg for 48 h. (A) Total cell lysates (10-40 µg) were used for Western blot analysis of the proteins indicated. Representative images from 3-10 experiments are shown. (B-F) 2-Deoxyglucose uptake (B), glucose oxidation (C), palmitate oxidation (D), lactate release (E) and glycogen synthesis (F) assays were performed as described in Methods. Results are mean ± SE of 3-12 experiments. * Significant difference vs. control (C) group at P <0.01.

Fig. 2. Neuregulins increase mitochondrial content. L6E9 cells were treated with 3 pM Hrg for 48 h. (A) Detection of porin, CPT-1 and COX-I content in total cell lysates (20 µg) by Western blot. Representative images and densitometries (mean values ± SE, normalized using β-actin as loading control protein) from 5 experiments are shown. (B) Analysis of relative mitochondrial mass using NAO dye staining. Results shown are relative values (mean ± SE) from 6 experiments. * Significant difference vs. control (C) group at P < 0.05.

Fig. 3. Neuregulins increase the expression of subunits of the OXPHOS system and the mitochondrial membrane potential. L6E9 cells were treated with 3 pM Hrg for 48 h. (A) Western blot analysis of the proteins indicated in mitochondrial fractions (20 µg). Representative images from three experiments are shown. (B) Measurement of the relative mitochondrial membrane potential using the JC-1 potentiometric dye. Results are shown as mean ± SE from 12 experiments. * Significant difference vs. control (C) group at P < 0.01.

Fig. 4. Neuregulins increase PGC-1α and PPARδ levels. L6E9 cells were treated with 3 pM Hrg for 48 hours. (A) Detection of PGC-1α protein in total cell lysates (120 µg) by Western-blot. A representative image and the relative densitometries (relative to β-actin content) from 9 experiments (mean ± SE) are shown. Results were confirmed by immunoprecipitation assays (not shown). (B) Relative quantification of PGC-1α RNA expression by Real-time PCR. Results from 3 experiments are shown as mean ± SE. (C) Detection by Western blot of PPARα and PPARδ protein levels in total cell lysates (100 µg). Control of loaded protein: α1(Na+/K+)ATPase (10 µg). Total lysates (100 µg) from gastrocnemius muscle were used as a positive control for PPARα detection. Representative images from 3-6 experiments are shown. * Significant difference vs. control (C) group at P < 0.01.

Fig. 5. PPARδ mediates neuregulin effects on mitochondrial protein levels. C2C12 control cells (-), cells over-expressing WT PPARδ or DN PPARδ were treated with 3 pM Hrg for 48 hours. Total cell lysates (20-80 µg) were used for Western blot analysis. Representative images and densitometries (mean ± SE, normalized using β-actin as loading control protein) from 3 experiments are shown. * Significant difference vs. untreated cells group (- Hrg) at P < 0.05.

Fig. 6. Neuregulins increase insulin sensitivity. L6E9 cells were treated with 3 pM Hrg for 48 h. (A) Results of glucose uptake for insulin concentration-response assays are shown as the mean ± SE of 4 experiments. * Significant difference between control (C; black triangles, continuous line) and neuregulin-treated (black circles, discontinuous line) groups at P < 0.01. Western blot of GLUT4 was done using 10 µg of plasma
membranes (PM) and representative images from 3 experiments are shown. **(B)** Western blots of indicated proteins were done using 40 µg of total cell lysates. Representative images from 3 experiments are shown. **(C)** Analysis of Hrg action on the submaximal insulin concentration effects (10 min for InsR or 15 minutes for others) regulating phosphorylation of InsR, PKB (Ser473) and PKCζ (Thr410), as well as on IRS-1 association to the p85 subunit of PI3K. Representative images from 3 experiments are shown.

**Fig 7. Neuregulin involvement in caffeine effects on PGC-1α.** L6E9 myotubes were incubated with dantrolene (10 µM) or ErbB3 blocking antibodies (10 µg/ml) for 30 minutes previous to caffeine (5 mM) or caffeine plus Hrg 3 pM treatments for 3 hours. **(A)** ErbB3 phosphorylation, in total cell lysates, and released neuregulins, in incubation medium, were analyzed by Western blot assays. **(B)** After indicated treatments, cells were washed in PBS and left for 15 hours with differentiation medium supplemented with dantrolene or anti-ErbB3 antibodies in the corresponding groups. PGC-1α was analyzed in total cell lysates by Western blot. Representative images from 3 experiments are shown.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
A) p-ErbB3

Neuregulin release

- 185 kDa
- 45 kDa

- + - + - + - + Anti-ErbB3
- - + + - - - + Dantrolene

Control Caff Caff + Hrg

B) PGC-1α

α1 (Na+/K+) ATPase

- 90 kDa
- 100 kDa

- + - + - + - + Anti-ErbB3
- - + + - - - + Dantrolene

Control Caff Caff + Hrg

Fig. 7