Original Article

Neuregulins Increase Mitochondrial Oxidative Capacity and Insulin Sensitivity in Skeletal Muscle Cells

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OBJECTIVE—Neuregulins are growth factors that are essential for myogenesis and regulate muscle metabolism. The addition of a recombinant neuregulin-1 isoform, heregulin-$\beta1_{177–244}$ (Hrg), containing 3 nmol/l of the bioactive epidermal growth factor–like domain, to developing L6E9 myocytes has acute and chronic effects on glucose uptake and enhances myogenesis. Here, we studied the metabolic adaptation of myocytes to chronic treatments with Hrg.

RESEARCH DESIGN AND METHODS—L6E9 and C2C12 myocytes were chronically treated with low concentrations of Hrg (3 pmol/l) that 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, whereas lactate release decreased. Hrg increased the abundance of oxidative phosphorylation (OXPHOS) subunits, enhanced mitochondrial membrane potential, and induced the expression of peroxisome proliferator–activated receptor (PPAR)γ coactivator1α 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. ***Diabetes*** **56:2185–2193, 2007**

Neuregulins are members of the epidermal growth factor (EGF) family, encoded by four different genes (*neuregulin-1* through *-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 transporter 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, involving cooperation between nuclear and mitochondrial genomes (rev. in 9), increases cell mitochondrial content. This, in turn, improves endurance performance by increasing the expression of components of the oxidative phosphorylation (OXPHOS) system (8) and 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 through similar mechanisms. In initial steps, the increase in cytosolic calcium induced by muscle contraction leads to the activation of calcium-dependent kinases, and calcium/calmodulin-dependent protein kinase II plays an essential role in calcium-induced mitochondrial biogenesis in L6 cells (12). Other kinases, such as the common and novel protein kinase C (PKC) and mitogen-activated protein kinase, are also activated by increased intracellular calcium concentrations (rev. in 13), although 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 and the transcription and replication of mitochondrial DNA. Among these, the transcriptional coactivator peroxisome proliferator–activated receptor γ coactivator (PGC)-1α drives and coordinates the initial steps in the regulation of mitochondrial biogenesis (rev. in 14). PGC-1α is mostly expressed 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). Overexpression of PGC-1α stimulates mitochondrial biogenesis in C2C12

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AMPK, AMP-activated protein kinase; COX, cytochrome c oxidase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FBS, fetal bovine serum; Hrg, heregulin-$\beta1_{177–244}$; IRS, insulin receptor substrate; mAb, monoclonal antibody; NOA, nonyl acridine orange; NRF, nuclear respiratory factor; OXPHOS, oxidative phosphorylation; pAb, polyclonal antibody; PGC peroxisome proliferator–activated receptor γ coactivator; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PPAR, peroxisome proliferator–activated receptor.

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 peroxisome proliferator–activated receptor (PPAR) γ, such as nuclear respiratory factor (NRF)-1 and -2, estrogen-related receptor-α, 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 in transgenic mice overexpressing 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, mitochondrial transcription factor A, and NRF-1 and -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.

Because exercise, muscle contraction, or increases in cytosolic calcium trigger the release of neuregulins in rat skeletal muscle, and we have previously shown that heregulin-$\beta1_{177–244}$ (Hrg) modulates the expression of GLUT4, we examined the chronic effects of Hrg on the metabolic properties of L6E9 cells. 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. Nadal-Ginard (Harvard University, Boston, MA). C2C12 cells overexpressing wild-type or dominant-negative forms of PPARδ were provided by Dr. Grimaldi (Université de Nice-Sophia Antipolis, Nice, France). Dulbecco's modified Eagle's medium (DMEM), glutamine, and antibiotics were purchased from BioWhittaker (Walkersville, MD), and fetal bovine serum (FBS) was obtained from Gibco (Invitrogen). Purified porcine insulin was a gift from Eli Lilly (Indianapolis, IN), and Hrg was donated by Genentech (San Francisco, CA). Commonly used chemicals, along with caffeine, dantrolene, 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). Anti–caveolin-3 mAb was purchased from Transduction Laboratories. Anti-PKCζ (C-20), anti–PGC-1α (K-15), anti-ErbB2 (C-18), and anti-ErbB3 (C-17) polyclonal antibodies (pAbs) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–myosin heavy chain (MF-20) and anti–$\alpha_1(Na^+/K^+)$ ATPase (α6F) mAbs were from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti–protein kinase B (PKB), anti–phospho-Ser^{473} PKB, anti–phospho-Thr^{410}-PKCζ, anti–insulin receptor β-chain, and anti–insulin receptor substrate (IRS)-1 pAbs were from Cell Signaling (Danvers, MA). Anti-phosphotyrosine (4G10) mAb and the anti-p85 subunit of phosphatidylinositol 3-kinase (PI3K) subunit pAb were from Upstate Biotechnology (Charlottesville, VA). Anti-PPARδ and -PPARα pAbs were from Affinity Bioreagents (Golden, CO). pAb anti-GLUT4 antibody (raised against the 15 COOH-terminal residues from GLUT4) was produced in our laboratory. Anti-GLUT1 pAb was from Abcam (Cambridge, U.K.). 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 palmitoyl transferase 1, which are predominant in muscle cell cultures) pAbs were raised against amino acids 314–430 of the rat L-CPT-1 and were a gift from Dr. Prip-Buus (Université René Descartes, Paris, France). Nonyl acridine orange (NAO) 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), and the BCA Protein Assay Reagent Kit was from Pierce (Rockford, IL). Immobilon polyvinylidene difluoride membranes, enhanced chemiluminescence reagents, 2-deoxy-D-[^{3}H]glucose, D-[U-^{14}C]glucose, and [U-^{14}C]palmitic acid were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). 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 effects of neuregulins, we added 3 pmol/l Hrg 24 h after cells were changed to differentiation medium, and studies were carried out 48 h later. L6E9 cells were serum starved for 4 h before insulin treatments, which lasted 15 min to obtain total cell lysates or 30 min for 2-deoxyglucose uptake assays.

C2C12 cells were grown in a similar way as L6E9 cells, except that we used a differentiation medium consisting of DMEM supplemented with 5% horse serum, 1% (vol/vol) antibiotics (10,000 units/ml penicillin G and 10 mg/ml streptomycin), 2 mmol/l glutamine, and 25 mmol/l HEPES (pH 7.4). Stable retrovirally infected C2C12 cells overexpressing wild-type or dominant-negative PPARδ (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 cells were obtained as described elsewhere (4). Cellular fractions enriched in mitochondria were obtained by homogenizing L6E9 myotubes with a homogenization buffer (0.25 mol/l sucrose, 1 mmol/l EGTA, 10 mmol/l HEPES [pH 7.4], and freshly added protease inhibitors [0.2 mmol/l phenylmethylsulfonyl fluoride, 1 μmol/l leupeptin, 1 μmol/l pepstatin, and 1 unit/ml aprotinin]) and then further homogenized with a glass homogenizer and a motor pestle. The homogenate was then centrifuged at 4,500 rpm for 10 min at 4°C. The pellet was resuspended with homogenization buffer and centrifuged again. The resulting pellet, the mitochondrial-enriched fraction, was resuspended in homogenization buffer. Crude plasma membranes and low-density microsomal membranes were obtained as reported elsewhere (5).

Immunoprecipitation and immunoblotting. Immunoprecipitation assays for the insulin receptor, p85 subunit of the PI3K and PKC-ζ, were performed as described elsewhere (4). Protein samples containing Laemmli sample buffer were subjected to SDS-PAGE, transferred to polyvinylidene difluorid membranes, and immunoblotted as described (5). Proteins were detected by the enhanced chemiluminescence 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 mmol/l glucose) containing 0.32 μCi D-[U-^{14}C]glucose (306 mCi/mmol) for glucose oxidation or 0.1 mmol/l palmitate with 0.1 μCi [U-^{14}C]palmitic acid (60 mCi/mmol) for palmitate oxidation. Cells were incubated for 3 h, 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 incubation medium from cells incubated in the presence or absence of Hrg for 48 h. Glycogen synthesis was measured as the incorporation of glucose into glycogen, as described elsewhere (24). Protein was measured by the bicinchoninic 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 min at 37°C, washed twice in PBS, and trypsinized to measure mean cell fluorescence at 580 nm by flow cytometry.

Measurement of mitochondrial membrane potential. 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 (597 nm). The concentration of JC-1 green monomers in the mitochondria increases in proportion to the membrane potential, and red fluorescent aggregates are formed when membrane potential exceeds −240 mV. Thus, mitochondrial membrane potential can be analyzed by determining the ratio of red versus green fluorescence, independently of the number of mitochondria measured. To this end, cells were incubated with 1 μmol/l JC-1 diluted in DMEM without FBS for 30 min at 37°C. 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 (carbonyl cyanide *m*-chlorophenylhydrazone) (10 μmol/l) (not shown).

RNA extraction and quantitative real-time PCR analysis. Total RNA was isolated using Trizol Reagent according to the manufacturers' instructions (Invitrogen, Carlsbad, CA). Reverse transcription was performed from 3 μg total RNA at 42°C for 90 min using Oligo dT and SuperScript II reverse transcriptase (Invitrogen). First-strand cDNA was subjected to real-time PCR

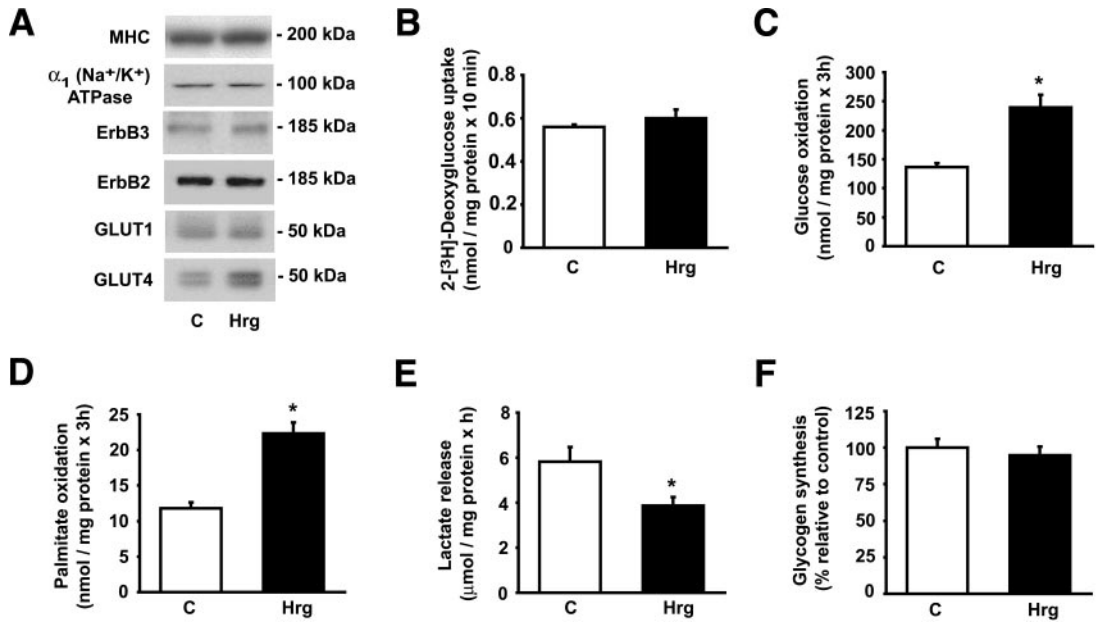

FIG. 1. Neuregulins increase oxidative metabolism. L6E9 cells were treated with 3 pmol/l Hrg for 48 h. *A*: Total cell lysates (10–40 μg) were used for Western blot analysis of the proteins indicated. Representative images from 3–10 experiments are shown. 2-deoxyglucose uptake (*B*), glucose oxidation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

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 using the manufacturer's protocol (comparative threshold cycles method) with normalization to ARP (acidic ribosomal phosphoprotein P0) as endogenous control. The sets of primers used were as follows: PGC-1 (5′-AAAGAGGGCCCGGTA CAGTGAGT-3′ and 5′-GGCCCTTTCTTGGTGGAGTGG-3′) and ARP (5′-GAGC CAGCGAAGCCACACT-3′ and 5′-GATCAGCCCGAAGGAGAAGG-3′). Real-time PCR was performed with the following cycling parameters: activation at 95°C for 10 min, PCR cycling at 40 cycles at 95°C for 15 s (denaturation), and annealing/extension at 60°C for 1 min.

Neuregulin detection in incubation medium. For the detection of released neuregulins in the incubation medium, 3 ml was concentrated ×10 by centrifugation using centricon. 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 h and then immunoprecipitated overnight using strepatavidin-agarose beads (Pierce, Rockford, IL). The resulting immunoprecipitate was resuspended in 50 μl Laemmli sample buffer and used for Western blot analysis. Neuregulins were detected using a pAb against their EGF domain (Ab2).

Statistical analysis. Data are shown as means ± 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 at >100 pmol/l (recombinant neuregulin-α1) in L6 (3) or >30 pmol/l (Hrg) in L6E9 (not shown), we treated L6E9 cells with a lower concentration (3 pmol/l) of Hrg for 48 h. At this concentration, Hrg-treated cells did not display any change in myotube formation (online appendix Fig. 1 [available at http://dx.doi.org/10.2337/db06-1726]) in the protein levels of myogenic markers, such as myosin heavy chain and caveolin-3, or in the expression of neuregulin receptors ErbB2 and ErbB3 (Fig. 1*A* and online appendix Table). Chronic treatment with 3 pmol/l Hrg increased GLUT4 levels, while GLUT1 levels were not significantly different from those of control cells (Fig. 1*A* and online appendix Table 1). The increase in total GLUT4 levels was detected in intracellular low-density microsomal membranes, where this transporter is located in basal conditions, whereas abundance in plasma membrane fraction remained unaffected (online appendix Table 1). Consequently, we observed no changes in basal glucose uptake between control cells and cells treated with 3 pmol/l Hrg (Fig. 1*B*). However, Hrg treatment increased glucose (85%) and palmitate (89%) oxidation (Fig. 1*C* and *D*, respectively), whereas lactate release decreased (Fig. 1*E*). Under these circumstances, glycogen synthesis was similar in control and Hrg groups (Fig. 1*F*).

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 acid oxidation, and cytochrome c oxidase (COX)-I, a subunit of OXPHOS complex IV that is encoded by mitochondrial DNA (Fig. 2*A*). NAO staining confirmed the increase in mitochondrial content induced by Hrg (Fig. 2*B*). Immunofluorescence confocal microscopy using COX-I antibody showed that Hrg treatment did not alter the mitochondrial network architecture of L6E9 cells (not shown).

The increase in COX-I induced by Hrg was proportionally higher than that of porin or CPT-1. To test whether mitochondria were enriched in OXPHOS complexes in Hrg-treated cells compared with controls, we analyzed the abundance of different OXPHOS complex subunits in mitochondrial fractions from L6E9 cells incubated with Hrg for 48 h. Hrg increased the abundance of all OXPHOS complexes in mitochondria, while porin and cytochrome c content per milligram of mitochondrial protein was similar (Fig. 3*A* and online appendix Table 2). Furthermore,

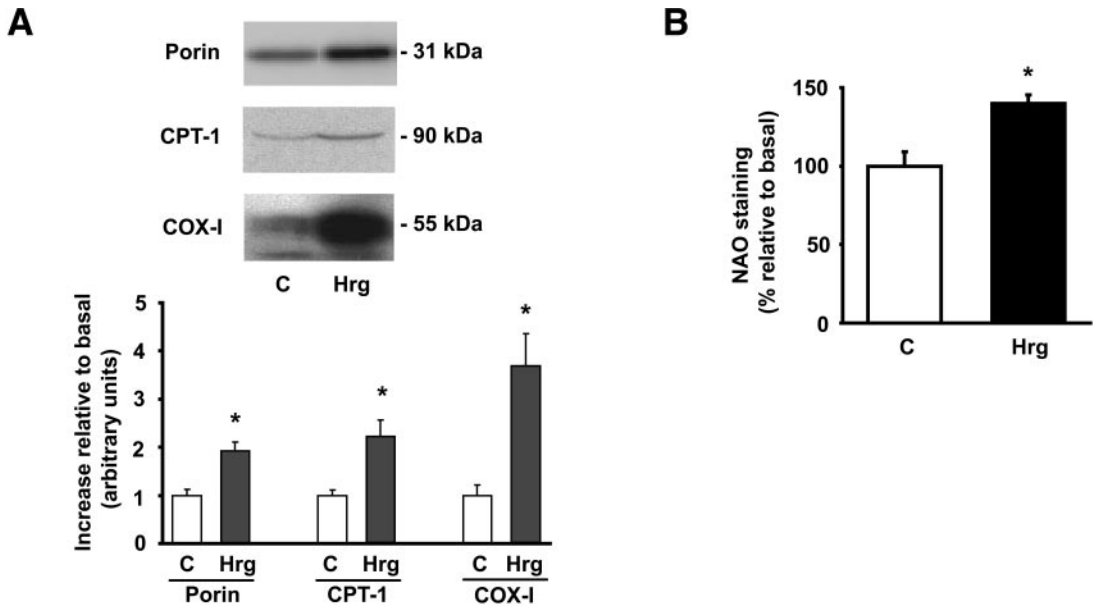

FIG. 2. Neuregulins increase mitochondrial content. L6E9 cells were treated with 3 pmol/l Hrg for 48 h. *A*: Detection of porin, CPT-1, and COX-I content in total cell lysates (20 μg) by Western blot. Representative images and densitometries (mean values ± SE, normalized using β-actin as loading control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

mitochondrial membrane potential, measured with JC-1 staining, was increased in Hrg-treated cells (Fig. 3*B*). 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. 4*A*) and a threefold increase in PGC-1α RNA expression (Fig. 4*B*). Next, we determined whether Hrg affects the expression of PPARα or -δ. These two members of the PPAR family regulate lipid metabolism and oxidative metabolism in skeletal muscle and are coactivated by PGC-1α (rev. in 25–28). As previously reported (29,30), no protein expression of PPARα was detected in L6E9 cells in control or in Hrg-treated cells. In contrast, there was a clear increase in PPARδ protein levels in response to Hrg in L6E9 cells (Fig. 4*C*)

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 wild-type or dominant-negative C2C12 cells (22). Transfected cells did not show any difference in the levels of late myogenic markers, caveolin-3, and myosin heavy chain (online appendix Fig. 2). As in L6E9, Hrg increased the protein levels of mitochondrial markers, subunits of OXPHOS complexes and PPARδ in

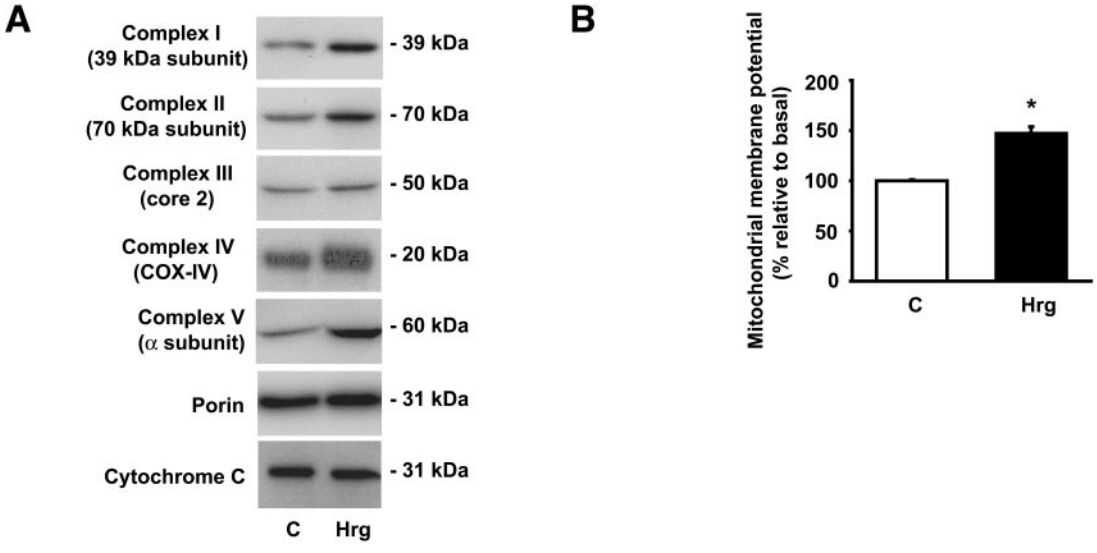

FIG. 3. Neuregulins increase the expression of subunits of the OXPHOS system and mitochondrial membrane potential. L6E9 cells were treated with 3 pmol/l Hrg for 48 h. *A*: Western blot analysis of the proteins indicated in mitochondrial fractions (20 μg). Representative images from three experiments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

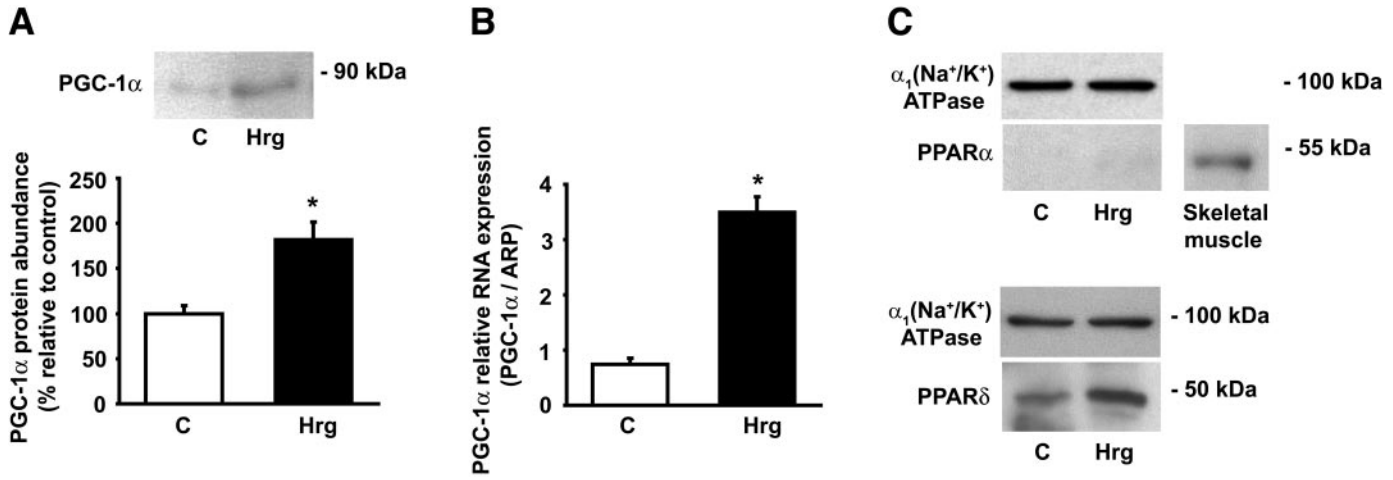

FIG. 4. Neuregulins increase PGC-1α and PPARδ levels. L6E9 cells were treated with 3 pmol/l Hrg for 48 h. *A*: Detection of PGC-1α protein in total cell lysates (120 μg) by Western blot. A representative image and the relative densitometries (relative to β-actin content) from nine experiments (means ± 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 three experiments are shown as means ± SE. *C*: Detection by Western blot of PPARα and PPARδ protein levels in total cell lysates (100 μg). Control of loaded protein: $\alpha_1(Na^+/K^+)$ATPase (10 μg). Total lysates (100 μg) from gastrocnemius muscle were used as a positive control for PPARα detection. Representative images from three to six experiments are shown. *Significant difference vs. control (C) group at $P < 0.01$.

control C2C12 cells (Fig. 5). Whereas wild-type PPARδ only showed a tendency to potentiate Hrg effects on the protein levels of the mitochondrial markers tested, dominant-negative 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 dominant-negative PPARδ cells (online 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, wild-type, or dominant-negative C2C12 cells, although impairment of PPARδ activity decreased PPARγ levels (online 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 1 μmol/l of the antagonist compound GW-9662 (online 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 pretreated or untreated for 48 h with 3 pmol/l Hrg. Hrg increased insulin sensitivity by almost one order of magnitude; however, while higher total GLUT4 levels were observed in Hrg-treated cells, maximal insulin response was not altered (Fig. 6*A*). The increase in insulin sensitivity was also observed in GLUT4 recruitment at the plasma membrane (Fig. 6*B*). To test whether the 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 insulin receptor, IRS-1, p85 subunit of PI3K, PKB, and PKCζ, at a submaximal insulin concentration (100 nmol/l) in L6E9 cells chronically pretreated or not with Hrg. Hrg-treated cells demonstrated increased expression of insulin receptor IRS-1 p85 regulatory subunit of PI3K, PKB, and PKCζ (Fig. 6*C* and online appendix Table 3*A*) and also enhanced insulin-stimulated activation of these proteins (Fig. 6*D* and online appendix Table 3*B*).

Next, we examined whether the effect of Hrg on insulin sensitivity was dependent on PPARδ, using dominant-negative C2C12 cells. Because insulin does not stimulate glucose transport in C2C12 cells due to the low expression of GLUT4, we analyzed Hrg effects on insulin signaling (online 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 Ser^{473}-PKB phosphorylation at a submaximal insulin concentration (10 nmol/l). These Hrg effects were abrogated in PPARδ dominant-negative C2C12 cells, indicating that Hrg required PPARδ to increase insulin sensitivity. In contrast with the observations made in L6E9, Hrg did not increase total insulin receptor protein or insulin-stimulated insulin receptor 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 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, we first analyzed caffeine effects on neuregulin release and ErbB3 activation in L6E9 myotubes (Fig. 7*A*). Incubation of L6E9 cells with caffeine for 3 h led to neuregulin 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. 7*A*), 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 h induced PGC-1α protein levels in differentiated myotubes (Fig. 7*B*), and both

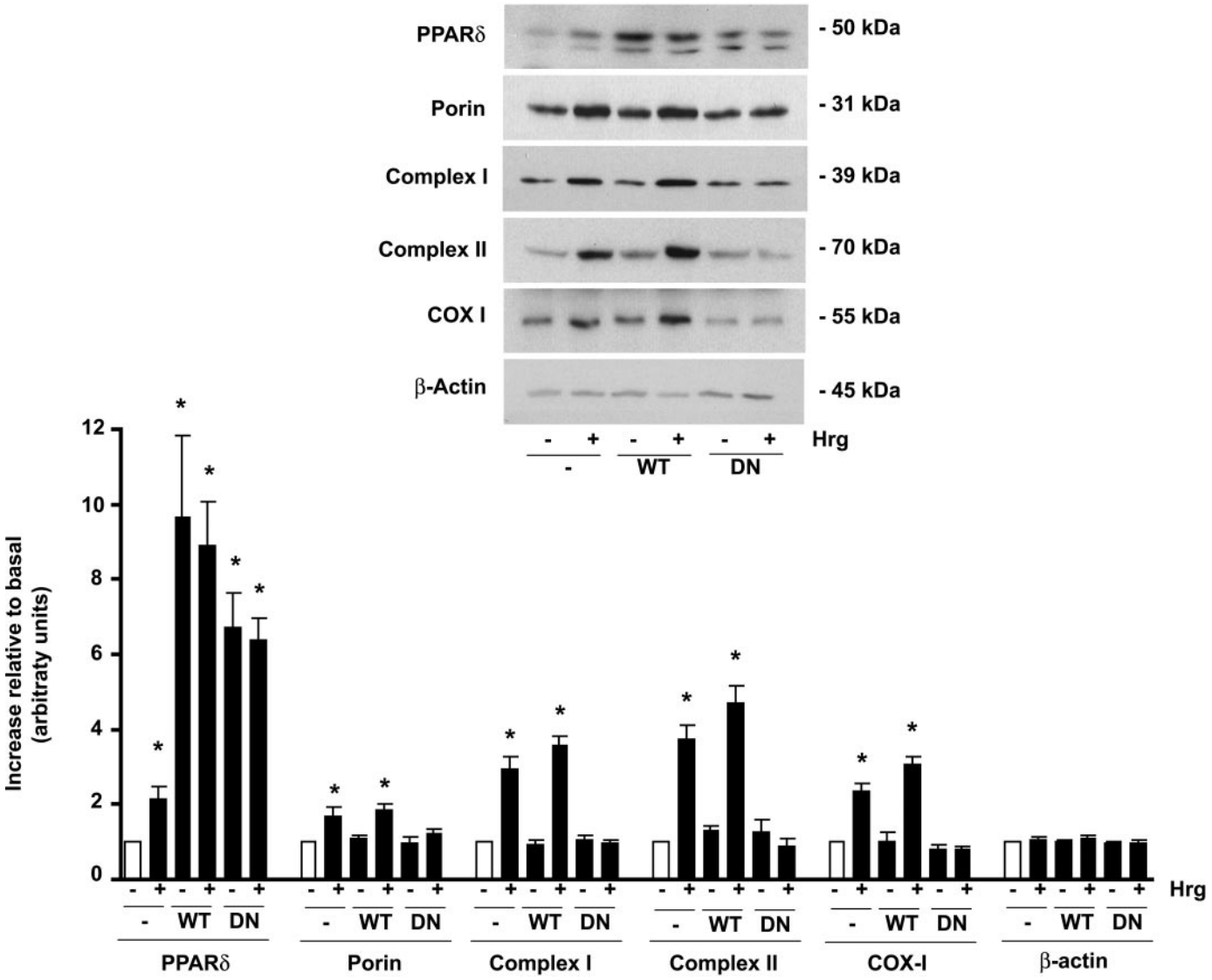

FIG. 5. PPARδ mediates neuregulin effects on mitochondrial protein levels. C2C12 control cells (-), cells overexpressing wild-type (WT), PPARδ, or dominant-negative (DN) PPARδ were treated with 3 pmol/l Hrg for 48 h. Total cell lysates (20–80 μg) were used for Western blot analysis. Representative images and densitometries (means ± SE, normalized using β-actin as loading control protein) from three experiments are shown. *Significant difference vs. untreated cells group (- Hrg) at $P < 0.05$.

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 because 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 adaptative 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 AMP-activated protein kinase (AMPK). 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 appendix Fig. 7).

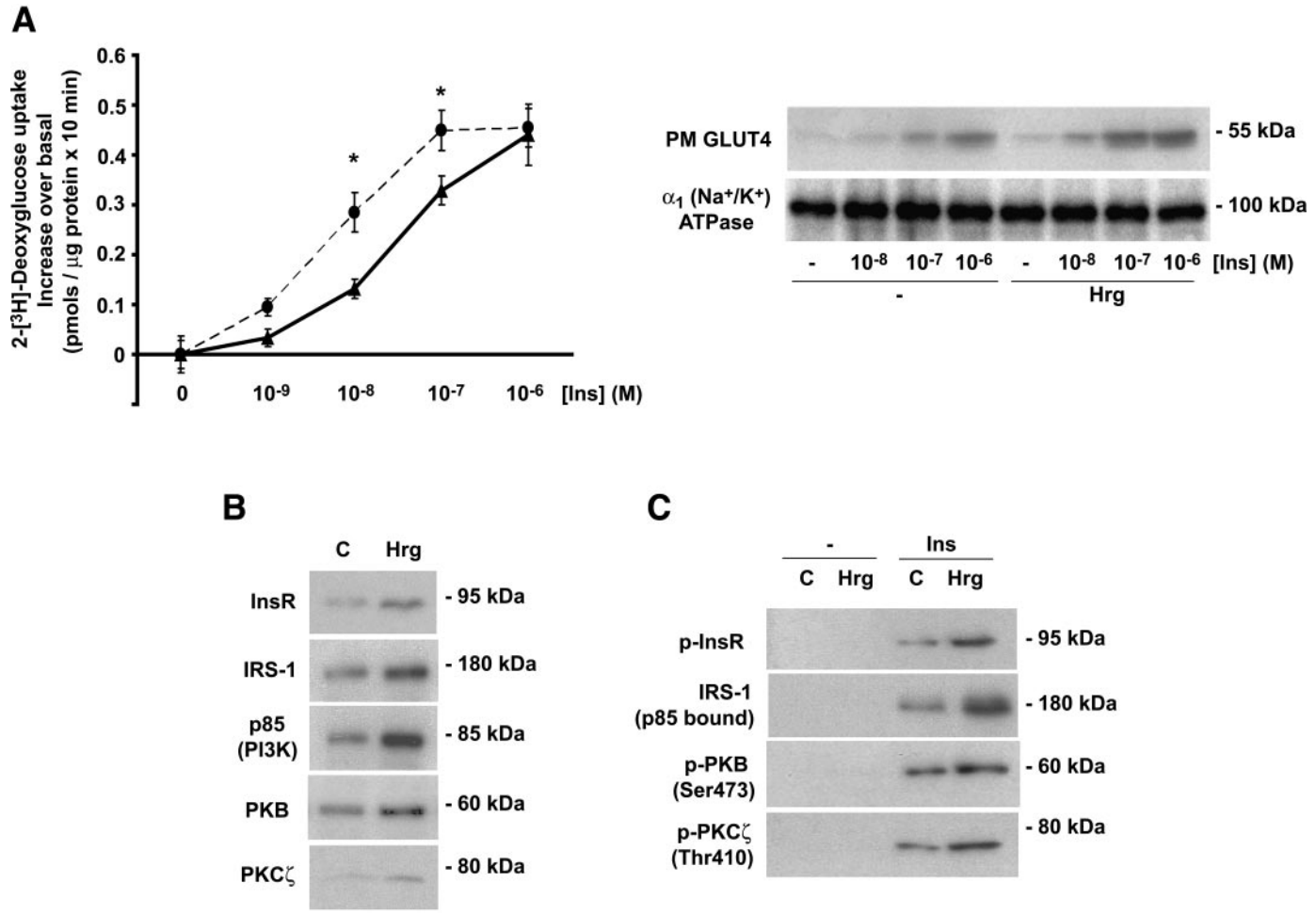

FIG. 6. Neuregulins increase insulin sensitivity. L6E9 cells were treated with 3 pmol/l Hrg for 48 h. *A*: Results of glucose uptake for insulin concentration response assays are shown as the means ± SE of four experiments. *Significant difference between control (C) (▲, continuous line) and neuregulin-treated (●, dashed line) groups at $P < 0.01$. Western blot of GLUT4 was conducted using 10 μg of plasma membrane (PM), and representative images from three experiments are shown. *B*: Western blots of indicated proteins were condcuted using 40 μg total cell lysates. Representative images from three experiments are shown. *C*: Analysis of Hrg action on the submaximal insulin concentration effects (10 min for InsR or 15 min for others) regulating phosphorylation of InsR, PKB (Ser^{473}), and PKCζ (Thr410) and also on IRS-1 association to the p85 subunit of PI3K. Representative images from three experiments are shown.

However, there is evidence that intermittent increases in cytosolic calcium induce mitochondrial biogenesis in L6 cells by increased expression of the coactivator PGC-1α (12,20). Because 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 cells 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 that indicate 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 GA-binding protein (GABP)-α and -β (their human heteromeric binding form also known as NRF-2), whose transcriptional activity is increased by neuregulins in muscle cells (40).

More than 20 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 downregulated (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 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 (rev. in 48), further studies should analyze whether neuregulins can improve muscle metabolic alterations associated with insulin-resistant states.

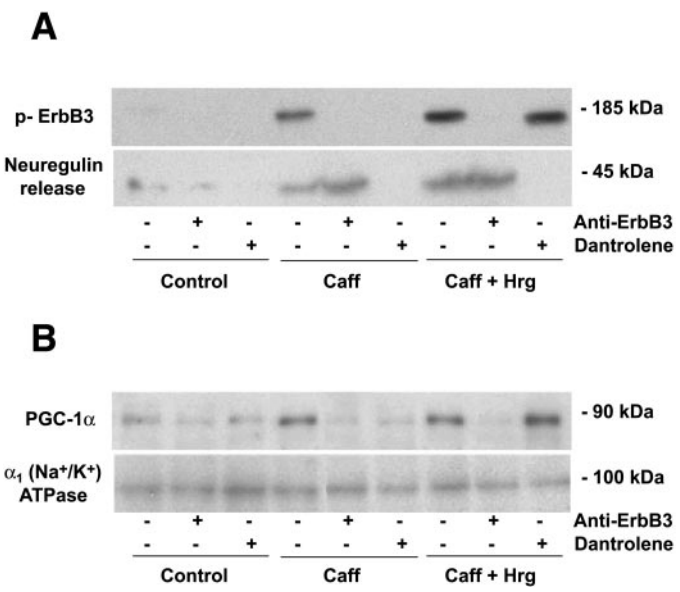

FIG. 7. Neuregulin involvement in caffeine effects on PGC-1α. L6E9 myotubes were incubated with dantrolene (10 μmol/l) or ErbB3-blocking antibodies (10 μg/ml) for 30 min previous to caffeine (5 mmol/l) or caffeine + Hrg 3 pmol/l treatments for 3 h. *A*: ErbB3 phosphorylation (in total cell lysates) and released neuregulins (in incubation medium) were analyzed by Western blot assays. *B*: After indicated treatments, cells were washed in PBS and left for 15 h 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 three experiments are shown.

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