Neuregulin Signaling on Glucose Transport in Muscle Cells*

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Neuregulin-1, a growth factor that potentiates myogenesis induces glucose transport through translocation of glucose transporters, in an additive manner to insulin, in muscle cells. In this study, we examined the signaling pathway required for a recombinant active neuregulin-1 isoform (rhHeregulin-β1, 177–244, HRG) to stimulate glucose uptake in L6E9 myotubes. The stimulatory effect of HRG required binding to ErbB3 in L6E9 myotubes. PKB activity is required for HRG action in both muscle cells and tissue. In L6E9 myotubes, HRG stimulated PKBα, PKBγ, and PKCζ activities. TPCK, an inhibitor of PDK1, abolished both HRG- and insulin-induced glucose transport. To assess whether PKB was necessary for the effects of HRG on glucose uptake, cells were infected with adenoviruses encoding a dominant negative mutants of PKBα. Dominant negative PKB reduced PKB activity and insulin-stimulated glucose transport but not HRG-induced glucose transport. In contrast, transduction of L6E9 myotubes with adenoviruses encoding a dominant negative kinaseinactive PKCζ abolished both HRG- and insulin-stimulated glucose uptake. In soleus muscle, HRG induced PKCζ but not PKB phosphorylation. HRG also stimulated the activity of p70S6K, p38MAPK, and p42/p44MAPK and inhibition of p42/p44MAPK partially repressed HRG action on glucose uptake. HRG did not affect AMPKα, or AMPKβ2 activities. In all, HRG stimulated glucose transport in muscle cells by activation of a pathway that requires PKB, PDK1, and PKCζ, but not PKBα, and that shows cross-talk with the MAPK pathway. The PK3, PDK1, and PKCζ pathway can be considered as an alternative mechanism, independent of insulin, to induce glucose uptake.

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* The abbreviations used are: NRGs, neuregulins; AChR, acetylcholine receptor; AMPK, 5′-AMP-activated protein kinase; DNP, 2,4-dinitrophenol; EGFR, epidermal growth factor; HRG, rhHeregulin-β1, 177–244; IR, insulin receptor; MAPK, mitogen-activated protein kinase; MAPKK, MAPK-activated protein kinase 1; MAPKAP-K2, MAPK-activated protein kinase 2; MOI, multiplicity of infection; p70S6K, p70 ribosomal S6 protein kinase; PDK1, 3-phosphoinositide-dependent kinase-1; PK3, phosphatidylidyinositol-3-kinase; PKC, protein kinase C; TK, N,N-tosyl-l-phenylalanine chloromethyl ketone; WT, wild type; PVDF, polyvinylidene difluoride.

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effects in various cell types (25, 26) and in cancer cells (27), while PKBαMAPK is involved in the effects of NGFα on morphological changes associated with cell transformation in breast cancer (28–30). No information is available on the effects of NGFα on PKBα and PKBαMAPK in muscle.

NGFα induces glucose uptake in myocyte cultures and muscle fibers (31). This effect is fast and requires translocation of glucose transporters to the plasma membrane (31). NGFα and insulin induce glucose uptake in an additive manner and both require PI3K activity (31).

The action of insulin on glucose uptake in adipose and muscle cells involves both PKBα (32–36) and PKCβ (37, 38). PKBαMAPK has also been implicated in acute insulin action on glucose uptake through activation, but not translocation, of glucose transporters (39).

A recent study indicates that muscle contraction, caused by either acute exercise or electrical stimulation, induces release of NGFs, to the extracellular milieu that results in ErbB-induced-activity (40). Both exercise and oxidative stress induce glucose uptake through activation, but not translocation, of glucose transporters (39).

2-Deoxy-D-[14C]glucose, D-[3H]mannitol, and the tissue solubilizer were purchased from Calbiochem (San Diego, CA). Anti-ErbB3 (Ab-5) monoclonal antibody, anti-insulin receptor phosphorylase monoclonal antibody, anti-phosphotyrosine monoclonal antibody, anti-ErbB3 (Ab-5) monoclonal antibody OSCRX (raised against the 15 C-terminal amino acid residues of h Her-2/neu) were purchased from Upstate Biotechnology Inc. Polyclonal antibodies were purchased from Cell Signaling. Anti-ErbB4 and anti-p85 PI3K subunit polyclonal antibodies were purchased from Pierce (Rockford, IL). Immobilon polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corp (Bedford, MA). ECL reagents were purchased from Amersham Biosciences. 2-Deoxy-o-[^32P]glucose was obtained from American Radiolabeled Chemicals, Inc. [3H]glucose was obtained from American Radiolabeled Chemicals, Inc. [3H]glucose was obtained from American Radiolabeled Chemicals, Inc.

**EXPERIMENTAL PROCEDURES**

**Cells, Reagents, and Materials**—The **L6E9** rat skeletal muscle cell line was kindly provided by Dr. B. Nadal-Ginard (Harvard University, Boston, MA). Dulbecco’s Modified Eagle Medium and fetal bovine serum were purchased from Invitrogen Life Technologies. Glutamine and antibiotics were purchased from Pierce. Insulin, culture medium was depleted of serum and replaced by 0.2% bovine serum albumin, which was added for 4.5 h.

**Cell Culture**—**L6E9** myoblasts were grown and differentiated to myotubes as previously described (30). Muscle homogenates were obtained from strips of rat soleus muscles. Muscles were frozen, collected into an Eppendorf, and placed on ice containing 200 μl of fresh lysis buffer, and then homogenized with an Eppendorf homogenizer. Homogenates were taken to a final volume of 500 μl by adding lysis buffer and then centrifuged at 15,000 rpm for 10 min at 4 °C. Finally, supernatant was collected and it was stored at −80 °C. Protein was measured by the Bradford method. Preparations of fractions enriched in plasma membranes (PM) or intracellular membranes (LDM) were obtained from two 10-mm culture dishes as described (31).

**Preparation of Extracts from L6E9 Myotubes and from Strips of Rat Soleus Muscle**—Homogenates were prepared from **L6E9** myotubes. They were placed on ice and washed twice in ice-cold phosphate-buffered saline before adding 300 μl (6-well plates) or 1 ml (100-mm dishes) of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, containing PH inhibition: 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin and 1 μM pepstatin). Cells were then scraped, collected into an Eppendorf tube, and then homogenized by repetitive passages through a 25-gauge needle before being centrifuged at 15,000 rpm for 10 min at 4 °C. Finally, supernatant was collected and it was stored at −80 °C. Protein was measured by the Bradford method.

**Immunoprecipitation Assays**—Immunoprecipitation was performed by conjugating 30 μg of protein G-Sepharose beads with 2–5 μg of the corresponding antibody, except when it is indicated, shaking for 1 h at 4 °C, then washing twice in lysis buffer and incubating with 500–1,000 μg of lystate overnight with constant shaking at 4 °C. After brief centrifugation the supernatant was discarded. The pellet was washed several times with the lysis buffer and boiled with 50 μl of Laemmli sample buffer (LBS) for Western blot as described below. PVDF membranes were blotted with the corresponding antibodies. For PKCα immunoprecipitation, 500 μg of homogenate were incubated overnight with 1 μg of an anti-αPKC-α antibody (C-20, Santa Cruz Bio-technology), with constant shaking at 4 °C, and 30 μg of protein G-Sepharose beads were then added. Beads were then washed twice in lysis buffer, boiled at 95 °C for 5 min in 50 μl LSB, and the supernatant was used for Western blot as described below. PVDF membranes were blotted with anti-phospho-PKCα/β (Thr410/405) antibody.

**PKB Activity Assay**—**L6E9** myotubes were washed once in phosphate-buffered saline and lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 100 mM NaF, and 1% Triton X-100 supplemented with 2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 10 mM orthovanadate. 100 μg of lystate was incubated with 1.5 μl of mouse monoclonal anti-phosphoarginine antibody (FP99, Santa Cruz Biotechnology) for 90 min at 4 °C, and then immunoprecipitated with 2.5 μg of rehydrated protein A-Sepharose beads (Sigma) for 60 min. The immunoprecipitates were used for the kinase assay, which was conducted with phosphatidylinositol as substrate in the presence of γ-[^32P]ATP (44). Lipids were separated by TLC, and the radioactivity corresponding to phosphatidylinositol 3-phosphate was analyzed using a Fuji FLA-2000 phosphomager.

**PKB, p70S6K, p85MAPK, and p42/p44MAPK Activity Assays**—5 μg of protein was incubated in a buffer containing 0.1% P8ase K and α (45), p70S6K (46), MAPK-αMAPK (47), or p90rsk (48) were conjugated to 5 μl of protein G-Sepharose beads for 30 min at 4 °C. After washing twice in lysis buffer, 500 μg of total lystate in the case of p70S6K or 100 μg otherwise were added and the mixture was incubated for 1 h at 4 °C on a shaking platform. It was then centrifuged briefly at 15,000 rpm. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl, and twice with 1 ml of reaction buffer (50 mM Tris-HCl, pH 7.5, 0.1% (v/v) β-mercaptoethanol, 0.1 mM EDTA). The standard assay, which is employed unless stated, otherwise contained 50 μl of total volume: washed protein G-Sepharose immunoprecipitated, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1% (v/v) β-mercaptoethanol, 2.5 μM PKI, 2 μM TRIF (Tyr1058/Akt1 catalytic subunit that inhibits the effect of PKCα/β/δ), 10 mM magnesium acetate, 0.1 mM γ-[32P]ATP (~300 cpm/pmol) and the corresponding substrate: 30 μM Crodide peptide (GRPRTRSSFAEG) in all cases except for measuring MAPK-αMAPK activity (30 μM MAPK-αMAPK substrate KKLKRTLSVIA). The protein was measured by the Bradford method.

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assays were carried out for 30 min at 30°C, the assay tubes being agitated continuously to keep the immunoprecipitated in suspension. Incorporation of [γ-32P]phosphate into each peptide substrate was determined using p81 phosphocellulose paper. Papers were washed in 0.5% orthophosphoric acid, dried, and Cherenkov radiation was measured. 1 milliunit of activity is the amount of enzyme that catalyzes the phosphorylation of 1 pmol of substrate in 1 min.

AMPK Activity Assay—AMPK α1 and β2 activities were measured in L6E9 myotubes lysates as described previously (48). Briefly, cells were incubated in HEPES-buffered saline (HBS) containing 5 mM glucose for 30 min at 37°C. After washing the cells in phosphate-buffered saline, they were lysed into a minimal volume of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithioretil, 10% glycerol, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100), cell debris was spun down in a bench top centrifuge at 12,000 rpm for 5 min, and the supernatant was taken for assay. Protein concentration was determined using the Bradford reagent. 100–200 μg of protein was then sequentially immunoprecipitated using sheep antibodies against either α1 or α2 (49) conjugated to protein G-Sepharose beads. AMPK activity within the immune complex was measured by the phosphorylation of synthetic SAMS peptide using radiolabeled [γ-32P]ATP.

Immunoblotting—Protein samples containing LSB were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes as previously described (31). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline solution (TBS) for 1 h at room temperature and then incubated overnight with the corresponding primary antibodies at recommended dilutions in 1% bovine serum albumin. PVDF membranes were then washed, incubated with appropriate secondary antibodies, containing horseradish peroxidase, for 1 h at room temperature and washed again. Proteins were detected by the ECL method and quantified by scanning densitometry.

Statistical Analysis—Data are presented as means ± S.E. Unpaired Student’s t test was used to compare two groups and one-way analysis of variance, posthoc Duncan’s t test, was used to compare more than two groups.

RESULTS

Neuregulins-induced Glucose Transport Requires an Intact ErbB3 Binding Activity—In order to prove the necessity of the ErbB3 binding activity in the NRG action on glucose uptake in L6E9 myotubes, we used an antibody, Ab5, that binds to ErbB3 and blocks the NRG-binding domain. When Ab5 (10 μg/ml) was added to the medium 30 min before HRG, it abolished the stimulation of glucose transport (Fig. 1A). Ab5 did not alter the insulin effect on glucose uptake (Fig. 1A).

HRG enhanced tyrosine phosphorylation of ErbB3 receptors but it did not induce tyrosine phosphorylation of the β subunit of insulin receptors (Fig. 1B). Moreover, chronic insulin treatment that generated insulin resistance characterized by a deficient induction of glucose uptake, did not alter HRG action (Fig. 1, C and D). In all, HRG required ErbB3 to stimulate glucose uptake in L6E9 myotubes and this effect did not involve activation of the insulin receptor and it was not modified by induction of insulin resistance.

Neuregulins Activate PI3K, PKB, and PKCζ in L6E9 Myotubes—Tyrosine-phosphorylated proteins were immunoprecipitated (PY99) and assayed for PI3K activity (Fig. 2A). HRG (3 nM) induced PI3K activity in L6E9 myotubes, and maximal effects were observed between 5 and 10 min.

Increase in phosphatidylinositol (3,4,5)-trisphosphate involves activation of PKD1, which in turn phosphorylates and activates PKB, Thr308 (50), and PKCζ, Thr410 (51). HRG (3 nM) induced PKB activity in L6E9 myotubes (Fig. 2B). PKBα showed maximal activity at 10–15 min, whereas PKBβ showed maximal activity at 30 min, which was maintained for 90 min. HRG did not stimulate PKBβ activity in this cell line (data not shown).

HRG (3 nM) activated PKCζ in L6E9 myotubes, with a maximum at 30 min (Fig. 2C). HRG and insulin did not show additive effects (Fig. 2, A–C).

FIG. 1. ErbB3 requirement on HRG-induced glucose uptake. A, L6E9 myotubes incubated in the absence or presence of 10 μg/ml Ab5 for 120 min were treated with 3 nM HRG (H) for 90 min or 1 μM insulin (I) for 30 min before 2-deoxyglucose uptake was assayed. Data are presented as means ± S.E. of three different experiments. * indicates a significant difference due to Ab5 antibody at p < 0.001. B, L6E9 myotubes were treated with either 3 nM HRG (10 min) or 1 μM insulin (5 min). Then, cell lysates were immunoprecipitated (IP) with anti-ErbB3 (C-17) or anti-insulin receptor β subunit antibodies. The immunoprecipitates were immunoblotted (IB) for tyrosine-phosphorylated residues. Results are representative scanned autoradiograms of three different experiments. C, L6E9 myotubes were treated with insulin (1 μM, 5 min) or HRG (3 nM, 10 min), lysed, immunoprecipitated with anti-p85 subunit of PI3K, and immunoblotted for IRS-1 or ErbB3. Results are representative autoradiograms of three different experiments.
PDK1 Is Involved in the Effect of Neuregulins on Glucose Uptake in L6E9 Myotubes—To study the role of PKB in the HRG action on glucose uptake, we used dominant negative mutants of PKB. Initially, we transduced L6E9 myocytes with adenovirus containing mutated forms of PKBα, AA-PKB, and AAA-PKB (MOI 300, 48 h), in order to inhibit endogenous PKB activity. L6E9 myocytes transduced with double mutant viruses showed a partial inhibition of insulin- or HRG-induced PKB activity (Fig. 4A). Almost complete inhibition of the PKB activity was obtained with the triple mutant AAA-PKB (Fig. 4A). The overexpression of the double mutant AA-PKB induced a significant inhibition of insulin-stimulated glucose transport, which was even greater in cells expressing the AAA-PKB mutant (Fig. 4B). In these conditions the dominant negative forms of PKB did not inhibit HRG-induced glucose transport or HRG-induced GLUT4 translocation (Fig. 4, B and C).

PKCζ Activity Is Necessary for Neuregulin-induced Glucose Uptake—The next step was to determine the involvement of PKCζ in the HRG-induced glucose uptake. Transduction of L6E9 myotubes with KI-PKCζ viruses (MOI 100, 48 h) blocked PKCζ activity (Fig. 5A) and repressed the effects of HRG and insulin on glucose uptake (Fig. 5B).

P70S6K, p38MAPK, and p42/p44MAPK Are Not Directly Involved in the Action of Neuregulin on Glucose Uptake—We analyzed HRG action on p70S6K, p38MAPK, or p42/p44MAPK activities in L6E9 myotubes and their possible involvement in the effects of HRG on glucose uptake using specific inhibitors.

HRG-induced p70S6K activity (Fig. 6A), which was completely abolished by the specific inhibitor rapamycin. However, rapamycin did not alter the effect of HRG on glucose uptake (Fig. 6B).

p38MAPK was moderately activated by HRG in comparison with the hyperosmotic effects of 0.5 M sorbitol (Fig. 6A). The specific inhibitor SB203580 (10 μM) abolished HRG activation of p38MAPK activation by HRG action (Fig. 6A) but did not interfere with its action on glucose uptake (Fig. 6B).

HRG stimulated p42/p44MAPK activity (Fig. 6A). The specific inhibitor PD098059 (50 μM) abolished this activation (Fig. 6A), and caused a partial repression of HRG action on glucose uptake (Fig. 6B).

Effects of Neuregulins on AMPK Activity—Due to the lack of a specific inhibitor of AMPK, many studies have used 5-amino-4-imidazolecarboxamide riboside, AICAR, as an AMPK activator. However, AICAR does not lead to activation in many cell types since its efficacy is dependent on its uptake by the cell. Another AMPK activator is DNP, an oxidative phosphorylation uncoupler. DNP stimulated AMPK activity. Under these conditions, HRG did not alter the AMPK activity at any
time or dose studied (Fig. 7A). Identical results were obtained in the H-2Kβ myocytes (48) (not shown).

DNP, HRG, and insulin showed additive effects on glucose transport (Fig. 7B) indicating that they activate at least partially different intracellular pathways to induce glucose transport in muscle cells.

**Neuregulin Signaling Cascade on Glucose Transport in Rat Soleus Muscle.** Strips of rat soleus muscle were incubated with HRG (5 nM, 15 min) or insulin (100 nM, 10 min) and lysates were obtained, immunoprecipitated with an anti-phosphotyrosine antibody, and the pellets were immunoblotted with antibodies against each neuregulin receptor. Results indicated that HRG, but not insulin, activated all the expressed neuregulin receptors in muscle fiber, ErbB2, ErbB3, and ErbB4 (Fig. 8A).

Treatment with wortmannin (2 μM, 150 min) abolished both insulin and HRG action on glucose uptake in incubated strips of rat soleus (Fig. 8B), indicating that PI3K is also required in the neuregulins signaling cascade at the muscle fiber.

Next, strips of rat soleus muscle were treated with HRG or insulin during 30 min, and lysates were obtained to analyze phosphorylation levels of PKB and PKCε. Whereas insulin induced phosphorylation of both kinases, HRG was unable to induce PKB phosphorylation (Fig. 8, C and D) at any time ranging from 10 to 60 min (not shown).

**DISCUSSION**

In this study we have demonstrated that NRGs stimulate PI3K, PKB, PKCε, p70S6K, p38MAPK, and p42/p44MAPKs in L6E9 myotubes, that the pathway PI3K, PDK1, and PKCε is essential to induce glucose transport and that p42/p44MAPKs also contribute to a maximal glucose transport stimulation. Studies done in rat soleus muscles also support the implication of the PI3K pathway on NRG-stimulated glucose transport. This is the first report indicating that NRGs induce a PDK1/PKCε pathway. In L6E9 myotubes ErbB3 is required to initiate this signaling cascade. NRGs do not transactivate the insulin receptors, so the NRG signaling pathway can be considered as an alternative mechanism to induce glucose transport in muscle cells which, like insulin, is initiated by PI3K activation (31, 53, 54). ErbB3 contains six consensus sequences, YXXM motifs that bind to the SH2 domain of the PI3K p85 subunit after ligand-induced tyrosine phosphorylation (19). ErbB3 has been characterized as the main mediator of heregulin-dependent PI3K activation pathway (55) and expression of mutated forms of ErbB3 at the YXXM motifs in COS7 cells abolishes PI3K and PKB activation by heregulin binding to the heterodimer ErbB2/ErbB3 (56). Since ErbB4 is also expressed and activated by HRG in skeletal muscle, differences on the insulin and the HRG action in soleus and cultured myocytes could be consequence of a different pattern of receptors expression.

PI3K activity is essential for HRG action on glucose transport (31), although HRG induces lower maximal PI3K activities than insulin. Differences on PI3K activation could be a consequence of the expression levels of ErbB3 and insulin receptors in L6E9 myotubes, and they also suggest that PI3K activity might not be limiting for signaling on glucose uptake. This view is also supported by the lack of additivity of HRG and insulin on PI3K activity. Otherwise, HRG requires more time than insulin to reach maximal effects on glucose transport (31), so a critical amount of phosphatidylinositol 3-phospho-min). Then, the 2-deoxyglucose uptake assay was performed. Results are obtained from three different experiments. Basal uptake: 0.62 ± 0.03 pmol/μg of protein × 10 min. B, basal; I, insulin action; H, HRG action. * indicates significant differences with the control group, at p < 0.001.
phate may be needed to trigger HRG stimulation of glucose transport. It is notable that HRG action is not impaired in muscle cells made insulin resistant by chronic exposure to insulin. Interestingly, soleus muscle insulin responsiveness was reduced in the adult rat compared with 1-month-old rats (2–3-fold increase in response to maximal insulin in adult rats and 5–9-fold increase in young rats, respectively); under these conditions, heregulin-induced stimulation of glucose transport remained unaltered (1.5–2-fold increase both in adult and in young rats).

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2 E. Suárez, J. Ryder, A. Zorzano, J. R. Zierath, and A. Guma, unpublished observations.

**FIG. 4.** Effect of PKB inhibition on HRG-induced glucose uptake. A, L6E9 myotubes were treated with 3 nM HRG (H) for 15 min or 1 μM insulin (I) for 10 min in the absence or the presence of adenovirus (MOI 100, 48 h) containing cDNA for LacZ, as a control of viral infection, AA-PKB or AAA-PKB. Total PKB was immunoprecipitated from lysates and PKB activity was assayed. Results were obtained from two experiments, assayed in triplicate. *, p < 0.05 versus corresponding LacZ group. †, p < 0.01 versus corresponding LacZ and AA-PKB groups. B, L6E9 myotubes were treated with 3 nM HRG (H) for 90 min or 1 μM insulin (I) for 30 min in the absence or presence of adenovirus as stated previously. Afterward, 2-deoxyglucose uptake was assayed. Results are the mean of three different experiments run in duplicate. Basal uptake: 0.64 ± 0.02 pmol/μg of protein × 10 min, no significant changes were observed with adenoviral transduction. I, insulin action; H, HRG action. *, p < 0.01 versus corresponding LacZ groups. †, p < 0.001 versus corresponding LacZ and WT-PKCζ groups.

**FIG. 5.** Effect of PKCζ inhibition on HRG-induced glucose uptake. A, L6E9 myotubes were treated with 3 nM HRG (H) for 30 min or 1 μM insulin (I) for 10 min in the absence or the presence of adenovirus (MOI 100, 48 h) containing cDNA for LacZ, as a control of viral infection, wild type PKCζ (WT-PKCζ), or kinase inactive PKCζ (KI-PKCζ). Total PKCζ was immunoprecipitated from lysates, immunoblotted using an antibody that recognizes PKCζ phosphorylated at Thr410-PKCζ and densitometrically quantified. Results were obtained from three different experiments. B, L6E9 myotubes were treated with 3 nM HRG (H) for 90 min or 1 μM insulin (I) for 30 min in the absence or presence of adenovirus as stated previously. Afterward, 2-deoxyglucose uptake was assayed. Results are the mean of three different experiments run in duplicate. Basal uptake: 0.64 ± 0.02 pmol/μg of protein × 10 min, no significant changes were observed with adenoviral transduction. I, insulin action; H, HRG action. *, p < 0.01 versus corresponding LacZ groups. †, p < 0.001 versus corresponding LacZ and WT-PKCζ groups.

It is notable that HRG action is not impaired in muscle cells made insulin resistant by chronic exposure to insulin. Interestingly, soleus muscle insulin responsiveness was reduced in the adult rat compared with 1-month-old rats (2–3-fold increase in response to maximal insulin in adult rats and 5–9-fold increase in young rats, respectively); under these conditions, heregulin-induced stimulation of glucose transport remained unaltered (1.5–2-fold increase both in adult and in young rats). Current
SB203580 (120 min), or 50 μM PD098059 (120 min).

A, kinase activities were assayed immunoprecipitating each kinase from lysates, as stated under “Experimental Procedures.” High concentration of sorbitol was used as a positive control for p38MAPK activity, and EGF was used as a positive control for p42/p44MAPKs activities. Results were obtained from two different experiments performed in duplicate. *, p < 0.001 versus basal values. B, 2-deoxyglucose uptake was assayed in L6E9 myotubes treated with 3 nM HRG (90 min) and/or insulin, in the absence or presence of DNP. Results were obtained from three different experiments run in duplicate. B, basal; H, HRG; I, insulin. *, p < 0.05 versus basal values. †, p < 0.05 versus corresponding non-DNP-treated groups. ¶, p < 0.05 versus both corresponding I and H groups.

Fig. 6. HRG stimulates P70S6K, p38MAPK, and p42/p44MAPKs. L6E9 myotubes were treated with 3 nM HRG (90 min), 1 μM insulin (30 min), 0.5M sorbitol (20 min) or 0.1 ng/ml rhEGF (20 min) in the absence or the presence of 1 ng/ml rapamycin (120 min), 10 μM

Fig. 7. HRG does not activate AMPK. Effect of DNP on HRG- and insulin-stimulated glucose uptake. L6E9 myotubes were treated with 3 or 30 nM HRG (5, 30, or 90 min), 1 μM insulin (30 min), or 0.5 mM DNP (60 min). A, AMPKζ1 and ζ2 activities were assayed in L6E9 myotubes immunoprecipitated lysates (see “Experimental Procedures”). Results were obtained from three different experiments and kinase activity assays performed in duplicate. *, p < 0.001 versus basal values. B, 2-deoxyglucose uptake was assayed in L6E9 myotubes treated with 3 mM HRG (90 min) and/or insulin, in the absence or presence of DNP. Results were obtained from three different experiments run in duplicate. B, basal; H, HRG; I, insulin. *, p < 0.05 versus basal values. †, p < 0.05 versus corresponding non-DNP-treated groups. ¶, p < 0.05 versus both corresponding I and H groups.
studies are being addressed to investigate the extent of neuregulin action in muscle in animal models of type 2 diabetes, obesity or aging.

HRG induces both PKBα and PKBγ in L6E9 myotubes. Similar results were previously reported for insulin action in L6 myotubes (45). HRG induces lower PKBα activity than insulin, as for PI3K activity. This is in accordance with the lower ability of HRG to phosphorylate Ser473, which is involved in the activation of PKB. In contrast, HRG has stronger maximal effects than insulin on PKCζ, but HRG requires longer time than insulin to reach them (maximal insulin effect on PKCζ is reached within 10 min, not shown). These differences might explain the potentiated effects observed on glucose transport since insulin and neuregulins do not have additive effects on PKB or PKCζ activation levels. Nonetheless, an alternative pathway to PI3K activation cannot be ruled out. In fact, a second insulin pathway is involved in the stimulation of glucose transport in adipose cells (reviewed in Ref. 57). This second pathway requires phosphorylation of the adaptor protein Cbl by the insulin receptor and activation of TC10, a small GTP-binding protein located in lipid rafts, which modulates actin structure. At present we do not know whether neuregulin stimulates the Cbl pathway in muscle cells and, if so, whether it is involved in neuregulin-induced glucose uptake.

Several lines of evidence implicate PKB activity in insulin-induced glucose uptake both in adipocytes and muscle (32–36) although some controversy exists (58, 59). PKBβ has been implicated in insulin action on glucose transport in 3T3-L1 adipocytes (36). Our results indicate that PKB blockage alters only partially insulin action on glucose uptake, suggesting that PKB might not be essential in L6E9 myotubes. Moreover, there is no dependence on PKB activity in the NRGs action on glucose transport in muscle cells and HRG does not stimulate PKB phosphorylation in soleus. Then, PKB does not seem to be required in the pathway inducing glucose uptake in response to NRGs. One interesting possibility that should be explored is that a different subcellular localization of PI3K is responsible for the different downstream activities of insulin or neuregulin that trigger glucose transport.

The effect of HRG on glucose transport is also independent of AMPK activity. DNP induces glucose uptake in an additive manner to NRGs action, so AMPK might constitute another pathway, independent of insulin and NRGs, to activate glucose uptake. Both insulin and exercise induce the MAPK pathway, in a PI3K-dependent manner and an AMPK-dependent manner, respectively (60, 61). Whereas PD98059 treatment does not affect insulin action on glucose uptake in muscle cells (62), it impairs AICAR stimulation of glucose transport in EDL muscle (61). Thus, the partial impairment of HRG action on glucose uptake under PD98058 treatment suggests that the PI3K-PDK1-PKB pathway activated by NRGs is modulated by the MAPK pathway. We conclude that NRGs require a signaling pathway to induce glucose uptake in L6E9 myotubes that involves ErbB3, PI3K, PDK1, and PKCζ and is not dependent on PKB activity.

**Fig. 8.** Effect of HRG on incubated strips of rat soleus muscle. A, strips were incubated with insulin (100 nM, 10 min) or HRG (5 nM, 15 min). Lysates were obtained and immunoprecipitated with anti-phosphotyrosine antibody. Pellets were immunoblotted for ErbB2, ErbB3 and ErbB4. Results are shown as representative autoradiograms of three different samples. B, strips were incubated with insulin (100 nM, 60 min) or HRG (5 nM, 120 min), in the absence or presence of wortmannin (2 μM, 150 min). 2-Deoxyglucose uptake was determined during the last 20 min of these periods. Results are the mean ± S.E. of four to five different samples. Basal 2-deoxyglucose uptake: 36.7 ± 8.2 nmol/g of muscle × 20 min. *p < 0.01 versus corresponding control groups. C, strips were incubated with insulin (100 nM, 30 min) or HRG (5 nM, 30 min), and then homogenized. 200 μg of total homogenate were loaded on SDS-page gel electrophoresis for Western blot assays using anti-PKB, anti-phospho-Ser473-PKB, anti-PCKζ, and anti-phospho-Thr410-PKCζ antibodies. Autoradiograms were densitometrically quantified, and results are shown as the mean ± S.E. obtained from three different samples. *p < 0.001 versus basal group.
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