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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-β_1, 177–244, HRG) to stimulate glucose uptake in L6E9 myotubes. The stimulatory effect of HRG required binding to ErbB3 in L6E9 myotubes. PI3K 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 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 kinase-inactive 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_1 or AMPKα_2 activities. In all, HRG stimulated glucose transport in muscle cells by activation of a pathway that requires PI3K, PDK1, and PKCζ, but not PKB, and that shows cross-talk with the MAPK pathway. The PI3K, PDK1, and PKCζ pathway can be considered as an alternative mechanism, independent of insulin, to induce glucose uptake.

Neuregulin1–4 gene products are involved in developmental differentiation of multiple tissues (1). They are closely related proteins that receive the generic name of neuregulins (NRGs),[1] but initially they were known as heregulin (2), *neu* differentiation factor, NDF (3, 4), glial growth factor, GGF (5, 6) or sensory and motor neuron derived factor, SMDF (7).

During adult life, NRGs are highly expressed in neurons and they act on the juxtaposed tissues contributing to the maintenance of differentiated patterns, such as the neuromuscular junction, NMJ (8–12). They are expressed as either membrane or soluble forms, mainly as a consequence of differential splicing of the primary transcript (reviewed in 13 and 14). Soluble forms attach to the basal lamina through proteoglycans (*e.g.* agrin) and, by regulated proteolysis, they are released and bind to their receptors. NRGs bind to type I tyrosine kinase receptors that belong to the family of EGF receptors, ErbB3 and ErbB4. Binding generates homo- and heterodimerization between them and with ErbB2, which lacks a binding domain, whereas ErbB3 does not have kinase activity (reviewed in Ref. 15). Cultured myotubes express ErbB2 and ErbB3, but not ErbB4 (10, 11), so the main NRG receptor is the heterodimer ErbB2/ErbB3. All three receptors are expressed in muscle tissue although only ErbB2 and ErbB4 accumulate at the postsynaptic site (16).

NRG binding and receptors dimerization induce tyrosine phosphorylation of receptors which activates several signaling cascades such as the PI3K-PKB, the mitogenic MAPKs and the stress-inducible p38MAPK pathways (17, 18). Tyrosine-phosphorylated ErbB3 recruits and activates PI3K in several cell types (19, 20). In muscle cells, heregulin induces at least two pathways, one dependent on PI3K activation (21, 22), in which p70S6K has been implicated (22), and the other stimulating the Ras/Raf/MEK/MAPK cascade (21–24). The NRG-induced MAPK pathway is required for the regulation of AChR subunits expression at the neuromuscular junction, NMJ (21, 22, 24), but the role of the PI3K/p70S6K-dependent pathway at this level is controversial (22).

The PI3K/PKB pathway is involved in NRG anti-apoptotic

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[1] The abbreviations used are: NRGs, neuregulins; AChR, acetylcholine receptor; AMPK, 5′ AMP-activated protein kinase; DNP, 2,4-dinitrophenol; EGF, epidermal growth factor; HRG, rhHeregulin-β_1 177–244; IR, insulin receptor; MAPK, mitogen-activated protein kinase; MAPKAP-K2, MAPK-activated protein kinase 2; MOI, multiplicity of infection; p70S6K, p70 ribosomal S6 protein kinase; PDK1, 3-phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; TPCK, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone; WT, wild type; PVDF, polyvinylidene difluoride.

effects in various cell types (25, 26) and in cancer cells (27), while p38MAPK is involved in the effects of NRGs on morphological changes associated with cell transformation in breast cancer (28–30). No information is available on the effects of NRGs on PKB and p38MAPK in muscle.

NRGs induce glucose uptake in myocyte cultures and muscle fibers (31). This effect is fast and requires translocation of glucose transporters to the plasma membrane (31). NRGs 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 PKB (32–36) and PKCζ (37, 38). P38MAPK 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 NRGs, to the extracellular milieu that results in ErbBs-induced-activity (40). Both exercise and oxidative stress induce glucose uptake through translocation of glucose transporters, in an additive and independent manner to the action of insulin, and AMPK has been suggested to be involved in this effect (41–43) although controversy exists in this aspect.

Here we examine the signaling cascade triggering the action of HRG on glucose uptake in L6E9 myotubes. PI3Ks, PDK1, and PKCζ, but not PKB, are involved in HRG-stimulated glucose uptake. The additive effect of HRG and insulin on glucose uptake did not involve AMPK activation.

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 BioWhittaker (Walkersville, MD) Purified porcine insulin was a kind gift from Eli Lilly Co. (Indianapolis, IN). Recombinant heregulin (heregulin-β1-(177–244), HRG) was donated by Genentech, Inc. (South San Francisco, CA). Most commonly used chemicals as well as wortmannin, *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and 2,4-dinitrophenol (DNP) were purchased from Sigma. PD098059, rapamycin, SB203580, and rhEGF were purchased from Calbiochem (San Diego, CA). Anti-ErbB3 (Ab-5) monoclonal antibody was purchased from Neomarkers (Fremont, CA). Anti-phosphotyrosine monoclonal antibody, anti-insulin receptor β-chain and anti-IRS-1 polyclonal antibodies were purchased from Transduction Laboratories. Anti-ErbB2 (C-18), anti-ErbB3 (C-17), and anti-PKCζ (C-20) polyclonal antibodies were purchased from Santa Cruz Inc. Anti-PKB, anti-phospho-Thr^{308}-PKB, anti-phospho-Ser^{473}-PKB, and anti-phospho-Thr^{410}-PKCζ polyclonal antibodies were purchased from Cell Signaling. Anti-ErbB4 and anti-p85 PI3K subunit polyclonal antibodies were purchased from Upstate Biotechnology Inc. Polyclonal antibody OSCRX (raised against the 15 C-terminal amino acid residues from GLUT4) was produced in our laboratory. Bradford reagent and all electrophoresis agents and molecular weight markers were obtained from Bio-Rad (Hercules, CA). BCA Protein Assay Reagent Kit was purchased from Pierce (Rockford, IL). Immobilon polyvinylidene difluoride (PVDF) was obtained from Millipore Corp (Bedford, MA). ECL reagents were purchased from Amersham Biosciences. 2-Deoxy-D-[^{3}H]glucose was obtained from American Radiolabeled Chemicals, Inc. 2-Deoxy-D-[^{14}C]glucose, D-[^{3}H]mannitol, and the tissue solubilizer were obtained from PerkinElmer Life Sciences. All chemicals were of the highest purity available.

AA-PKB (S473A/T308A) adenoviruses were kindly provided by Dr. M. Kasuga (Kobe University School of Medicine, Kobe, Japan). AAA-PKB (S473A/T308A/K179A), WT-PKC-ζ and kinase-inactivated PKC-ζ (K281W), KI-PKCζ, adenoviruses, were kindly provided by Dr. R. V. Farese (University of South Florida College of Medicine, Tampa, Florida).

Cell Culture—L6E9 myoblasts were grown and differentiated to myotubes as previously described (30). To analyze the effect of HRG or insulin, culture medium was depleted of serum and replaced by 0.2% bovine serum albumin, which was added for 4.5 h.

2-Deoxy-D-[^{3}H]Glucose Uptake—L6E9 cells were cultured on 6-well plates. 2-Deoxyglucose uptake assays were performed as previously described (31). Radioactivity was determined by scintillation counting. Protein was measured by BCA protein reagent assay (Pierce). Each condition was run in duplicate or triplicate. 2-Deoxyglucose transport was linear during the period (10 min) assayed (not shown).

2-Deoxyglucose uptake assays were performed in strips of rat soleus muscles as previously described (31). Protein was measured by Bradford method.

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, 50 mM sodium fluoride, containing freshly added protease inhibitors: 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 passes 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. Preparations of fractions enriched in plasma membranes (PM) or in intracellular membranes (LDM) were obtained from two 10-mm culture dishes as reported (31).

Muscle homogenates were obtained from strips of rat soleus. Muscles were frozen, collected into an Eppendorf, 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 1 ml/strip by adding lysis buffer and then centrifuged at 15,000 rpm for 10 min at 4 °C. The pellet was discarded, and supernatant was collected and stored at −80 °C until used. Protein was measured by the Bradford method.

Immunoprecipitation Assays—Immunoprecipitation was performed by conjugating 30 μl 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 lysate 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 assays as described below. PVDF membranes were blotted with the corresponding antibodies.

For PKC-ζ immunoprecipitation, 500 μg of homogenate were incubated overnight with 1 μg of an anti-nPKC-ζ antibody (C-20, Santa Cruz Biotechnology), 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-PKCζ/λ (Thr$^{410/403}$) antibody.

PI3K 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 mg/ml aprotinin, 1 mM pepstatin A, 1 ng/ml leupeptin, 2 mM phenylmethylsulfonylfluoride, and 10 mM sodium orthovanadate. 100 μg of lysate protein was incubated with 1.5 μl of mouse monoclonal anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology) for 90 min at 4 °C, and then immunoprecipitated with 2.5 mg of rehydrated protein A-Sepharose beads (Sigma) for 60 min. The immunoprecipitates were used for the kinase assay, which was performed with phosphatidylinositol as substrate in the presence of [γ-^{32}P]ATP (44). Lipids were separated by TLC, and the radioactivity corresponding to phosphatidylinositol 3-phosphate was analyzed using a Fuji FLA-2000 phosphoimager.

PKB, p70S6K, p38MAPK, and p42/p44MAPK Activity Assays—5 μg of sheep antibodies raised against PKBα or γ (45), p70S6K (46), MAPKAP-K2 (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 lysate 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 13,000 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 EGTA). 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, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 2.5 μM PKI (TTYADFIASGRTGRRNAIHD, peptide inhibitor of the cyclic AMP-dependent protein kinase), 10 mM magnesium acetate, 0.1 mM [γ-^{32}P]ATP (~300 cpm/pmol) and the corresponding substrate: 30 μM Crosstide peptide (GRPRTSSFAEG) in all cases except for measuring MAPKAP-K2 activity (30 μM MAPKAP-K2 substrate KKLNRTLSVA). The

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-^{32}P]phosphate into each peptide substrate was determined using p81 phosphocellulose paper. Papers were washed in 0.5% orthophosphoric acid, dried, and Cherenkov radiation was counted. 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 α2 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 M EDTA, 1 mM dithiothreitol, 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 raised 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-^{32}P]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. 1*A*). Ab5 did not alter the insulin effect on glucose uptake (Fig. 1*A*).

HRG enhanced tyrosine phosphorylation of ErbB3 receptors but it did not induce tyrosine phosphorylation of the β subunit of insulin receptors (Fig. 1*B*). 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. 2*A*). 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 PDK1, which in turn phosphorylates and activates PKB, Thr^{308} (50), and PKCζ, Thr^{410} (51). HRG (3 nM) induced PKB activity in L6E9 myotubes (Fig. 2*B*). PKBα showed maximal activity at 10–15 min, whereas PKBγ 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. 2*C*). HRG and insulin did not show additive effects (Fig 2, *A–C*).

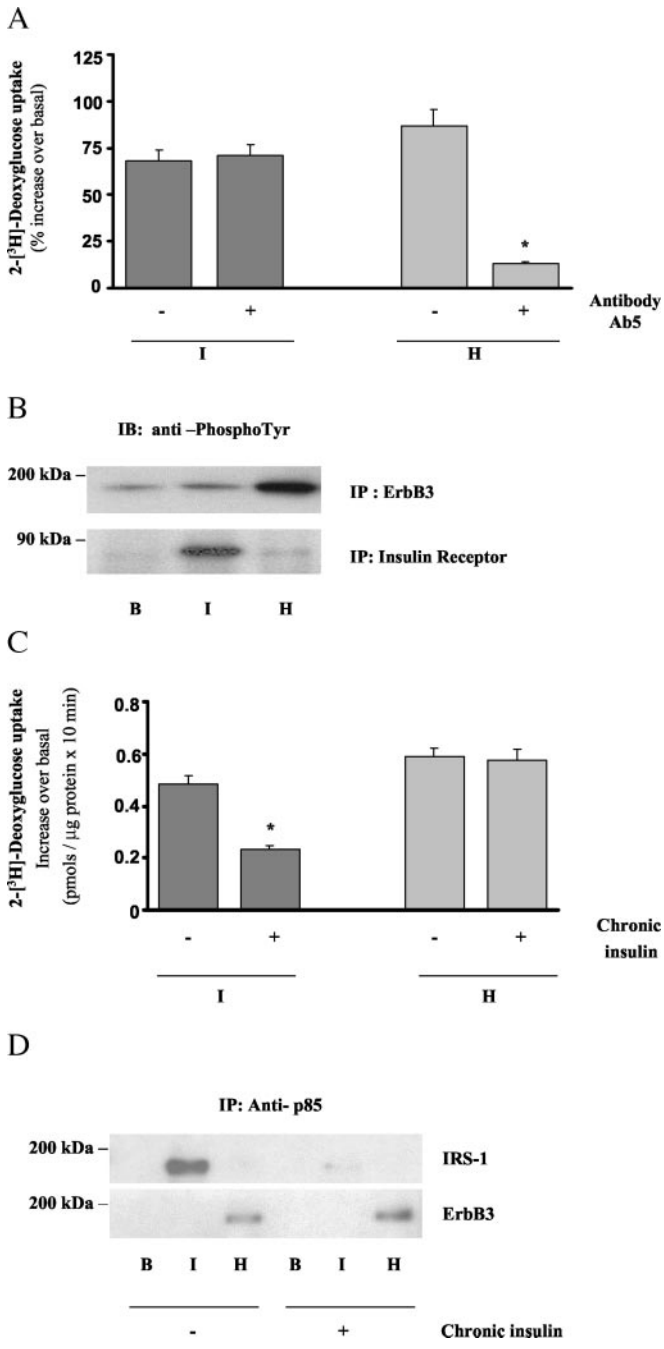

FIG. 1. **ErbB3 requirement on HRG-induced glucose uptake.** *A*, L6E9 myotubes incubated in the absence or presence of 10 μg/ml Ab5 for 120 min were treated with 3 nM HRG (*H*) for 90 min or 1 μM insulin (*I*) for 30 min before 2-deoxyglucose uptake was assayed. Results are the mean ± S.E. of three different experiments run in duplicate. Basal (*B*) 2-deoxyglucose uptake: 0.71 ± 0.07 pmol/μg protein × 10 min. * 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 for 24 h (chronic insulin) previously to acute insulin or HRG treatments done as stated above. Results are the mean ± S.E. of three different experiments. * indicates a significant difference, due to chronic insulin, at $p < 0.001$. *D*, L6E9 myotubes in the presence or absence of insulin 1 μM for 24 h 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.

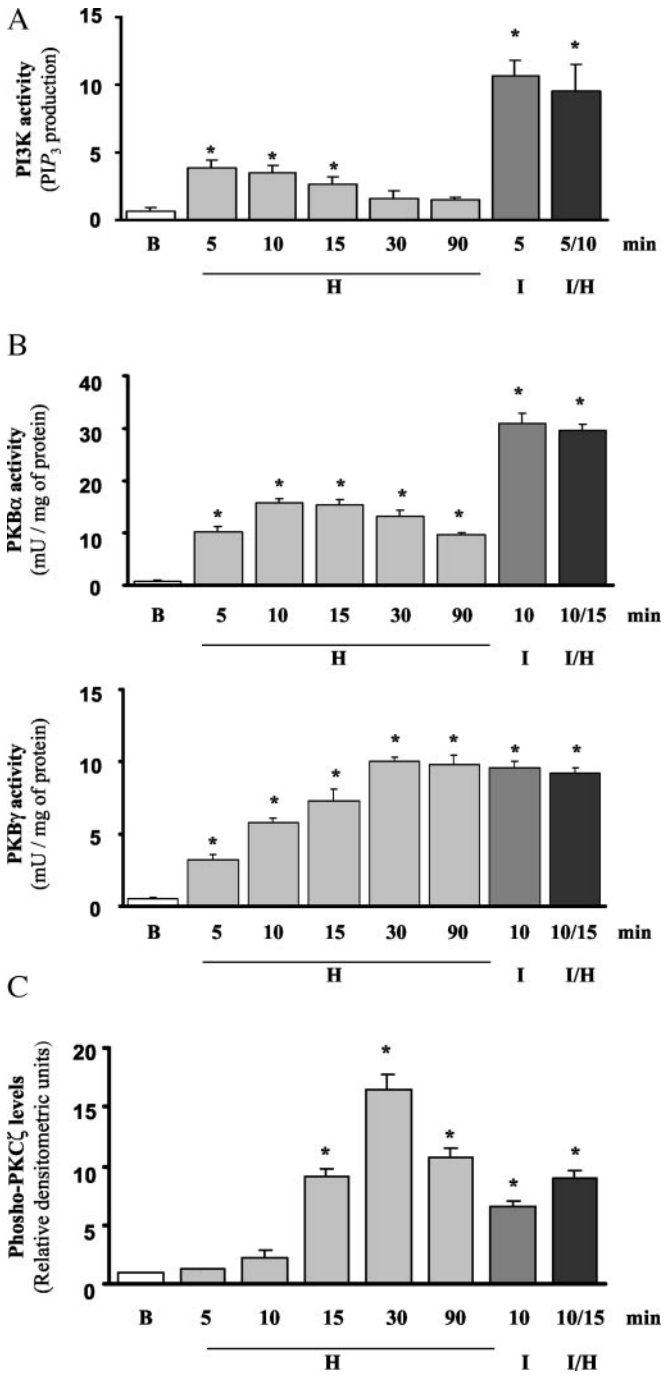

FIG. 2. **HRG stimulates PI3K, PKBα/γ, and PKCζ.** L6E9 myotubes were treated with 3 nM HRG or/and 1 μM insulin for the indicated times, and each kinase was immunoprecipitated from the lysates and assayed. *A*, PI3K activity was assayed and phosphatidylinositol 3-phosphate (PIP_3) production was quantified densitometrically. Results are relative values that correspond to a representative experiment from two independent experiments, each one run in duplicate and assayed twice. *B*, PKBα or PKBγ were immunoprecipitated, and activities were assayed as described under "Experimental Procedures." Results correspond to a representative experiment from two different experiments, each one run in duplicate and assayed in triplicates. *C*, total PKCζ was immunoprecipitated from lysates and phospho-Thr^{410}-PKCζ was immunodetected and quantified by densitometric analysis. Results, expressed as relative to basal values, were obtained from three different experiments. *B*, basal; *H*, HRG, *I*, insulin. * indicates significant differences with the basal group, at $p < 0.01$.

PDK1 Is Involved in the Effect of Neuregulins on Glucose Uptake in L6E9 Myotubes—PI3K activity is required in the HRG-induced pathway on glucose uptake in L6E9 myotubes, based in our previous studies using the specific PI3K inhibitor wortmannin (31). In the present study, we analyzed the role of PDK1 on the NRGs effects, based on the use of TPCK, a PDK1 inhibitor (52). 50 μM TPCK inhibited both PKB and PKCζ activities induced by HRG or by insulin (Fig. 3, *A* and *B*). TPCK inhibited PKB Thr^{308} phosphorylation without affecting Ser^{473} phosphorylation (Fig. 3*A*). Interestingly, Ser^{473} was consistently phosphorylated less under HRG action than under insulin action while no remarkable differences were observed at Thr^{308} phosphorylation. 50 μM TPCK also repressed both HRG- and insulin-induced glucose transport (Fig. 3*C*).

PKB Is Not Involved in the Action of Neuregulins on Glucose Uptake in L6E9 Myotubes—In order 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. 4*A*). Almost complete inhibition of the PKB activity was obtained with the triple mutant AAA-PKB (Fig. 4*A*). 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. 4*B*). 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. 5*A*) and repressed the effects of HRG and insulin on glucose uptake (Fig. 5*B*).

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/p44MAPKs activities in L6E9 myotubes and their possible involvement in the effects of HRG on glucose uptake using specific inhibitors.

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

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

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

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 and accumulation as a monophosphorylated form, ZMP, which acts as an AMP-mimetic, promoting allosteric activation of AMPK and phosphorylation by an upstream kinase (43). L6E9 myotubes are not a good cellular model for AICAR trials since it appears to have low effect on AMPK α_1 and α_2 (not shown), so AMPK was activated by increasing intracellular AMP with DNP, an oxidative phosphorylation uncoupler. DNP stimulated AMPKα_1 and AMPKα_2 activities in L6E9 myotubes. Under these conditions, HRG did not alter the AMPK activity at any

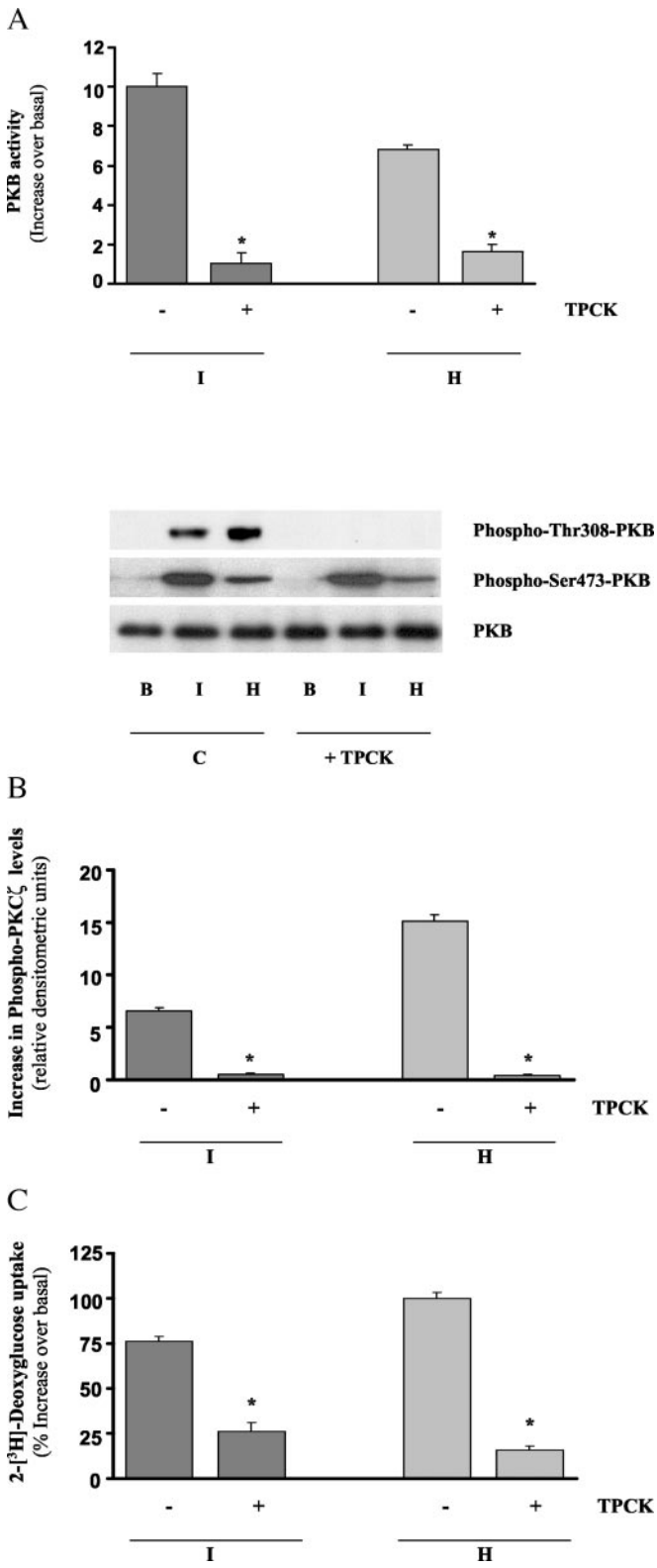

FIG. 3. **HRG-induced glucose uptake requires PDK1 activity.** L6E9 myotubes were treated with 3 nM HRG (15 min) or 1 μM insulin (10 min), in the absence or presence of the PDK1 inhibitor, 50 μM TPCK (35 min). *A*, total PKB activity was determined from one experiment assayed in triplicate. Cell lysates were also immunoblotted with antibodies that recognize total PKB (C-terminal) or PKB phosphorylated at Ser^{473} (hydrophobic motif) or Thr^{308} (activation loop). Similar results were obtained in three separate experiments. *B*, phospho-Thr^{410}-PKCζ was immunodetected and densitometrically quantified. Relative phospho-Thr^{410}-PKCζ levels are the mean of three independent experiments. *C*, L6E9 myotubes were treated with 3 nM HRG (90 min) or 1 μM insulin (30 min), in the absence or the presence of 50 μM TPCK (110 min). Then, the 2-deoxyglucose uptake assay was performed. Results are obtained from three different experiments. Basal uptake: 0.62 $\pm$ 0.03 pmol/μg of protein $\times$ 10 min. *B*, basal; *I*, insulin action; *H*, HRG action. * indicates significant differences with the control group, at $p <$ 0.001.

time or dose studied (Fig. 7*A*). Identical results were obtained in the H-$2K^b$ myocytes (48) (not shown).

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

Neuregulins 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. 8*A*).

Treatment with wortmannin (2 μM, 150 min) abolished both insulin and HRG action on glucose uptake in incubated strips of rat soleus (Fig. 8*B*), 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 PKCζ. 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, PKCζ, p70S6K, p38MAPK, and p42/p44MAPKs in L6E9 myotubes, that the pathway PI3K, PDK1, and PKCζ 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/PKCζ 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, Y*XX*M motifs that bind to the SH_2 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 Y*XX*M 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-phos-

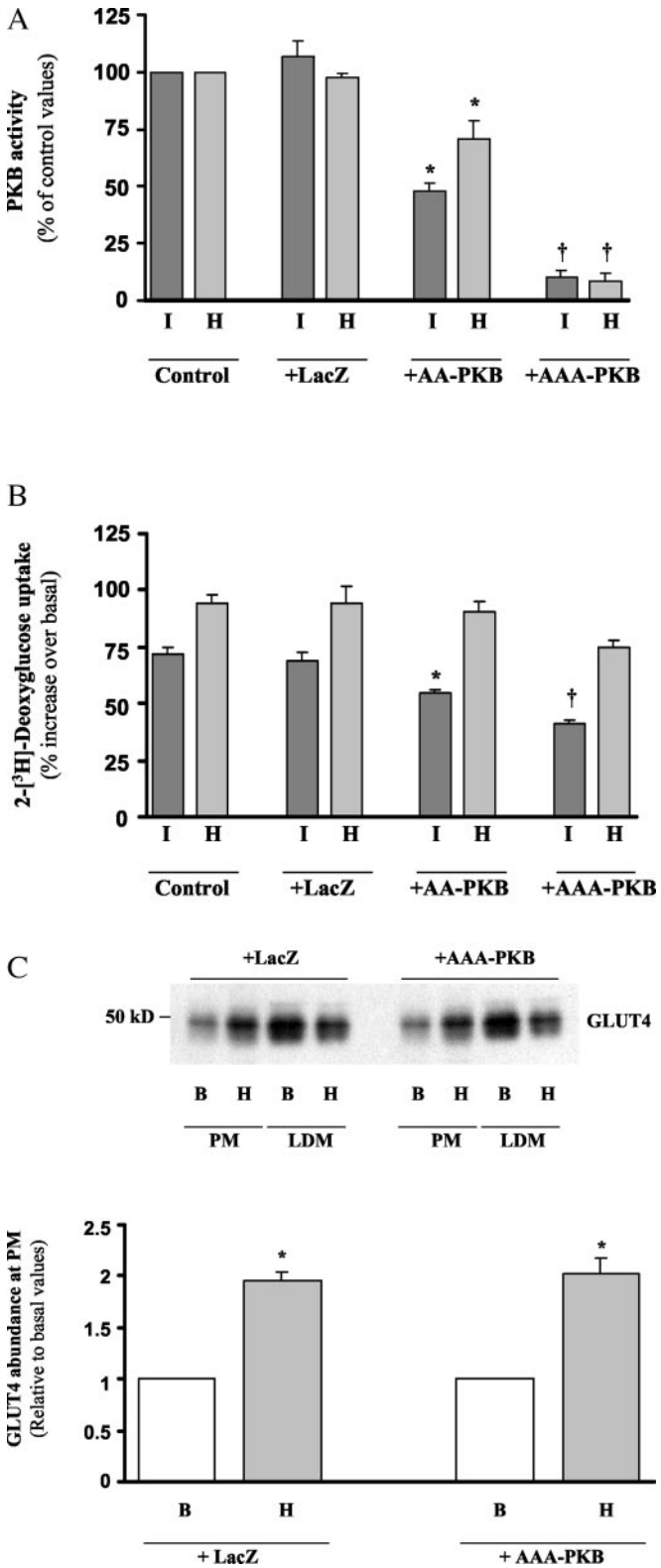

FIG. 4. **Effect of PKB inhibition on HRG-induced glucose uptake.** *A*, L6E9 myotubes were treated with 3 nM HRG (*H*) for 15 min or 1 μM insulin (*I*) for 10 min in the absence (*C*) or the presence of adenovirus (MOI 300, 48 h) containing cDNA for LacZ, as a control of viral infection, AA-PKB or AAA-PKB. 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 five different experiments performed in duplicate. Basal uptake (*C*): 0.64 ± 0.03 pmol/μg of protein × 10 min, no significant changes were observed with adenoviral transduction. *, $p < 0.05$ *versus* corresponding LacZ group. †, $p < 0.01$ *versus* both corresponding LacZ and AA-PKB groups. *C*, L6E9 myotubes were treated with HRG (3 nM, 90 min), in the absence or presence of LacZ or AAA-PKB adenoviruses (300 MOI, 48 h). Afterward, cells were scraped and homogenized for plasma membrane (*PM*) and intracellular light density microsomes (LDM) isolation. Membrane fractions were immunoblotted for GLUT4 and recruitment at the PM fraction was densitometrically quantified from three different experiments. *B*, basal; *H*, HRG action. *, $p < 0.001$ *versus* corresponding basal groups.

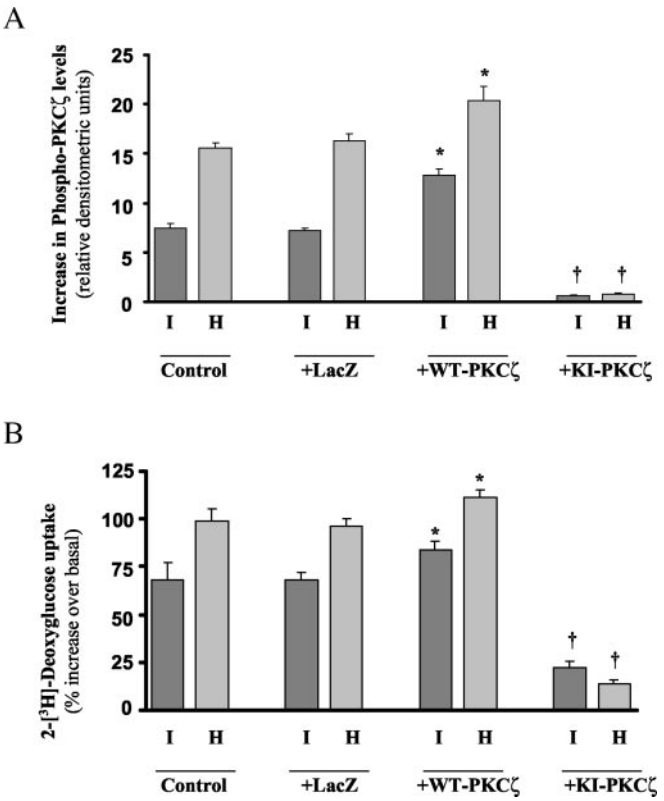

FIG. 5. **Effect of PKCζ inhibition on HRG-induced glucose uptake.** *A*, L6E9 myotubes were treated with 3 nM HRG (*H*) for 30 min or 1 μM insulin (*I*) for 10 min in the absence or the presence of adenovirus (MOI 100, 48 h) containing cDNA for LacZ, as a control of viral infection, wild type PKCζ (WT-PKCζ), or kinase inactive PKCζ (KI-PKCζ). Total PKCζ was immunoprecipitated from lysates, immunoblotted using an antibody that recognizes PKCζ phosphorylated at Thr^{410}-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.

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).[2] Current

[2] E. Suárez, J. Ryder, A. Zorzano, J. R. Zierath, and A. Gumà, unpublished observations.

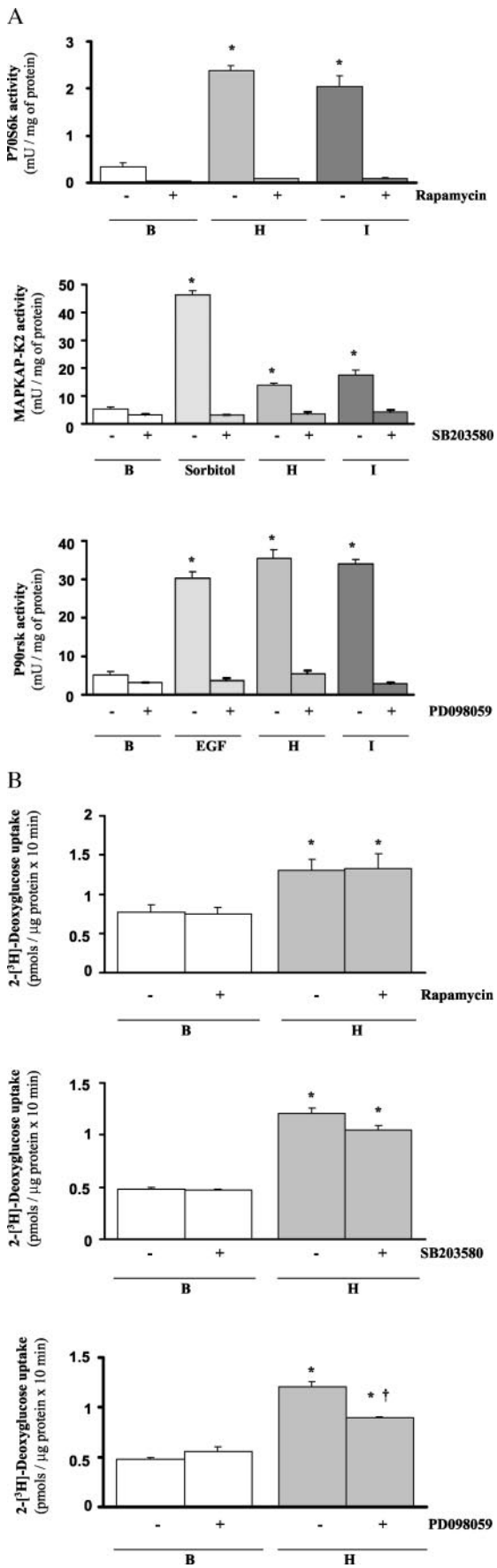

FIG. 6. **HRG stimulates P70S6K, p38MAPK, and p42/p44MAPKs.** L6E9 myotubes were treated with 3 nM HRG (90 min), 1 μM insulin (30 min), 0.5 M sorbitol (20 min) or 0.1 ng/ml rhEGF (20 min) in the absence or the presence of 1 ng/ml rapamycin (120 min), 10 μM SB203580 (120 min), or 50 μM PD098059 (120 min). *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, and assayed in triplicate. *B*, 2-deoxyglucose uptake was assayed in the absence or presence of rapamycin (five different experiments), SB203580 (four different experiments), or PD098059 (four different experiments), run in duplicate. Statistical significance: Student's paired *t* test referred to the control group. * indicates significant differences with the basal group, at $p < 0.01$. † indicates significant differences with the respective control, at $p < 0.05$.

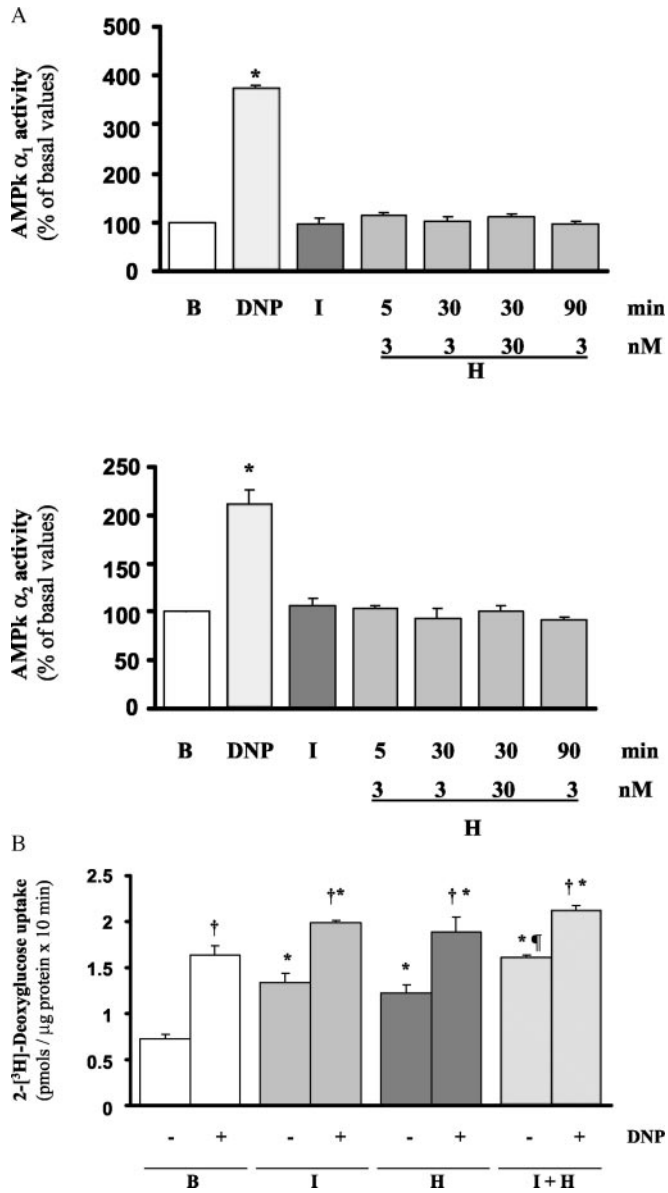

FIG. 7. **HRG does not activate AMPK.** Effect of DNP on HRG- and insulin-stimulated glucose uptake. L6E9 myotubes were treated with 3 or 30 nM HRG (5, 30, or 90 min), 1 μM insulin (30 min), or 0.5 mM DNP (60 min). *A*, AMPKα_1 and α_2 activities were assayed in L6E9 myotubes immunoprecipitated lysates (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 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.

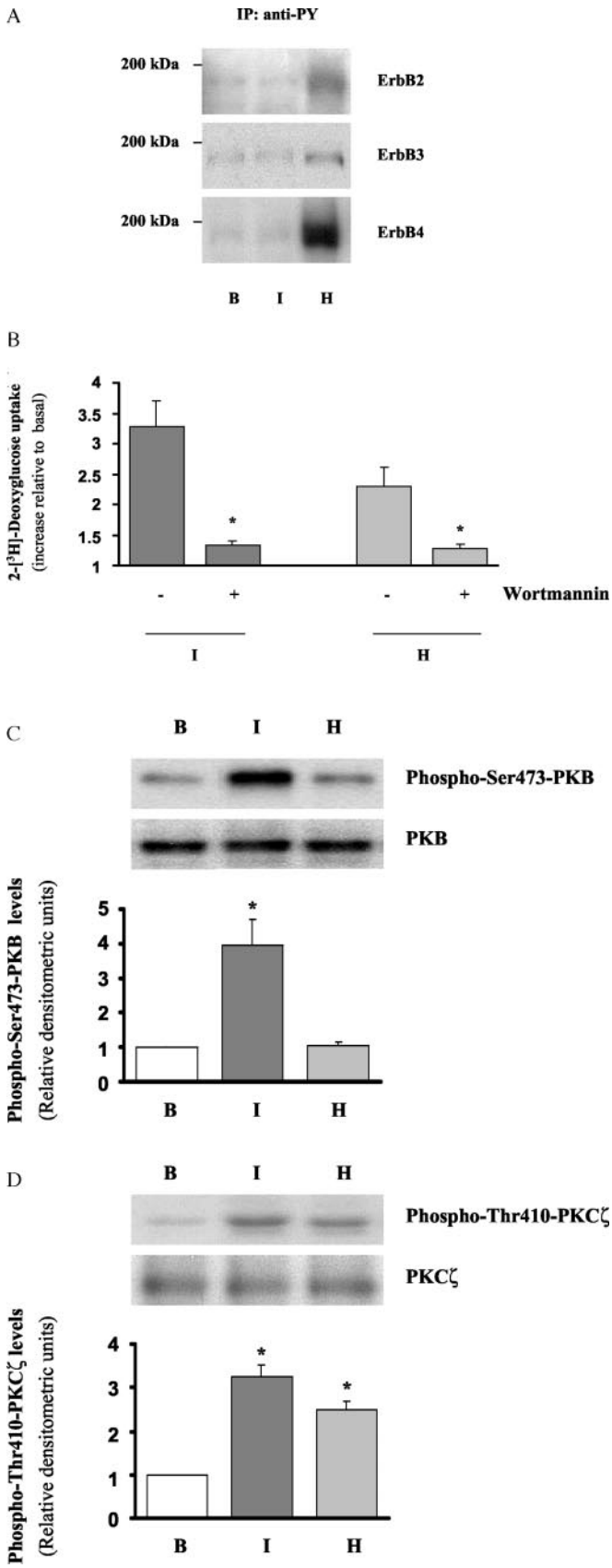

FIG. 8. **Effect of HRG on incubated strips of rat soleus muscle.** *A*, strips were incubated with insulin (100 nM, 10 min) or HRG (5 nM, 15 min). Lysates were obtained and immunoprecipitated with anti-phosphotyrosine antibody. Pellets were immunoblotted for ErbB2, ErbB3 and ErbB4. Results are shown 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* and *D*, 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-Ser^{473}-PKB, anti-PCKζ, and anti-phospho-Thr^{410}-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

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 Ser^{473}, 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.

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