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A Novel Role of Neuregulin in Skeletal Muscle

NEUREGULIN STIMULATES GLUCOSE UPTAKE, GLUCOSE TRANSPORTER TRANSLOCATION, AND TRANSPORTER EXPRESSION IN MUSCLE CELLS*

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Neuregulins regulate the expression of acetylcholine receptor genes and induce development of the neuromuscular junction in muscle. In studying whether neuregulins regulate glucose uptake in muscle, we analyzed the effect of a recombinant neuregulin, ᵣheregulin-β1-(177–244) (HRG), on L6E9 muscle cells, which express the neuregulin receptors ErbB2 and ErbB3. L6E9 responded acutely to HRG by a time- and concentration-dependent stimulation of 2-deoxyglucose uptake. HRG-induced stimulation of glucose transport was additive to the effect of insulin. The acute stimulation of the glucose transport induced by HRG was a consequence of the translocation of GLUT4, GLUT1, and GLUT3 glucose carriers to the cell surface. The effect of HRG on glucose transport was dependent on phosphatidylinositol 3-kinase activity. HRG also stimulated glucose transport in the incubated soleus muscle and was additive to the effect of insulin. Chronic exposure of L6E9 cells to HRG potentiated myogenic differentiation, and under these conditions, glucose transport was also stimulated. The activation of glucose transport after chronic HRG exposure was due to enhanced cell content of GLUT1 and GLUT3 and to increased abundance of these carriers at the plasma membrane. However, under these conditions, GLUT4 expression was markedly down-regulated. Muscle denervation is associated with GLUT1 induction and GLUT4 repression. In this connection, muscle denervation caused a marked increase in the content of ErbB2 and ErbB3 receptors, which occurred in the absence of alterations in neuregulin mRNA levels. This fact suggests that neuregulins regulate glucose transporter expression in denervated muscle. We conclude that neuregulins regulate glucose uptake in L6E9 muscle cells by mechanisms involving the recruitment of glucose transporters to the cell surface and modulation of their expression. Neuregulins may also participate in the adaptations in glucose transport that take place in the muscle fiber after denervation.

Skeletal muscle is the main tissue that contributes to glucose disposal in absorptive conditions. A limiting step in this process is glucose transport, which is mediated by different glucose transporters; GLUT1 is responsible for basal transport and GLUT4 is responsible for insulin- or exercise-stimulated glucose transport through translocation to the plasma membrane (1). GLUT1 is highly expressed in fetal muscle, but during perinatal life it is markedly repressed, whereas GLUT4 is induced (2). This effect is temporally coincident with the process of innervation of the muscle fiber (3). Therefore, denervated muscle shows a decrease in GLUT4 and an increase in GLUT1 expression (3–7). A program of electrical stimulation of the denervated muscle prevents GLUT4 gene repression (3), which suggests that basal contractile activity dependent on innervation regulates the expression of GLUT4 in skeletal muscle. Furthermore, GLUT4 is more sensitive than GLUT1 to the lack of muscle contraction (5, 8), and the extent of induction of GLUT1 depends mainly on the fiber type (6, 7, 9).

Neuregulins are a family of closely related products encoded by a single gene, neuregulin-1 (10). In the last few years, other related genes have been identified (neuregulin-2, -3, and -4). They were isolated initially from *ras*-transformed mouse fibroblasts (*neu* differentiation factor (NDF)) (11, 12) and human breast cancer cells (heregulin) (13). Three other factors were isolated from neural sources: acetylcholine receptor-inducing activity from chicken brain (14), glial growth factor from bovine brain (15, 16), and sensory and motor neuron-derived factor (17). More than 15 distinct isoforms arise by alternative splicing and cell type-specific transcription initiation sites (reviewed in Refs. 18 and 19). Two major groups can be distinguished on the basis of whether they are membrane-associated or soluble isoforms. The first group of neuregulins contain a transmembrane domain and a cytosolic tail, reside as membrane proteins, and are released to the extracellular milieu after proteolytic cleavage. Most of the released forms contain an N-terminal Ig-like domain that binds to the glycosaminoglycan portion of proteoglycans in the extracellular matrix. Common to all isoforms, there is a C-terminal EGF[1]-like domain defined by six cysteine residues that fold the domain into compact, protease-resistant β-sheets by forming three disulfide bonds. The EGF-like domain is sufficient to elicit biological responses. After proteolysis, the EGF-like domain is released

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[1] The abbreviations used are: EGF, epidermal growth factor; HRG, ᵣheregulin-β1-(177–244); IRAP, insulin-responsive aminopeptidase; SCAMPs, secretory component-associated membrane proteins; VAMPs, vesicle-associated membrane proteins; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SN, supernatant; PM, plasma membrane; LDM, low density vesicle membrane; PCR, polymerase chain reaction.

and binds to members of the ErbB family of tyrosine kinase receptors, ErbB3 (HER3) and ErbB4 (HER4). ErbB4 shows ligand-stimulated tyrosine kinase activity. ErbB3, however, is a tyrosine kinase-deficient receptor because an aspartate and a glutamate in the kinase domain that are critical for autophosphorylation are replaced by other residues. Neuregulin binding to ErbB3 signals through heterodimerization with ErbB2 (HER2, c-*neu*), which displays tyrosine kinase activity (20, 21).

Neuregulins have major effects on the growth and development of epithelial cells (22), and generation of knockout mice has demonstrated that they are essential for the development of cranial nerve, ganglia, and Schwann cell precursors along peripheral nerves in the trunk (23). In the hearts of mutant embryos for neuregulin, ErbB2 and ErbB4, ventricular trabeculation does not occur, which results in developmental arrest and embryo death at embryonic day 10.5 (23–25). Neuregulins also affect the biology of skeletal muscle; they are potent activators of the expression of acetylcholine receptors (26–28). In addition, the neuregulins GGF2 and NRGα1 activate myogenic differentiation (29, 30). There is also evidence for the operation of a neuregulin-ErbB3 autocrine signaling pathway during an early stage of myoblast differentiation (30). In the mature muscle fiber, ErbB2 and ErbB3 are concentrated at the neuromuscular junction, and therefore it is thought that neuregulins regulate the protein composition and the functioning of the neuromuscular junction (26, 27, 31–33).

Here we examined the effects of the neuregulin ,heregulin-β1-(177–244) on glucose uptake in L6E9 muscle cells and the mechanism involved. Our results indicate that HRG stimulates glucose transport and translocates glucose transporters to the cell surface in muscle cells. Additionally, chronic exposure to HRG also stimulates glucose transport and causes alteration of the expression pattern of glucose transporters in muscle cells. Our data are compatible with a model in which neuregulins regulate glucose disposal in or near the neuromuscular junction in innervated muscle fiber. In addition, neuregulin may also participate in the adaptations in glucose uptake that take place in the muscle fiber after denervation.

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). Recombinant heregulin (,heregulin-β1-(177–244) (HRG)) was obtained from Genentech, Inc. (South San Francisco, CA). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), glutamine, and antibiotics were purchased from BioWhittaker (Walkersville, MD). Most commonly used chemicals and wortmannin were from Sigma. Pepstatin, leupeptin, and aprotinin were from ICN (Costa Mesa, CA). Immobilon polyvinylidene difluoride was obtained from Millipore Corp. (Bedford, MA). Purified porcine insulin was a kind gift from Lilly Co. ECL reagents were from Amersham Pharmacia Biotech UK (Little Chalfont, Buckinghamshire, UK). 2-Deoxy-D-[^{3}H]glucose was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). 2-Deoxy-D-[^{14}C]glucose, D-[^{3}H]mannitol, and the tissue solubilizer Protosol were obtained from PerkinElmer Life Sciences. All chemicals were of the highest purity grade available. Bradford reagent and all electrophoresis agents and molecular weight markers were obtained from Bio-Rad (Hercules, CA). Polyclonal antibody OSCRX (raised against the 15 C-terminal amino acid residues from GLUT4) was produced in our laboratory by Dr. Conxi Mora. Anti-GLUT1 antibody was purchased from Diagnostic International (Karlsdorf, Germany). Anti-rat/mouse GLUT3 antibody was kindly provided by Dr. Gwyn W. Gould (University of Glasgow, UK). Anti-ErbB3 (C-17) and ErbB2 (Neu) (C-18) antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Monoclonal antibodies against myosin heavy chain (MF20) and against α_1-Na^+/K^+-ATPase (α6F) were purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-IRAP and anti-SCAMPs antibodies (3F8) were kindly provided by Dr. Paul Pilch (Boston University, Boston, MA). Anti-VAMP2 and anti-cellubrevin antibodies were kindly provided by Dr. Joan Blasi (Universitat de Barcelona). Tissue culture material was purchased from Corning Inc.

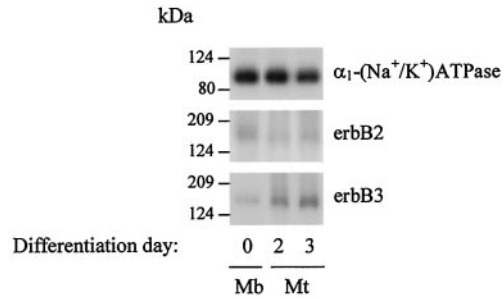

FIG. 1. **ErbB2 and ErbB3 expression in L6E9 cells.** Total membranes from L6E9 cells at different stages of differentiation were obtained and processed for Western blot assay. 25 μg of protein was loaded to each *lane*. The abundance of ErbB2 and ErbB3 was determined by using specific polyclonal antibodies. The specificity of the recognition in Western blot was assayed by incubation of the antibodies in the presence or absence of antigenic peptides (data not shown). Cells at day 0, pre-confluent myoblasts (*Mb*) (80–90% of confluence); day 2, young myotubes (*Mt*); day 3, mature myotubes. Autoradiograms representative of three different experiments are shown.

Cell Culture—L6E9 myoblasts were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics (10,000 units/ml of penicillin G and 10 mg/ml streptomycin), 2 mM glutamine, and 25 mM Hepes (pH 7.4). Pre-confluent myoblast (80–90%) were induced to differentiate by lowering FBS to final concentration of 2% (v/v).

To analyze the acute effect of HRG on glucose transport and transporters, fully differentiated myotubes were depleted of serum containing 0.2% bovine serum albumin for 4.5 h. 90 min before the end of this period, HRG was added (except in the time course experiments), and when insulin action was studied, it was added for the last 30 min of this period. For the chronic HRG treatment, HRG was added 24 h after the cells were changed to the low serum medium, and studies were carried out after 1, 2, or 3 days of HRG treatment.

2-Deoxy-D-[^{3}H]Glucose Uptake—Cells were cultured on 6-well plates. Transport (34) was initiated by washing the cells twice in a transport solution (20 mM Hepes, 137 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 2 mM pyruvate, pH 7.4). Cells were then incubated for 10 min with a transport solution that contained 0.1 mM 2-deoxy-D-glucose and 1 μCi of 2-deoxy-D-[^{3}H]glucose uptake (10 mCi/mmol). To determine background labeling, we incubated the cells for 10 min with ice-cold 50 mM glucose in phosphate-buffered saline buffer (PBS) containing the same specific activity of 2-deoxy-D-[^{3}H]glucose. Uptake was stopped by the addition of 2 volumes of ice-cold 50 mM glucose in PBS. Cells were washed twice in the same solution and disrupted with 0.1 M NaOH, 0.1% SDS. Radioactivity was determined by scintillation counting. Protein was determined by the Bradford method. Each condition was run in duplicate or triplicate. Glucose transport was linear during the period assayed (data not shown).

Preparation of Homogenates and Membrane Fractions from L6E9 Myocytes—Homogenates were obtained from cells cultured on 6-well plates at 2, 3, or 4 days of differentiation with one plate from each group. Cells were placed on ice, washed twice in ice-cold PBS, and scraped into 2 ml of PBS. Cells were pelleted at 3,000 rpm for 5 min and resuspended in 300 μl of lysis buffer (20 mM Hepes, 350 mM NaCl, 20% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, pH 7.9) containing freshly added protease inhibitors (1 mM dithiothreitol, 0.1% (v/v) phenylmethylsulfonyl fluoride, 0.1% (v/v) aprotinin). The homogenate was shaken for 30 min at 4 °C and centrifuged at 1,000 rpm for 20 min, and the supernatant (SN) was collected and kept at −20 °C. For total membrane preparation (35), 3–4 10-cm dishes were used for each experimental group. Cells were washed and scraped into 10 ml of PBS as described previously and pelleted at 1,000 rpm for 5 min. They were then resuspended in 3 ml/dish of cold homogenization buffer (250 mM sucrose, 2 mM EGTA, 5 mM sodium azide, 20 mM Hepes, pH 7.4) containing freshly added protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin) and homogenized in a glass Dounce homogenizer (pestle A, 20 strokes). Homogenate was centrifuged at 2000 rpm for 5 min, and the supernatant (SN_1) was kept. The pellet was resuspended in 1.5 ml of homogenization buffer/dish, re-homogenized, and centrifuged in the same way. The new pellet was discarded, and the supernatant (SN_2) was pooled with SN_1 and centrifuged at 190,000 × *g* for 1 h 10 min. The pellet (total membranes) was collected and resuspended in 250 μl of 20 mM Hepes,

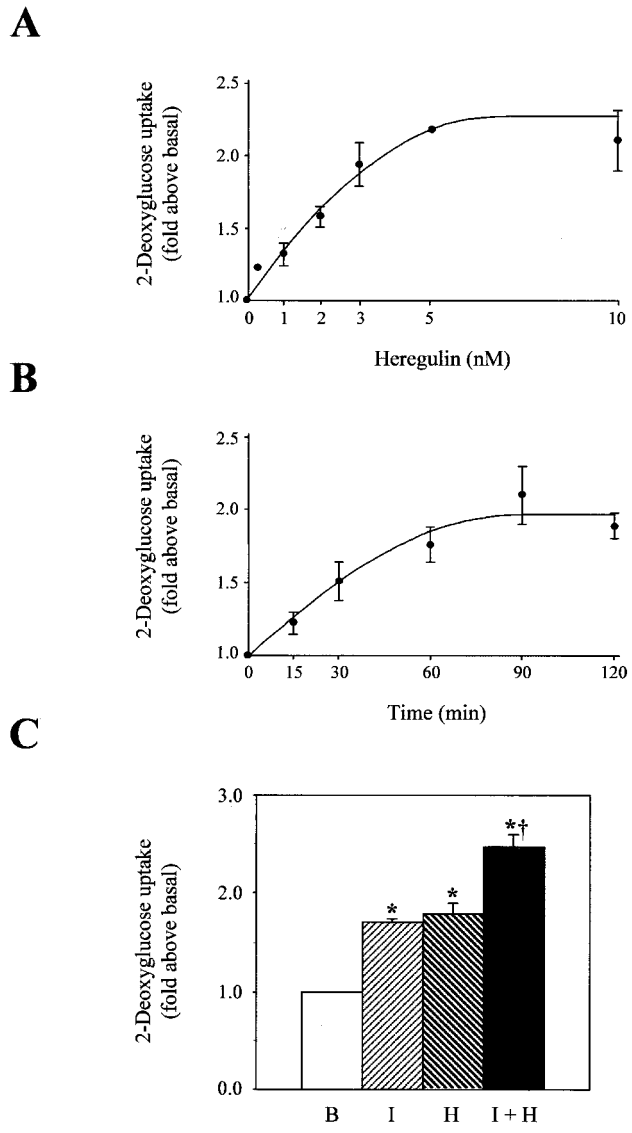

FIG. 2. **Acute effects of HRG on 2-deoxyglucose uptake in L6E9 myotubes.** Cells were seeded on 6-well plates for 2-deoxyglucose uptake studies. Fully differentiated L6E9 myotubes were serum-depleted for 4.5 h prior to 2-deoxyglucose uptake assays. *A*, cells were treated during the last 90 min with different HRG concentrations ranging from 0 to 10 nM. *B*, cells were assayed at 3 nM HRG during different times ranging from 15 to 120 min. 2-Deoxyglucose uptake values are expressed per μg of protein after subtraction of background values. Results are the mean ± S.E. of 3–12 observations per group and are expressed as relative values (treated above nontreated cells transport). Basal transport rates were (dose-response studies) 1.55 ± 0.21 and (time course studies) 1.13 ± 0.11 pmol of 2-deoxyglucose/μg protein × 10 min. *C*, cells were treated with 3 nM HRG (*H*; during the last 90 min of incubation), with 1 μM insulin (*I*; during the last 30 min of incubation), or with both insulin and HRG (*I* + *H*). 2-Deoxyglucose uptake values were expressed per μg of protein after subtraction of background values. Results are the mean ± S.E. of 3–4 observations/group. *, indicates significant differences with basal group at $p < 0.01$; †, indicates significant differences with the insulin- or HRG-treated groups at $p < 0.01$ (Student's *t* test). Basal transport rate was 0.97 ± 0.10 pmol of 2-deoxyglucose/μg protein × 10 min. *B*, basal.

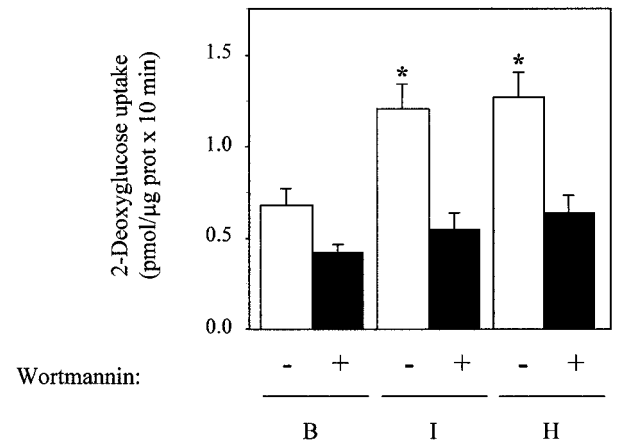

FIG. 3. **Effects of wortmannin on heregulin-stimulated glucose uptake in L6E9 myotubes.** Differentiated myotubes were serum-depleted for 4.5 h before to the assay of 2-deoxyglucose uptake. Cells were treated (*filled bars*) or not (*open bars*) with wortmannin (1 μM) for 120 min in basal conditions (*B*), in HRG-treated cells (*H*, 3 nM, 90 min), or in insulin-treated cells (*I*, 1 μM, 30 min). Results are the mean ± S.E. of 7–11 different experiments. *, indicates significant differences with basal group at $p < 0.01$. *prot*, protein.

pH 7.4. When membrane fractionation was required (35), cells were cultured on 2–4 15-cm dishes for each experimental group and treated initially as described above. The pooled SN_1 and SN_2 were centrifuged at 24,000 × *g* for 1 h. The pellet, a partially purified plasma membrane (PM) fraction, was resuspended in 500 μl of 20 mM Hepes, pH 7.4, and the SN was further centrifuged at 190,000 × *g* for 1 h. The new pellet, a low density fraction (LDM) of intracellular origin, was resuspended in 200 μl of Hepes solution.

Fusion Index—Cells were cultured in 10-cm dishes. After treatments, cells were washed twice in cold PBS, and 1 mM $ZnSO_4$ in 20% dimethyl sulfoxide was added at room temperature and incubated for 1 min. Cells were then washed gently in cold PBS and fixed with 2.5% glutaraldehyde in PBS for 2 min. Cells were then treated for 1 min with 50% ethanol and rinsed with PBS before staining with filtered 0.04% Giemsa in PBS, pH 6.8, overnight. Finally, cells were rinsed with tap water. Under an optical microscope, several randomly chosen fields were photographed, and the nuclei per cell were counted.

Animals and Tissue Sampling—For studies of incubated soleus muscle, male Wistar rats (250 g) were anesthetized with pentobarbital, 5–7 mg/100 g of body weight, and strips were isolated by a modification of the method of Crettaz *et al.* (36).

For denervation studies, the peroneal nerve of anesthetized male rats (ketamine, 20 mg/kg of body weight) was severed unilaterally. Three days after denervation, tibialis anterior and extensor digitorum longus muscles were dissected and frozen in liquid nitrogen. All procedures were reviewed and approved by the local ethics committee. Muscles were processed to obtain total membranes as reported previously (37).

Glucose Transport by Strips of Soleus Muscles—Isolated strips of soleus muscles were incubated as reported (38). HRG (3 nM, 120 min) was added after 30 min of muscle incubation, and 1 h later, insulin (100 nM, 60 min) was added to the medium. Previous to the uptake period, muscles were washed for 10 min with a glucose-depleted medium. Thereafter, muscles were incubated in the presence of 2-deoxy-D-[^{14}C]glucose uptake (1 mCi/mmol) and D-[^{3}H]mannitol (0.5 mCi/mmol), as an extracellular space marker, for 20 min, the time in which linear conditions are maintained. Muscles were then frozen and processed as reported (38).

Electrophoresis and Immunoblotting of Membranes—SDS-polyacrylamide gel electrophoresis was performed on membrane protein. Proteins were transferred to Immobilon in buffer consisting of 20% methanol, 200 mM glycine, 25 mM Tris, pH 8.3. After transfer, the filters were blocked with 5% nonfat dry milk in Tris-buffered saline solution for 1 h at room temperature and then incubated overnight at 4 °C with antibodies directed against GLUT4 (1:800), GLUT1 (1: 1300), GLUT3 (1: 500), ErbB3 (1:500), ErbB2 (1:500), myosin heavy chain (1:20), α_1-subunit of the Na^+/K^+ ATPase (1:100), IRAP (1/1000), SCAMPs (1/3000), VAMP2 (1/1000), and cellubrevin (1/500), all diluted in 1% (w/v) bovine serum albumin, 0.067% (w/v) sodium azide in Tris-buffered saline, 0.09% (v/v) Tween 20. The immune complex was detected using an ECL chemiluminescence system. The autoradiograms were quantified using scanning densitometry. Immunoblots were performed in conditions in which autoradiographic detection was in the linear response range.

Reverse Transcriptase-PCR from Total RNA—cDNA was synthesized from 2 μg of total RNA at 20 μl of final reaction volume the using SuperScript II Rnase H$^-$ Reverse Transcriptase System (Life Technologies, Inc.). $(dT)_{15}$ was used as the primer at 0.4 μM. Genomic contamination was monitored by enzyme-free controls. The resulting cDNA was diluted 1/10, and 1 μl of this dilution was amplified by PCR in an MJ Research PT-100 thermocycler at 25 μl final reaction volume. PCR primers 5′-TCAGAGCTTCGAATTAACAAAGC-′3 and 5′-GTGGTCATGGCTGATAGATAC CT-′3 (Life Technologies, Inc.), corresponding to

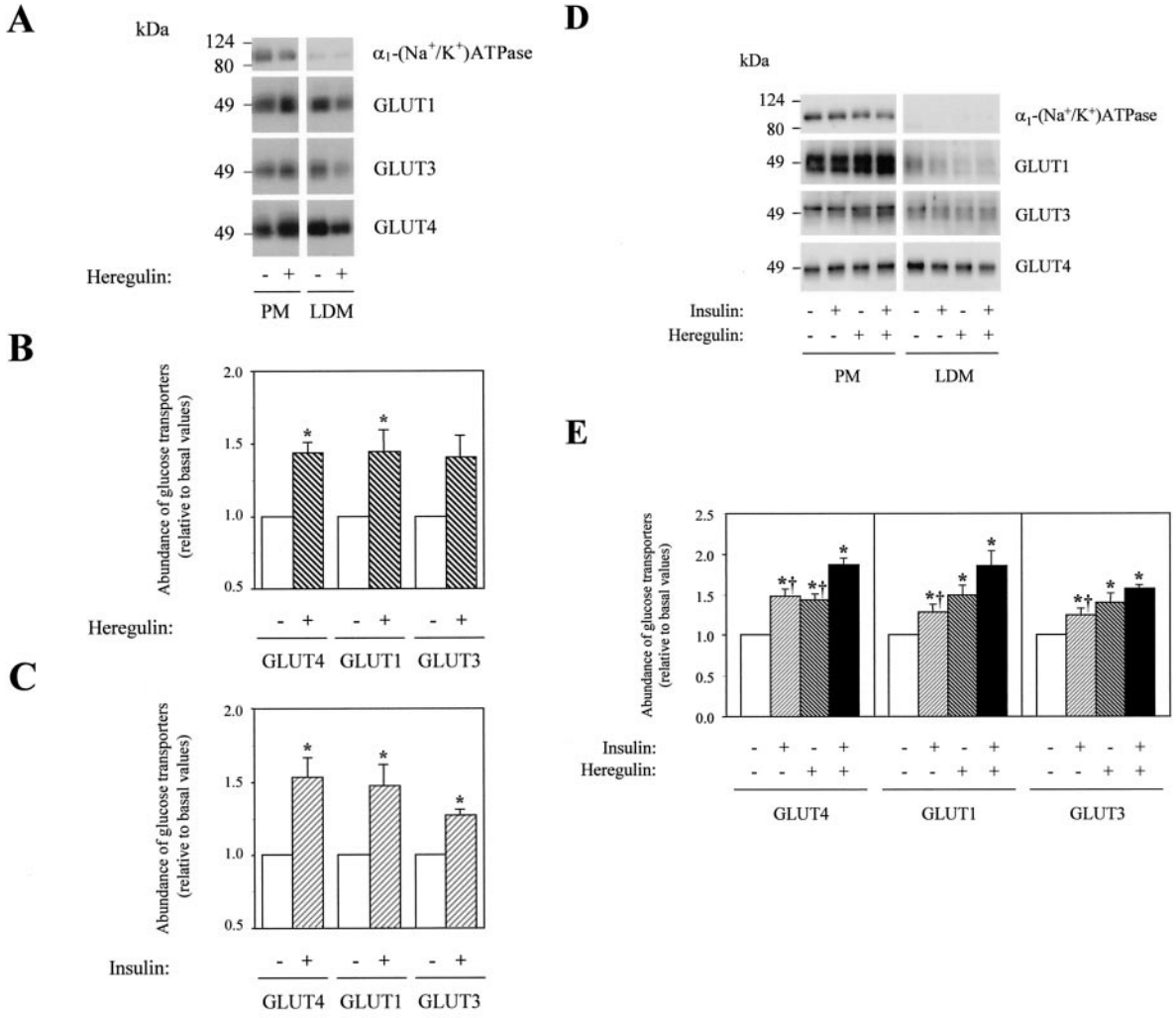

FIG. 4. **Effect of heregulin and insulin on redistribution of glucose transporters in L6E9 myotubes.** L6E9 myotubes were serum-depleted for 4.5 h and thereafter treated or not during the last 90 min with 3 nM HRG (*A* and *B*), during the last 30 min with 1 μM insulin (*C*), or with a combination of both effectors (*D* and *E*). PM and LDM fractions were obtained and assayed by Western blot to determine the abundance of GLUT1, GLUT3, and GLUT4 glucose transporters. *Panel A* shows autoradiograms from a representative experiment (25 μg of protein/lane). *Panel B* shows the values of the densitometry corresponding to the abundance of glucose transporters in plasma membrane fractions after incubation with HRG; the results are the mean ± S.E. of 3 observations per group. *Panel C* shows the values corresponding to the abundance of glucose transporters in plasma membrane fractions after incubation with insulin; the results are the mean ± S.E. of 4–5 observations per group. *Panel D* shows autoradiograms of a representative experiment (20 μg of protein/lane). *Panel E* shows the values of the densitometry corresponding to the abundance of glucose transporters in plasma membrane fractions after incubation with HRG and insulin; results are the mean ± S.E. of 6 observations per group. *, indicates significant differences compared with the basal group at $p < 0.05$ (Student's *t* test). †, indicates significant differences compared with the insulin + HRG group at $p < 0.05$ (Student's *t* test).

627–649 and 1608–1630 base pairs, respectively, of rat NDF (sequence with total identity to all neuregulin isoforms) were added at 0.4 μM and dNTPs at 0.2 mM. 1.25 units of *Taq* Expand High Fidelity and its corresponding buffer with Mg^{2+} were used (Roche Molecular Biochemicals). PCR was performed as follows: an initial step of 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 2 min at 72 °C; and a final step of 5 min at 72 °C. Nonsaturating conditions were ensured by previous assays with the same cDNAs samples subjected to different number of PCR cycles (20 to 35) and in which the maintenance of linearity was determined (data not shown). For electrophoretic analysis, 5 μl of the final reaction volume was loaded in 1.5% agarose gel.

RESULTS

Expression of ErbB2 and ErbB3 Receptors in L6E9 Muscle Cells—As a first step, we assayed the level of neuregulin receptors ErbB2 and ErbB3 in total membrane extracts obtained from L6E9 myoblasts and cells after 2 and 3 days of differentiation induced by exposure to low serum conditions (Fig. 1). The ErbB2 content was maximal in myoblasts, and expression was somewhat reduced during differentiation. In contrast, ErbB3 levels were low in myoblasts but rose during differentiation (a 2-fold increase on day 3 of differentiation) (Fig. 1). Under these conditions, the abundance of the α_1 subunit of the Na^+/K^+-ATPase remained unaltered, indicating that the pattern of changes detected for neuregulin receptors was specific. Our results indicate the presence of neuregulin receptors in L6E9 muscle cells, which is consistent with reports in different muscle cell lines (25, 32).

Heregulin Acutely Stimulates Glucose Transport and Glucose Transporter Translocation in Muscle—Fully differentiated L6E9 myotubes were incubated with different concentrations of HRG for 90 min (Fig. 2*A*). HRG stimulated glucose transport with half-maximal effects detected at 2 nM HRG and maximal effects (2–2.5-fold stimulation of glucose transport) observed between 3 and 5 nM HRG, which is consistent with other reports on the effects of neuregulins (26, 30, 39). Time course studies were also performed at maximal concentrations of HRG (3 nM) (Fig. 2*B*). The maximal effect of HRG on glucose transport was reached between 60 and 90 min of incubation.

The effect of a maximal concentration of HRG (3 nM for 90

min) on glucose transport was comparable with the effect caused by a supramaximal concentration of insulin (1 μM for 30 min) (Fig. 2*C*). Furthermore, the combination of HRG and insulin caused an additive stimulation of glucose transport (Fig. 2*C*).

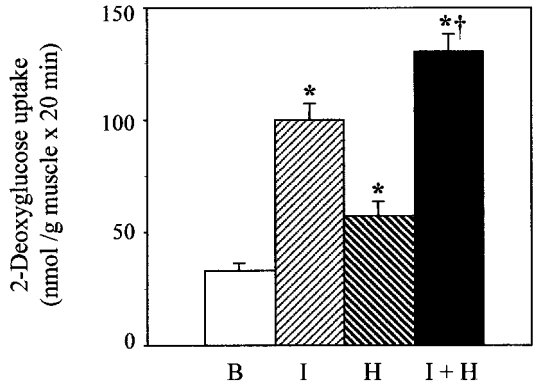

FIG. 5. **Effect of HRG on glucose uptake in the incubated soleus muscle.** Muscle strips were incubated in the presence of insulin (*I*, 100 nM, 60 min), HRG (*H*, 3 nM, 120 min), or both (*I* + *H*). Following 2-deoxyglucose uptake, muscles were digested, and radioactivity was measured in a β-counter. Results are the mean ± S.E. of 3–5 separate experiments. *, indicates significant differences with basal group at $p < 0.05$; †, indicates significant differences between the insulin and HRG groups at $p < 0.05$ (Student's *t* test).

To determine whether the effect of HRG on glucose transport required phosphatidylinositol 3-kinase activity, wortmannin (1 μM) (40) was added 30 min prior to HRG. Insulin-stimulated glucose transport was inhibited by wortmannin, and under these conditions wortmannin also blocked HRG-induced glucose transport (Fig. 3).

Incubation of L6E9 myotubes for 90 min in the presence of HRG did not alter the cell content of GLUT4, GLUT1, or GLUT3 (data not shown). In the next step, L6E9 myotubes were incubated in the absence or presence of 3 nM HRG for 90 min and then were further subjected to subcellular fractionation of membranes. This yielded two membrane fractions: PM, which were highly enriched in plasma membrane markers such as the α_1 subunit of the Na^+/K^+-ATPase, and LDM, which are of intracellular origin. A typical experiment starting with four 15-cm dishes yielded ~3–5 mg of membrane proteins in the PM fraction and 0.5–1.5 mg of membrane proteins in the LDM fraction. Incubation of cells for 90 min in the presence of HRG caused a 43% increase in the abundance of GLUT4 in PM fractions and a 50% decrease in GLUT4 in LDMs (Fig. 4, *A* and *B*), consistent with HRG-induced GLUT4 translocation. HRG also caused a 45 and a 40% increase in the abundance of GLUT1 and GLUT3 proteins, respectively, in PM, which sug-

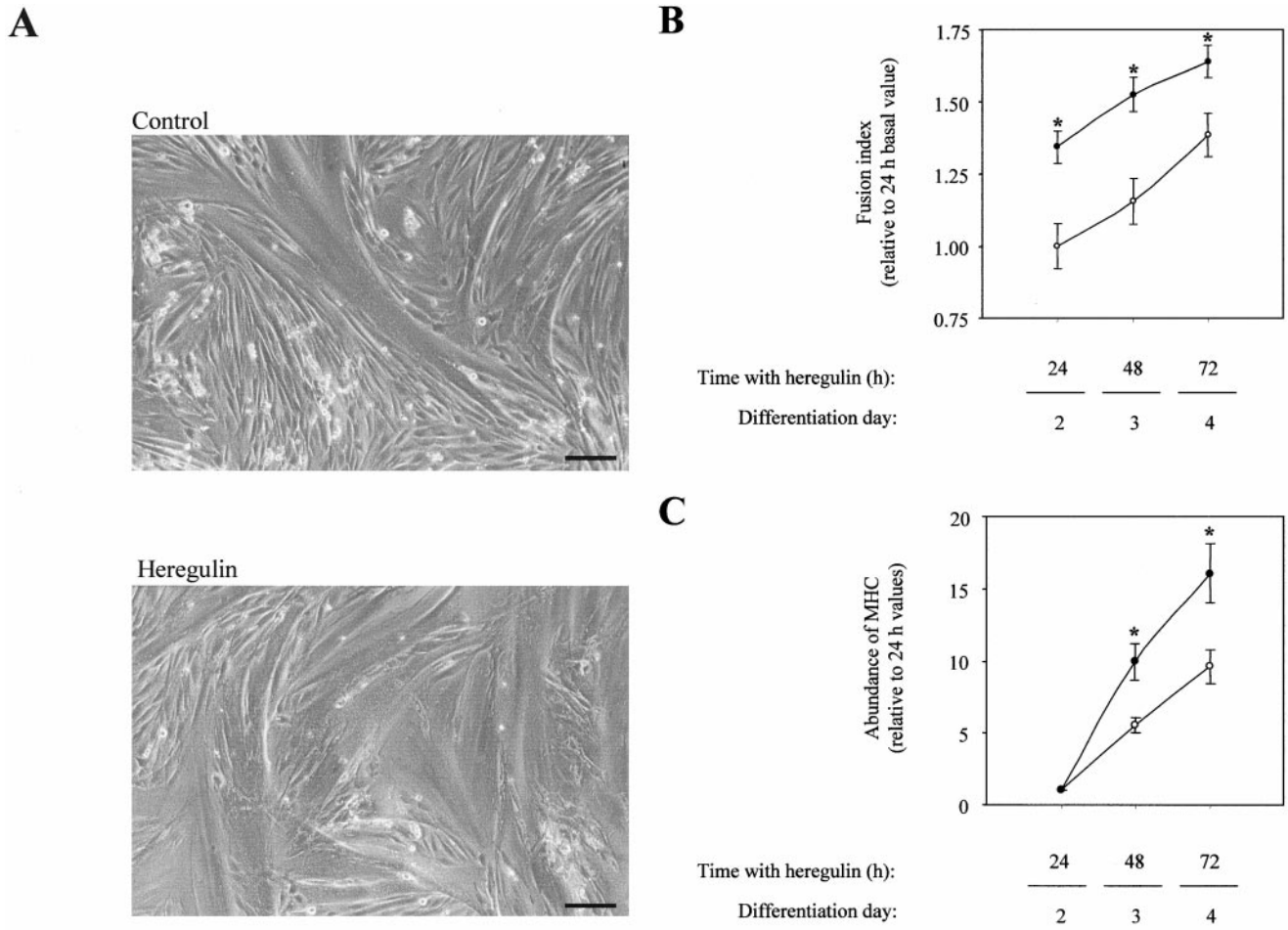

FIG. 6. **Effect on HRG on myotube formation.** L6E9 cells proliferated for 2 days at 10% FBS. Then the cells were cultured at 2% FBS to allow them to differentiate. After 24 h of differentiation, cells were incubated in the presence or absence of 3 nM HRG for different times. *A*, control cells and cells treated with HRG for 48 h are shown. The *bar* indicates 28 μm. *B*, fusion index studies were done in glutaraldehyde-fixed cultures where nuclei were stained with Giemsa. The fusion index was calculated as the number of nuclei/cell in a determined area. At least 10 different fields were randomly analyzed from each plate. *Open symbols*, nontreated cells; *filled symbols*, HRG-treated cells. Results are the mean ± S.E. of 3 independent experiments; they are shown as values relative to control cells on differentiation day 2. *C*, homogenates from control (*open circles*) and 3 nM HRG-treated (*filled circles*) cells were obtained and processed by Western blot to determine myosin heavy chain (*MHC*) expression. Three different experiments were run. Results are shown as absolute increments with respect to day 2 of differentiation. *, differences between control and HRG-treated groups were statistically significant at $p < 0.05$ (Student's *t* test).

gests that HRG also redistributed GLUT1 and GLUT3 to the cell surface (Fig. 4, *A* and *B*). The effects of HRG were specific, and the abundance of the α_1 subunit of Na^+/K^+-ATPase was unaltered after incubation with HRG (Fig. 4*A*). HRG and insulin (1 μM for 30 min) had a similar effect on all glucose transporters (Fig. 4, *B* and *C*). The combination of HRG and insulin (Fig. 4, *D* and *E*) caused an additional stimulation of GLUT4, GLUT1, and GLUT3 translocation in muscle cells (87, 86, and 57% increase at PM, respectively). In all, our data indicate that HRG causes translocation of glucose transporters to the cell surface and that HRG is at least as potent as insulin in activating glucose transport and glucose transporters translocation in L6E9 myotubes.

To determine whether HRG affects glucose transport in rat skeletal muscle, strips of soleus muscles were incubated in the absence or presence of HRG and/or insulin. HRG induced a 73% increase on glucose uptake. HRG and insulin showed additive effects on glucose transport (Fig. 5).

Chronic Incubation with Heregulin Stimulates Glucose Transport and Regulates Transporter Expression in Muscle Cells—Next, we studied the effect of chronic incubation with HRG on glucose transport in L6E9 cells. To this end, preconfluent cells were induced to differentiate by decreasing the serum content (2% FBS), and after 1 day, 3 nM HRG was added for 24, 48, or 72 h. Cells treated with HRG showed enhanced myotube formation (Fig. 6*A*), which was detected after 24 h of HRG addition (fusion index values were enhanced 34, 32, and 18% at 24, 48, or 72 h, respectively, after heregulin addition) (Fig. 6*B*). An enhanced rate of cell fusion induced by neuregulins is in agreement with previous reports in other muscle cell lines (29, 30). Chronic HRG caused an enhanced expression of myosin heavy chain (*MHC*, Fig. 6*C*), a late myogenic protein. GLUT4 is also induced during late myogenesis, showing high levels of expression after 3 days of differentiation (Fig. 7*A*). However, long-term incubation with HRG reduced GLUT4 expression 60% after 72 h (Fig. 7). In contrast, HRG increased the expression of GLUT1 and GLUT3 with maximal effects at 48 h of treatment (60 and 36% increases, respectively) (Fig. 7*A*).

The effect of HRG on GLUT4 expression in L6E9 was specific, and the abundance of other components of GLUT4 vesicles such as IRAP (41), SCAMPs (42), VAMP2 (43), or cellubrevin (44) remained unaltered by HRG (Fig. 7*B*).

Exposure to HRG for 48 h stimulated glucose transport in L6E9 myotubes to the same extent as insulin, and when both factors where tested together, the effect was additive (Fig. 8*A*). Western blot analysis of plasma membranes showed an increase in GLUT1 and GLUT3 and a decrease in GLUT4 from HRG-treated cells (Fig. 8*B*).

Expression of ErbB2 and ErbB3 Receptors in Denervated Muscle—The induction of GLUT1 and repression of GLUT4 in L6E9 muscle cells resemble the effects observed upon denervation of the muscle fiber (3), and therefore we examined the expression of the neuregulin receptors ErbB2 and ErbB3 in denervated muscles. There was a 2–3-fold increase in the abundance of both ErbB2 and ErbB3 in total membrane extracts from denervated muscle (Fig. 9*A*). We also determined neuregulin expression in control and denervated muscle. To this end, specific primers were generated to detect all possible neuregulin isoforms expressed by muscle. Neuregulin mRNAs were amplified by reverse transcriptase-PCR in a way that ensured nonsaturating concentrations. Muscle expressed low levels of neuregulins in either innervated or denervated muscle, and denervation caused no alteration in the expression level (Fig. 9*B*), which is in agreement with a previous report (33).

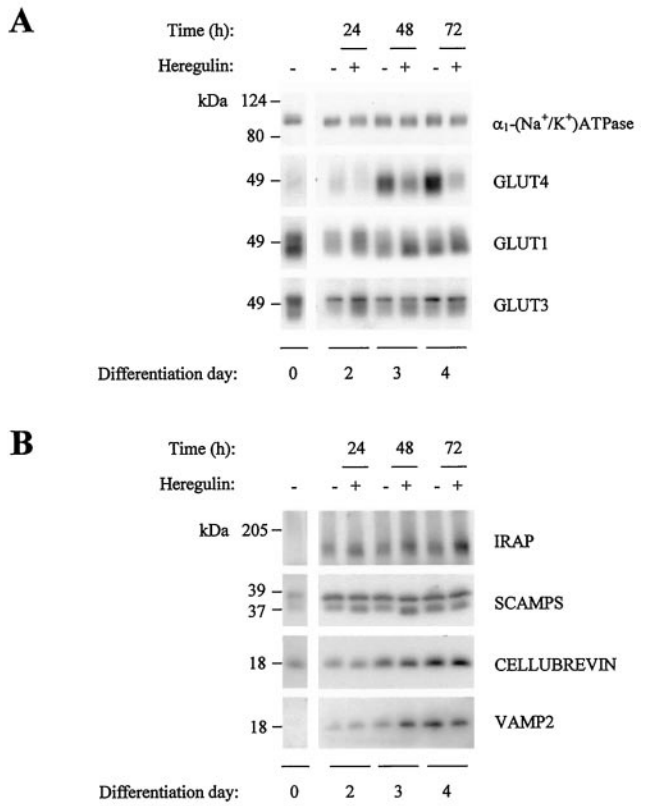

FIG. 7. **Effects of chronic HRG on the expression of glucose transporters, IRAP, SCAMPs, VAMP2, and cellubrevin.** L6E9 cells proliferated for 2 days at 10% FBS and then were cultured at 2% FBS to allow them to differentiate. After 24 h of differentiation, cells were incubated in the presence or absence of 3 nM HRG for different times. Total membranes were analyzed to determine GLUT1, GLUT3, and GLUT4 total content (*A*) or abundance of IRAP, SCAMPs, VAMP2, and cellubrevin (*B*). As a control we used cells at the myoblastic stage (10% FBS, 80–90% confluence, day 0). The α_1 subunit of Na^+/K^+-ATPase was used as a control of protein loading. Representative autoradiograms are shown (10 μg of protein/lane for GLUT1, GLUT3, and GLUT4 and 25–30 μg of protein/lane for IRAP, SCAMPs, cellubrevin, and VAMP2). At least three different experiments were performed.

DISCUSSION

Neuregulins play a central role in muscle biology. They are synthesized by myoblast cells and initiate an autocrine signaling pathway that promotes myogenic differentiation (30). In addition, neuregulins regulate the expression of acetylcholine receptors and utrophin in muscle (26–28, 45, 46). In the muscle, the neuregulin receptors ErbB2 and ErbB3 are found only in the neuromuscular junction, and it is thought that neuregulins maintain the protein composition, and therefore the functional properties, of the neuromuscular junction. In this study we provide evidence for a metabolic role of neuregulins in muscle, *i.e.* neuregulins stimulate glucose transport in muscle cells by various mechanisms. On the one hand, they promote rapid translocation of glucose transporters from an intracellular site to the plasma membrane. On the other hand, neuregulins cause up-regulation of GLUT1 and GLUT3 glucose transporters, which is concomitant with an enhanced abundance at the plasma membrane in conditions in which GLUT4 is markedly repressed. These results suggest that in the mature muscle fiber, neuregulins regulate glucose uptake in or near the neuromuscular junction through changes in glucose transporter distribution or in glucose transporter expression.

We have shown in this study that heregulin acutely stimulates glucose transport in muscle cells and tissue. To our knowledge, this is the first report of a rapid effect of neuregulins that is independent of changes in gene expression. The

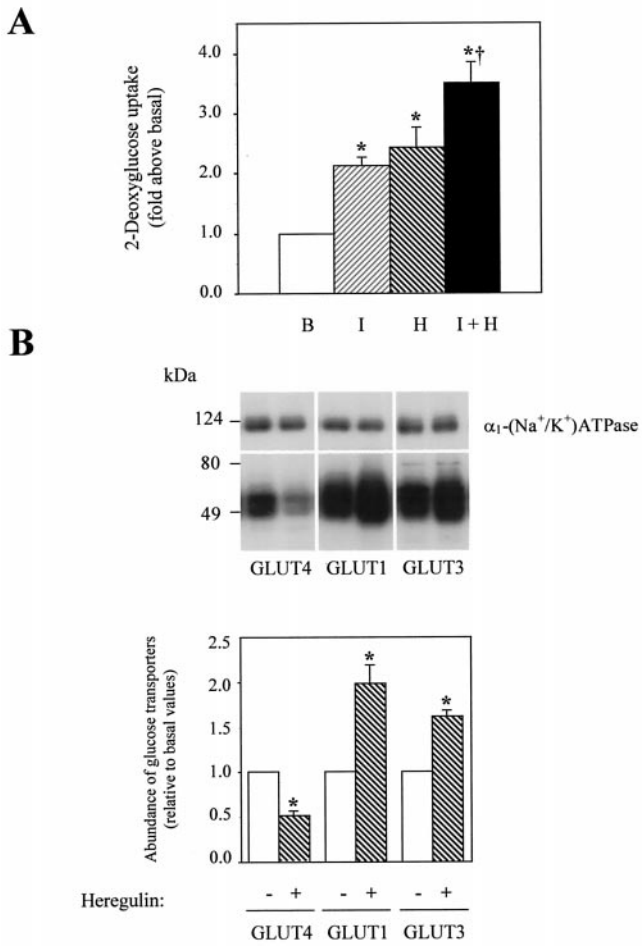

FIG. 8. **Glucose transport in heregulin-treated L6E9 muscle cells and abundance of glucose transporters at the plasma membrane.** L6E9 myotubes were treated or not for 48 h with 3 nM HRG. *A*, at term, cells were depleted of serum for 4.5 h, and some were treated with 1 μM insulin during the last 30 min. 2-Deoxyglucose uptake values were expressed per μg of protein after subtraction of background values. Results are the mean ± S.E. of 4 different experiments. Basal transport values were 0.73 ± 0.14 pmol of 2-deoxyglucose/μg protein × 10 min. *, indicates the existence of significant differences compared with the basal group at $p < 0.05$; †, indicates significant differences compared with insulin or HRG groups at $p < 0.05$ (Student's *t* test). *B*, basal; *I*, insulin; *H*, heregulin. *B*, at term, plasma membrane fractions were obtained and assayed by Western blot to determine the abundance of GLUT1, GLUT3, and GLUT4 glucose transporters. Autoradiograms from a representative experiment are shown (25 μg of protein/lane). Autoradiograms were subjected to densitometry; results are the mean ± S.E. of 3–5 independent experiments. *, indicates significant differences compared with the basal group at $p < 0.01$ (Student's *t* test).

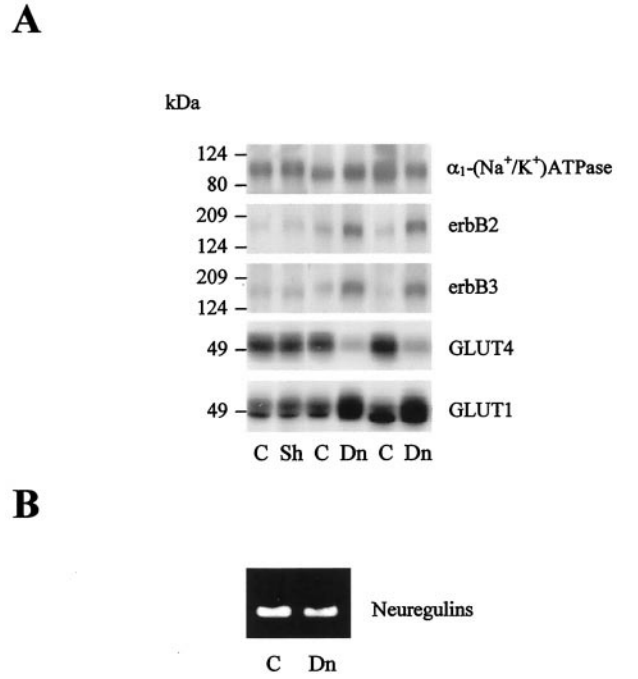

FIG. 9. **Expression of ErbB2 and ErbB3 proteins and neuregulin mRNA in denervated muscle.** Total membranes were obtained from 3-day denervated (*Dn*), contralateral rat tibialis anterior muscles (*C*), or sham-operated muscles (*Sh*). Membrane proteins were laid on gels. After blotting, GLUT4, GLUT1, ErbB2, and ErbB3 were detected by incubation with specific polyclonal antibodies. Representative autoradiograms are shown (25 μg of protein/lane). Total RNA was obtained from 3-day denervated and contralateral extensor digitorum longus muscle. Equal volumes of total RNA were subjected to reverse transcriptase-PCR with primers specific for the amplification of neuregulin mRNA. Original RNA aliquots were subjected to electrophoresis as a loading control (not shown). A representative image of six different samples is shown.

rapid effect of HRG on glucose transport was explained by the translocation of GLUT4, GLUT1, and GLUT3 glucose transporters, and it was independent of changes in glucose transporter expression. The translocation of the glucose transporter in response to HRG was comparable with the effect of insulin. Furthermore, the stimulation of glucose transport by insulin and HRG was additive, which suggests the activation of different or complementary mechanisms. It has been reported that ErbB3 neuregulin receptor activates phosphatidylinositol 3-kinase activity and that neuregulins activate Akt/protein kinase B (47–49). In this regard, we found that the effect of HRG on glucose transport was completely blocked by wortmannin, suggesting that it requires an intact phosphatidylinositol 3-kinase activity.

During myogenic differentiation, basal glucose uptake decreases as a consequence of the down-regulation of GLUT1 and GLUT3. Here, we have shown that heregulin blocks the down-regulation of both GLUT1 and GLUT3 associated with muscle cell differentiation and increases their abundance at the plasma membrane, which explains the stimulation of glucose transport in muscle cells subjected to chronic treatment with heregulin. For GLUT1 expression, we know that gene transcription is a crucial regulatory step in muscle cells (50); therefore, it is likely that heregulin changes GLUT1 gene transcription. In connection with the factors that regulate GLUT1 gene transcription, we have previously established that Sp1 transactivates GLUT1 gene transcription (50), whereas Sp3 represses the transcriptional activity of the GLUT1 promoter in L6E9 cells (50, 51). Furthermore, myoblasts have high levels of Sp1 and Sp3, and during onset of myogenesis there is a decrease in the Sp1 content, so that the Sp1/Sp3 ratio falls, which is concomitant to GLUT1 repression (51). In this connection it has been reported that neuregulins phosphorylate Sp1, which is involved in the activation of the expression of acetylcholine receptor ϵ-subunit (52). Thus, HRG might up-regulate GLUT1 expression through changes in Sp1, increasing either its total cellular content or its active form.

HRG potentiates both myoblast proliferation (an increase of 44% in L6E9 cells; data not shown) and myotube formation, the latter being in keeping with previous observations (30). In this regard, the effect of heregulin is similar to the effects of insulin-like growth factors, activating both the proliferation and differentiation of muscle cells (53). The finding that HRG represses expression of GLUT4 at the time that myotube formation is induced indicates that both effects are not dependent on each other. In addition, this effect is specific for GLUT4 and HRG does not compromise the expression of other proteins

such as IRAP, VAMP2, SCAMPs, or cellubrevin that colocalize with GLUT4 in intracellular compartments of the muscle.

Muscle denervation causes GLUT1 induction and GLUT4 repression (3–7), but the mechanisms are largely unknown. Thus, denervation increases muscle cAMP (54), and chronic incubation with permeable cAMP analogues down-regulates GLUT4 and up-regulates GLUT1 in L6E9 myotubes (55), so cAMP may participate in the effects of denervation. Furthermore, muscle denervation enhances Sp1 and Sp3 binding activity (51), which indicates that denervation-mediated enhancement in GLUT1 gene transcription may be also explained by activation of the Sp1 site of the proximal GLUT1 promoter. Muscle denervation represses GLUT4 gene transcription, which requires a DNA fragment encompassing 730 base pairs from the transcription initiation site (56); however, the regulatory sites and the transcription factors involved are unknown. We show that muscle denervation in the rat up-regulates the neuregulin receptors ErbB2 and ErbB3, which is concomitant with unaltered levels of neuregulin mRNA. This suggests that the effects of muscle denervation on glucose transporter expression are mediated by enhanced neuregulin action in the muscle fiber.

Another important aspect refers to the cellular distribution of neuregulin receptors in the muscle fiber. In innervated muscle, neuregulin receptors are limited to the neuromuscular junction (31, 32); therefore, the biological effects of neuregulin may be restricted to this domain of the muscle fiber. Immunocytochemical evidence indicates that after muscle denervation, ErbB2 abundance diminishes, whereas ErbB3 does not change at the neuromuscular junction (31). As a whole, this suggests that newly synthesized ErbB2 and ErbB3, in the muscle fiber after denervation, are not concentrated at the neuromuscular junction but are spread over the membrane surface. Thus, neuregulins may alter muscle physiology in extrajunctional areas after muscle denervation and may have an impact on glucose uptake in the muscle fiber.

In summary, HRG regulates glucose uptake in muscle cells and in rat muscle by mechanisms that involve, at least in L6E9 cells, either the rapid redistribution of glucose transporters or the regulation of glucose transporter expression. Neuregulins may regulate glucose disposal in or near the neuromuscular junction in the innervated muscle fiber. Based on the fact that HRG up-regulates GLUT1 and down-regulates GLUT4 in muscle cells and that the abundance of neuregulin receptors increases after denervation, we also postulate that neuregulins may participate in the adaptations in glucose uptake that take place in the muscle fiber after denervation.

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