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, heregulin-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-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, heregulin-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.
Neuregulin Action on Glucose Transport in Muscle Cells

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 kindly provided by Dr. B. Nadal-Ginard (Harvard University, Boston, MA). 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. Antibiotics were purchased from BioWhittaker (Walkersville, MD). Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), glutamine, and antibiotics were purchased from BioWhittaker (Walkersville, MD). Recombinant heregulin (heregulin-β1(177–244) (HRG)) was kindly provided by Dr. B. Nadal-Ginard (Harvard University, Boston, MA). 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. Antibiotics were purchased from BioWhittaker (Walkersville, MD). Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), glutamine, and antibiotics were purchased from BioWhittaker (Walkersville, MD). Recombinant heregulin (heregulin-β1(177–244) (HRG)) was kindly provided by Dr. B. Nadal-Ginard (Harvard University, Boston, MA).

Preparation of Homogenerates 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 sonicated in 1 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 MgCl2, 0.5 mM EDTA, 0.1 mM dithiothreitol, 0.1% (v/v) phenylmethylsulfonyl fluoride, 0.1% (v/v) pepstatin) and 1 μg of 2-deoxy-β-[3H]glucose and 1 μCi of 2-deoxy-β-[3H]glucose uptake (10 μCi/ml). To determine background labeling, we incubated the cells for 10 min with ice-cold 50 μM glucose in phosphate-buffered saline buffer (PBS) containing the specific activity of 2-deoxy-β-[3H]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% 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).

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Basal transport rates were expressed as relative values (treated above nontreated cells transport).

In time course studies, cells were washed twice in cold PBS, and 1 mM ZnSO₄ in 20% 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-β-[14C]glucose uptake (1 μCi/ml) and [3H]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, 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 (1:200), GLUT5 (1:500), ErbB3 (1:100), and ErbB2 (1:1000), myosin heavy chain (1:2000), VAMP2 (1/1000), and cellubrevin (1/500), all diluted in 1% (w/v) dimethyl sulfoxide was added at room temperature and incubated for 1 h. 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.

Reverse Transcriptase-PCR from Total RNA—cDNA was synthesized from 2 μg of total RNA at 20 °C in a 20-μl mixture containing 1 unit Superscript II RNAse H (Life Technologies, Inc.), 0.4 μM dNTPs, 200 mM mHglycine, 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 (1:200), α-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, 0.087% (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 °C in a 20-μl mixture containing 1 unit 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'-TCAGACGTCTTGATATAACAAAGC-3' and 5'-GTGGTCATGGGCTGATAGATACCT-3' (Life Technologies, Inc.) corresponded to
627–649 and 1608–1630 base pairs, respectively, of rat NDF (sequence with total identity to all neuregulin isoforms) were added at 0.4 mM dNTPs at 0.2 mM. 1.25 units of Taq Expand High Fidelity and its corresponding buffer with Mg2+ 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 α1 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. 2A). 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. 2B). 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

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

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 α1 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-

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**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).

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**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 25 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).
suggests 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 α1 subunit of Na⁺/K⁺-ATPase was unaltered after incubation with HRG (Fig. 4A). 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. 6A), 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. 6B). 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. 6C), a late myogenic protein. GLUT4 is also induced during late myogenesis, showing high levels of expression after 3 days of differentiation (Fig. 7A). 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. 7A).

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. 7B).

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. 9A). 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. 9B), which is in agreement with a previous report (33).

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
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 trans-activates 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 suggests that heregulin changes GLUT1 gene transcription.

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 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 trans-activates 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 e-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.
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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 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. Immuno- cytochemical 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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