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Stimulation of glucose transport in L6 muscle cells by long-term intermittent stretch–relaxation

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Skeletal muscle stretch increases resting metabolism and causes hypertrophy. We have examined the effect of mechanical stretch *in vitro* on glucose transport activity and transporter contents in L6 muscle cells. Long-term (24–48 h) stretch-relaxation (25% maximal elongation at 30 cycles per min) of cell monolayers significantly increased glucose uptake by 1.6- to 2-fold in myotubes but not in myoblasts. The presence of serum was required for the stretch–relaxation induced increase in glucose uptake. Cycloheximide inhibited the mechanical stimulation of glucose uptake, and the latter response was not additive to the stimulatory effect of long-term exposure to insulin. GLUT1 and GLUT4 glucose transporter contents were not changed in total cell membranes from mechanically stimulated cells relative to controls. These results indicate that mechanical stimulation through passive stretch may be an important regulation of nutrient uptake in fetal myotubes independent of innervation.

Glucose transport; Glucose transporter; Mechanical activity; L6 muscle cell

1. INTRODUCTION

Skeletal muscle is a major tissue involved in glucose disposal in vivo, and this function is exquisitely regulated by hormones [1] and physical activity [2]. Physical activity (i.e. contractile activity) also stimulates muscle metabolic rates and lactate production in isolated and primary cultures of skeletal muscle [3,4]. On the other hand, passive muscle tension (i.e. unrelated to innervation) plays an important regulatory role in embryonic and neonatal muscle development [5]. However, the molecular and cellular mechanisms by which mechanical activity is transduced into these biochemical processes are unknown.

The L6 muscle cell line of day-old rat skeletal muscle origin [6] expresses many biochemical [7], morphological [8] and metabolic [9] characteristics of the mature skeletal muscle. These cells express both GLUT1 and GLUT4 glucose transporters [10,11], but their appearance differs during myogenesis. GLUT1 isoform content decreases during differentiation of myoblasts into myotubes, whereas GLUT4 transporters are barely detectable in myoblasts and increase in content in myotubes [11]. Differentiation-associated changes also occur in the glycosylation and cellular localization of the transporters [12]. Importantly, insulin sensitivity of glucose transport appears only in differentiated myotubes. Accordingly, this cell line provides a suitable model system to investigate the regulation of glucose transport by diverse stimuli.

In the present study we examined the effect of intermittent stretch–relaxation on glucose transport activity and transporter contents in L6 cells using a novel vacuum operated, passive stretch apparatus. This instrument causes deflections in the cell monolayer that effectively stretch the cell surface [13].

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2. MATERIALS AND METHODS

2.1. *Materials*

α-Minimal essential medium (α-MEM), fetal bovine serum and other tissue culture reagents were obtained from Gibco. Porcine insulin, 2-deoxy-D-glucose and cytochalasin B were obtained from Sigma Chemical Co. 2-Deoxy-D-[^{3}H]glucose was purchased from New England Nuclear. ^{125}I-labelled protein A was purchased from ICN. GLUT1 (RaGLUTRANS) and GLUT4 (RaIGRT) glucose transporter antibodies were obtained from East Acres Biologicals (Southbridge, MA).

2.2. *Cell culture*

L6 muscle cells were grown in α-MEM supplemented with 2% fetal bovine serum in an atmosphere of 5% CO_2 at 37°C and allowed to fuse and differentiate, essentially as reported earlier [11,12]. The myoblasts were plated in 6-well plates (Amino Plates P-1001A, Flexcell Corp., McKeesport, PA) with flexible bottoms at approximately 6×10^4 cells/ml. The maximum fusion (>90%) was attained at 7 days after seeding the cells.

2.3. *Mechanical stimulation*

The vacuum-operated stretch apparatus (F-100C Flexercell Strain Unit, Flexcell Corp.) is controlled by a computer with variable time frequency and stretch intensity. Culture wells are deformed to a known percent elongation by application of a precise vacuum. Upon release of the vacuum, culture wells return to their original conformation. The stretching regimen consisted of 25% maximal elongation (vacuum at 20 kPa) at 30 cycles per min in this study. According to

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Vandenburgh et al. [4] the mechanical activity used in this study can be expected to promote muscle cell maturation after myotube fusion. Mechanical activity began on day 7 or 8 after seeding the cells. Differentiated myotubes remained morphologically intact throughout the mechanical stretch used in these experiments, as observed by inverted phase microscopy.

2.4. *Glucose transport assay*

The cells were rinsed three times in HEPES buffered saline solution (140 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4) and carrier-mediated hexose uptake (cytochalasin B-inhibitable) of 10 μM 2-deoxy-D-[^{3}H]glucose (1 μCi/ml) in the above solution was measured for 10 min at room temperature [11]. This was followed by rinsing the cells three times with ice-cold saline solution and cell disruption with 0.05 N NaOH. Cell-associated radioactivity was determined by scintillation counting. Protein was determined by the Bradford method [14] and results were expressed in pmol/mg protein·min. Statistical analysis of the results was performed by Student's *t*-test.

2.5. *Preparation of total cell membranes and Western blot analysis*

Total cell membranes (i.e. post-nuclear particulate fraction without cytosolic components) were prepared as described [11,15]. Cells from 6-well plates were gently scraped, centrifuged at 700 × *g* for 10 min, resuspended in ice-cold buffer I (250 mM sucrose, 5 mM NaN_3, 2 mM EGTA, 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A, 10 μM E-64 and 20 mM HEPES, pH 7.4) and then homogenized using a Dounce type A homogenizer (20 strokes). The homogenate was centrifuged at 760 × *g* for 5 min, and the supernatant was centrifuged at 190,000 × *g* for 60 min to obtain total cell membranes. The final pellet was suspended in buffer I and frozen at −20°C. Membrane samples (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels according to the method of Laemmli [16]. GLUT1 and GLUT4 glucose transporter isoforms were detected by Western blotting essentially as described earlier [12,15] using C-terminus-specific antibodies to each isoform. Quantitative analysis of autoradiograms was carried out by laser scanning densitometry.

3. RESULTS

3.1. *Mechanical activity increases glucose uptake in myotubes but not in myoblasts*

Recently, it was reported that computerized mechanical cell stimulation provides a method for the analysis of skeletal muscle growth in response to passive mechanical activity [17]. We attempted to analyze the effect of passive mechanical activity on membrane glucose transport systems in L6 muscle cells which have been previously used to investigate the regulation of glucose transport by other stimuli [10,11,15,18]. Fig. 1A shows the time-course of stimulation of glucose uptake by passive mechanical activity in myotubes. Glucose uptake was significantly higher in mechanically stimulated cells relative to control cells. A 2-fold stimulatory effect was attained by 48 h. In cells mechanically stimulated for 24 h, total cell protein content did not change appreciably compared with control cells (130.29±12.75 μg/well vs. 142.43±10.68 μg/well, n=7, respectively). Moreover, the cytochalasin B-insensitive component of glucose uptake was not different in control and mechanically stimulated cells. Therefore, these cells are structurally strong enough to withstand repetitive mechanical stimulation without cell damage that could cause cellular detachment or membrane leakiness.

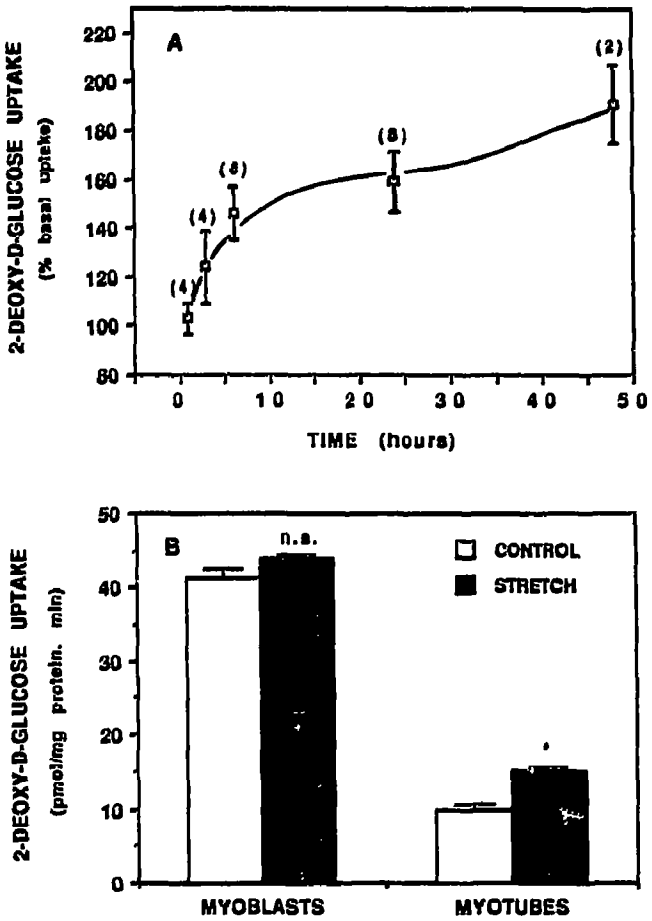

Fig. 1. (A) Time-course of glucose uptake by control and mechanically stimulated myotubes. At time 0, the culture medium was changed to the fresh medium and myotubes were incubated without or with mechanical stimulation. At each time point, 2-deoxy-D-glucose uptake was measured for 10 min as described in Materials and Methods. The mechanical stimulation was carried out as described in Materials and Methods. The number of independent experiments are indicated in brackets. Results are means ± S.E.M. of independent experiments each performed in quadruplicate. (B) Effect of mechanical activity for 24 h on glucose uptake in L6 myoblasts and myotubes. The mechanical stimulation initiated on 2 days for myoblasts and 8 days for myotubes after seeding the cells. Results are means ± S.E.M. of four determinations of a representative experiment. *P<0.025; n.s., not significant, relative to the corresponding uptake in control cells.

Fig. 1B compares the response of glucose uptake to 24 h of passive mechanical activity in myoblasts and myotubes. As seen previously [11], basal glucose uptake decreased during differentiation of L6 myoblasts into myotubes. Notably only the myotubes but not the myoblasts showed a significant increase in glucose uptake by passive stretch. Therefore, mechanical stimulation of glucose uptake appears to be a specific property of differentiated muscle cells.

3.2. *Effects of cycloheximide, insulin and serum deprivation on the stimulation of glucose uptake by mechanical activity*

Protein synthesis rates measured by [^{14}C]phenylalanine incorporation into the trichloroacetic acid-insolu-

ble fraction of cell extracts are elevated by mechanical activity in primary cultures of avian skeletal muscle [19]. Hence the possible participation of new protein synthesis in the response of glucose uptake to passive stretch was analyzed. When 5 μg/ml cycloheximide was added to the culture medium during mechanical stimulation of myotubes for 24 h, the stimulation of glucose uptake into myotubes did not reach statistical significance (Fig. 2A). Thus, stimulation of glucose uptake by mechanical activity appears to depend on ongoing protein synthesis in quiescent myotubes.

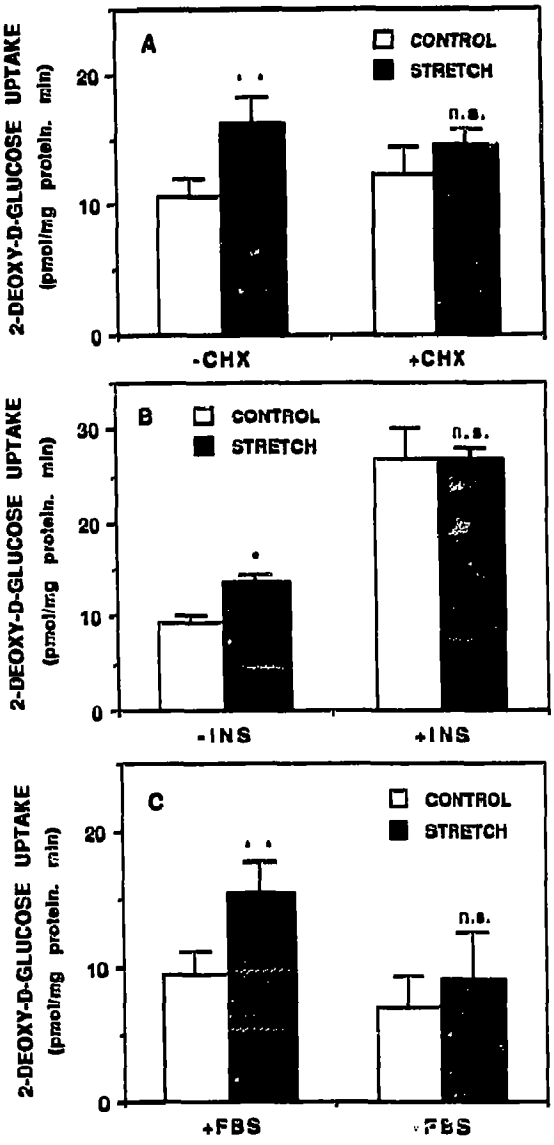

Fig. 2. Effect of cycloheximide (A), insulin (B) and serum deprivation (C) on glucose uptake in control and mechanically stimulated myotubes. Myotubes were incubated without or with mechanical stimulation for 24 h and 2-deoxy-D-glucose uptake was measured for 10 min as described in Materials and Methods. At the start of the mechanical stimulation, culture medium was changed to the fresh medium in the absence or presence of 5 μg/ml cycloheximide (CHX), 100 nM insulin (INS) or 2% fetal bovine serum (FBS). Results are the mean ± S.E.M. of three different experiments each performed in quadruplicate. *$P<0.025$; **$P<0.05$; n.s., not significant, relative to the corresponding uptake in control cells.

Previously we reported that chronic stimulation (24 h) of glucose transport by insulin [18] and insulin-like growth factor-I (IGF-I) [15] occurs by a complex mechanism which includes enhanced glucose transporter synthesis and a change in the subcellular distribution of glucose transporter proteins. It is possible that the stimulation of glucose transport by mechanical activity may be mediated by mechanisms similar to those with insulin or growth factors. To test this hypothesis, we examined the effects of insulin and serum during mechanical stimulation on glucose uptake. Prolonged treatment with insulin (24 h) increased glucose uptake by 3-fold. Mechanical activity had no further effect on insulin-stimulated glucose uptake (Fig. 2B). Furthermore, when serum was omitted from the culture medium, glucose uptake dropped by 27% in control cells and did not respond to mechanical activity (Fig. 2C). These observations suggest that mechanical activity can partly mimic the long-term insulin effect on glucose uptake, and that serum growth factors may be required for this response.

3.3 *Effect of mechanical activity on GLUT1 and GLUT4 transporter proteins*

L6 myotubes express both GLUT1 and GLUT4 glucose transporters and these isoforms are differentially regulated by insulin and IGF-I [10,15]. We therefore examined the effect of mechanical activity on both glucose transporter contents using isoform-specific antibodies. Fig. 3 shows a typical result of Western blot analysis of GLUT1 and GLUT4 transporter proteins (A) and densitometric values of the Western blots in each isoform in three independents preparations of total cell membranes from control and mechanically-stimulated cells (B). The results suggest that neither GLUT1 nor GLUT4 protein contents are affected by mechanical activity, and hence this treatment is not likely to increase the synthesis or decrease the degradation (i.e. change of turnover rate) of these transporters. This result also indicates that the inhibition of mechanical stimulation of glucose uptake by cycloheximide is not due to inhibition of biosynthesis of transporter proteins, but rather that ancillary proteins may be synthesized in response to passive stretch.

4. DISCUSSION

It has been long documented that the resting metabolism of a skeletal muscle increases proportionally with stretch [20]. Mechanical regulation is responsible for hypertrophia and involves rapid and long-term functions that may involve ionic changes and mechanogenetic regulation of gene transcription [21]. These responses occur independently of muscle innervation or of electrical activity [21]. Passive stretch alone is able to

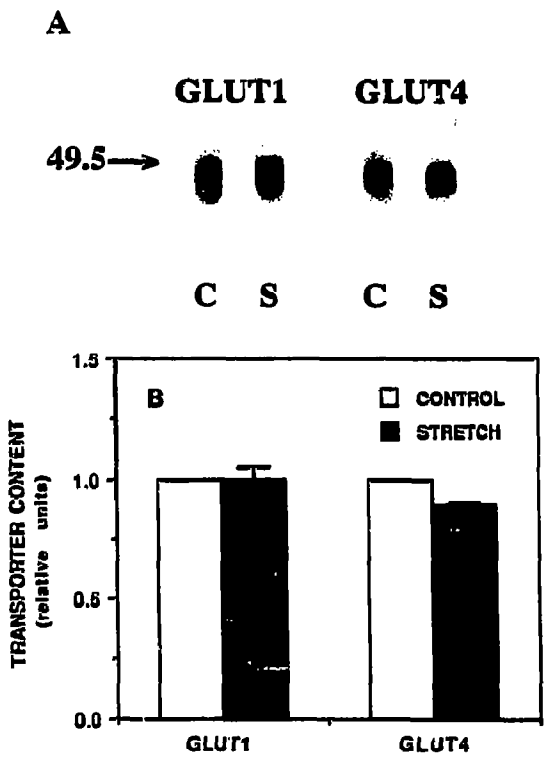

Fig. 3. (A) Effect of mechanical activity on GLUT1 and GLUT4 glucose transporters in myotubes. Total cell membranes were prepared from myotubes incubated for 24 h without (C) or with (S) mechanical stimulation. The number on the left indicates a molecular weight marker in kDa. (B) Densitometric analysis of GLUT1 and GLUT4 transporter contents in control and mechanically stimulated myotubes. The contents in control myotubes were assigned a value of 1.0 in each isoform and other values are expressed in relative units. Results are the means ± S.E.M. of three different preparations.

initiate the biochemical events that form the basis of hypertrophy in muscle cells. However, the precise mechanism by which this occurs and the molecular basis for the stretch-induced increase in resting metabolism are unknown. In this study we have attempted to approach these questions by investigating the effect of passive stretch on non-innervated muscle cells in culture, focussing on the glucose transport system by measuring its activity, glucose transporter protein expression and dependence on growth factors.

Mechanical activity through passive stretch effectively stimulated glucose uptake into differentiated L6 myotubes but not into the precursor myoblasts (Fig. 1). This result suggests that mechanical regulation of glucose transport is a muscle-specific property. The molecular events underlying the stimulation of hexose uptake by passive stretch in muscle cells in culture remain to be defined, but analogies can be found with other stimuli known to elevate glucose uptake. Passive stretch resembled the stimulation of hexose transport caused by prolonged exposure to serum [22], insulin [18] or IGF-I [15] because all these effectors, when presented for several hours, produced a protein synthesis-dependent response of glucose transport. Moreover, the stimulatory action of passive stretch appeared to involve some elements common to those participating in the long term response to insulin, since the concomitant exposure of cultures to passive stretch and insulin did not stimulate transport further from that caused by the hormone alone. However, in contrast with the actions of insulin or IGF-1, the effect of passive stretch was not accompanied by a rise in total cellular levels of GLUT1 transporters. Moreover, the stimulation caused by mechanical activity was of a smaller magnitude than that provoked by prolonged hormonal treatment.

The magnitude of the response to passive stretch was, rather, in keeping with the more modest response of glucose transport elicited by acute exposure to insulin or IGF-1 (i.e. 1.5 to 2-fold). The acute response to these hormones in L6 myotubes is largely ascribed to a redistribution of pre-formed glucose transporters (mostly the GLUT4 isoform) from an intracellular compartment to the cell membrane [15,18]. The possibility that transporter recruitment is triggered as well by mechanical activity is intriguing. Testing of this scenario will require implementation of immunofluorescence techniques, since subcellular fractionation cannot be applied to myotubes grown in the flexible material of the plates used in the Flexercell unit. However, it is already apparent that, if translocation of transporters is involved, this process will differ from that initiated by acute exposure to insulin or IGF-I because de novo protein synthesis appears to be required by the mechanical but not by the acute hormonal response. The newly synthesized proteins or factor contributing to the stimulation of glucose uptake by passive stretch are presently unknown, but they may play an important role as transducers of mechanical stimulation into biochemical processes.

In the present study, the response of transmembrane glucose transport to mechanical activity in L6 myotubes required the simultaneous presence of serum factors (although serum was not required for the elevation in glucose metabolism caused by mechanical activity in avian skeltetal muscle, [4]). It is possible that passive stretch sensitizes the L6 muscle cells to respond to macromolecules present in the medium, or that passive stretch and serum may result in a synergistic effect on a common signalling pathway.

In conclusion, the results presented indicate that mechanical activity can induce a time-dependent increase in hexose transport in L6 myotubes, which is dependent on protein synthesis but does not involve net changes in GLUT1 or GLUT4 glucose transporter levels. The response requires extracellular serum and may involve recruitment and/or activation of pre-existing transporter units. Since passive stretch is the main form of activity during fetal muscle development, this process may be of importance in the procurement of nutrients by the developing muscle fibers.

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