Contractile $C_2C_{12}$ myotube model for studying exercise-inducible responses in skeletal muscle

Taku Nedachi,1,2 Hideaki Fujita,2 and Makoto Kanzaki1,3

1Center for Research Strategy and Support, 2Division of Biomaterials, Tohoku University Biomedical Engineering Research Organization; and 3Graduate School of Biomedical Engineering, Tohoku University, Sendai, Japan

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It has been well documented that an appropriate amount of physical exercise exerts a wide variety of biological effects, such as an enhancement of glucose disposal, changes in muscle fiber types, induction of angiogenesis, and regulation of the immune system (35). In addition, adequate exercise is highly beneficial not only for preventing the development of type 2 diabetes but also for diabetic patients already suffering from insulin resistance (24, 32). However, the molecular basis of these beneficial effects of exercise/skeletal muscle contraction is still poorly understood, and elucidation of the mechanisms by which skeletal muscle cells decipher and respond to contractile stimuli is a major issue in understanding the beneficial and in some cases detrimental effects of exercise.

An excellent cell culture model would be an invaluable tool for investigating a wide array of biological phenomena. However, no such model that can be utilized for investigating these diverse effects of muscle contraction on muscle cells is available yet because a conventional cell culture system lacks the muscle contractile activity required for physiologically relevant energy expenditure and mechanical stress. We recently succeeded in endowing $C_2C_{12}$ myotubes with vigorous contractile activity as a result of de novo sarcomere assembly by applying electric pulse stimulation (EPS) (22). Thus, one of the aims of the present study is the establishment of a novel skeletal muscle contraction model based on the use of contractile $C_2C_{12}$ myotubes to explore contraction-inducible changes in contracting muscle cells, focusing especially on glucose metabolism and secreted proteins, which recently have been attracting considerable research attention.

One of the typical anticipated effects of exercise/skeletal muscle contraction is improvement of metabolic properties. For instance, an acute bout of exercise increases glucose transport into contracting muscles, leading to clinically significant decreases in blood glucose levels (32, 46). Several facilitative glucose transporters (GLUTs) are expressed in skeletal muscle (50), and an insulin-responsive glucose transporter 4 (GLUT4) was identified as a major GLUT responsible for exercise/contraction-induced glucose uptake achieved through its redistribution from intracellular storage compartments to the cell surface in skeletal muscle (9, 18). One of the signaling mechanisms evoked by exercise/contraction to regulate GLUT4 translocation is the activation of 5′-AMP-activated protein kinase (AMP kinase) (21, 41). However, since expression of a dominant negative version of the AMP kinase mutant only partially blocked contraction-induced glucose uptake in skeletal muscle, AMP kinase-independent pathways were also assumed to contribute to this response (49). The Ca$^{2+}$/calmodulin-dependent protein kinase II pathway was recently proposed to be a second pathway involved in contraction-dependent glucose uptake (80). Another important feature of exercise effects on muscle is that insulin sensitivity for glucose transport is amplified in working muscles, which is attributed, at least in part, to the increase in GLUT4 contents (26, 63). The other proposed mechanism underlying this improved insulin responsiveness is an enhancement of insulin receptor signaling pathway(s) resulting from responses elicited by exercise/contraction (16, 75); however, other reports have asserted that exercise does not affect insulin signaling (27, 73, 78); thus, the effect of exercise on insulin receptor signaling remains controversial.

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Secreted paracrine/endocrine factors, adipocytokines, produced by fat tissue have recently attracted attention (72). Adipocytokines such as tumor necrosis factor-α, interleukin-6 (IL-6), adiponectin, and so on are produced in response to metabolic states such that these adipocyte-derived factors control glucose and lipid metabolism in other tissues/organs (61). Like adipocytes, skeletal muscle also produces various secreted factors (58), and factors such as IL-6, IL-8, and IL-15 are known to be induced by exercise and are therefore termed “exercise factors” (54, 58). In particular, IL-6 reportedly has positive effects on skeletal muscle glucose metabolism (13, 66), and it is increasingly apparent that some of the health benefits of exercise might be mediated by these exercise factors, including IL-6, functioning in an autocrine, paracrine, and/or endocrine fashion. In this regard, effects of exercise are not limited to metabolic changes in working skeletal muscles but are also brought about by other responses such as the induction of lipolysis in adipocytes (5), body temperature increases (40), regulation of the immune system (59), and so on. The other exercise factor, IL-8, belongs to the ELR (glutamate-leucine-arginine)-containing CXC chemokine family (14, 54); however, whether the other ELR chemokines, such as CXC ligand 1/KC (CXCL1/KC) and CXC ligand 5/LIX (CXCL5/LIX), are also exercise factors remains unknown. Hence, although “exercise factors” could be important for explaining these functions of exercise, it has been difficult to identify exercise factors in vivo, since alterations in the systemic levels of these muscle-derived secreted factors in response to exercise are often minimal.

We herein report the establishment of an advanced in vitro contraction model derived from improving our contractile C2C12 myotube culture system by applying an appropriate EPS. This novel model allows successful induction of EPS-evoked contraction of C2C12 myotubes followed by increases in glucose transport strongly associated with enhanced GLUT4 recycling at the plasma membrane, which occurs concurrently with activation of the AMP kinase and MAP kinase signaling pathways. By using this advanced in vitro contraction system, we succeeded in identifying mouse CXCL1/KC and CXCL5/LIX as novel candidates for exercise factors that may contribute to mediating the effects of exercise.

MATERIALS AND METHODS

Materials. The Western blot detection kit (West super femto detection reagents) was from Pierce Biotechnology (Rockford, IL). Dulbecco’s modified Eagle’s medium (DMEM), MEM nonessential and essential amino acid solution, penicillin/streptomycin, and trypsin-EDTA were purchased from Sigma Chemical (St. Louis, MO). Cell culture equipment was from BD Biosciences (San Jose, CA). Calf serum and fetal bovine serum were obtained from BioWest (Nuaillé, France). Immobilon-P was from Millipore (Bedford, MA). Unless otherwise noted, all chemicals were of the purest grade available from Sigma Chemical or Wako Pure Chemical Industries (Osaka, Japan).

Cell culture. Mouse skeletal muscle cell lines, C2C12 myoblasts (passage no. 4–10) (83), were maintained in DMEM containing 1 g/l glucose supplemented with 10% FBS, 30 µg/ml penicillin, and 100 µg/ml streptomycin (growth medium) at 37°C under a 5% CO₂ atmosphere. For biochemical studies, cells were grown on four-well plates (Nalgen Nunc International, Rochester, NY) at a density of 1 × 10⁶ cells/well in 5 ml of growth medium or on six-well plates (BD Biosciences) at a density of 3 × 10⁴ cells/ml in 3 ml of growth medium. Three days after plating, the cells had reached ~80–90% confluence (day 0). Differentiation was then induced by switching the growth medium to DMEM supplemented with 2% calf serum, 200% amino acids, 30 µg/ml penicillin, and 100 µg/ml streptomycin (differentiation medium). The differentiation medium was changed every 24 h.

Animal experiments. Eleven-week-old male C57BL/6J mice were divided into two groups (5 animals in each group), and one group received treadmill training (15 cm/s, 30 min, 8% slope, once) followed by immediate euthanization. Extensor digitorum longus (EDL) and soleus muscles from each leg were isolated and cut into small pieces, and total RNAs were isolated using Trizol reagents according to the manufacturer’s protocol (Invitrogen). All protocols for animal care and use were performed according to the guidelines for animal experiments of Tohoku University and were approved by the Animal Care and Use Committee of Tohoku University School of Medicine.

EPS. Fully differentiated C2C12 myotubes in four- or eight-well dishes (Nalgen Nunc International) were placed in a chamber for electrical stimulation (C-Dish; IonOptix, Milton, MA). Electrical stimulation was applied to the cells in the C-Dish using a C-Pace pulse generator (C-Pace 100; IonOptix). DMEM containing 2% calf serum supplemented with 200% amino acids (Sigma) was used during the EPS treatments. In some cases, 50 µM PD-98059 (Sigma), 20 µM SP-600125 (Biosource International, Camarillo, CA), 5 µM STO-609 (Tocris Bioscience, Ellvissille, MO), 20 µM compound C (Calbiochem, La Jolla, CA), or 20–200 nM SB-225002 (Calbiochem) was added to the culture. The medium was changed every 12 h during EPS. For the last 4 h of EPS, the cell culture medium was changed to serum-free DMEM. After the full treatment, serum-starved cells were subjected to the following experiments. In additional experiments, after 24-h EPS treatment (1 Hz, 2 ms, 40 V/60 mm), the cells were incubated in fresh serum-free DMEM for ~6 h with 0.1 Hz EPS to achieve a rested state and then subjected to a second EPS challenge at 1 Hz and 40 V/60 mm with various pulse durations. To avoid diminishing contractile ability after complete discontinuation of EPS, application of a very low frequency of EPS (0.1 Hz) was necessary even during the resting period to obtain strong contractile activity in response to the second EPS challenge (Kanazaki M unpublished observations).

Calculation of index of movement. The index of movement was calculated as reported previously (22). Briefly, five high-quality images of cells (2,592 × 1,944 pixels, 1,250 × 938 µm) were taken sequentially with a digital camera (Olympus C-5060; Olympus, Tokyo, Japan) and a dissection microscope (Olympus CKX41) equipped with a ×10 objective lens at 700-ms intervals during EPS. Four images were created using the absolute value obtained by subtracting the first image from the second to fifth images, which were then overlaid to make one differential image. The calculated average intensity of the differential image indicates the amount of myotube movement (index of movement).

Western blot analysis. The expression and phosphorylation of each protein were analyzed by Western blotting. In brief, the harvested cell lysates were subjected to 5 or 12% SDS-polyacrylamide gel electrophoresis (1:30, bis-acrylamide). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P), and the membranes were then blocked for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20. Immunoblotting to detect each protein was achieved with 1-h incubation, with a 1:1,000 dilution of primary antibody [anti-LKB, anti-phospho-AMPK, anti-phospho-acetyl-CoA carboxylase (ACC), anti-phospho Erk5, anti-phospho Erk1/2, anti-phospho JNK, anti-JNK, anti-phospho p38, anti-phospho ERK1/2, anti-phospho ERK5, anti-phospho Akt (Ser473), anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-GSK-3β, anti-GSK3β, anti-myocyte enhancer factor 2 (MEF2; Iowa Hybridoma Bank, University of Iowa, Iowa City, IA)] and anti-BD living color antibody (BD Bio-
performed to assess the efficiency of protein transfer. Specific total or phosphoproteins were visualized after subsequent incubation with a 1:5,000 dilution of anti-mouse or rabbit IgG conjugated to horseradish peroxidase and a SuperSignal Chemiluminescence detection procedure (Pierce Biotechnology). Protein concentrations were determined using a bicinchoninic acid assay (Pierce). Three independent experiments were performed for each condition. Comassie blue staining was also performed to assess the efficiency of protein transfer.

2-Deoxyglucose uptake assay. A 2-deoxyglucose (2-DG) uptake assay was performed as described previously (37). Briefly, after serum starvation for 4 h, C2C12 myotubes were washed with Krebs-Ringer phosphate HEPES (KRPH) buffer (10 mM phosphate buffer, pH 7.4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, 10 mM HEPES [pH 7.4]) and then incubated without or with 100 nM insulin for 60 min in KRPH buffer. Glucose transport was determined by the addition of [3H]-2DG (0.1 mM, 0.5 μCi/ml; PerkinElmer Life and Analytical Science, Boston, MA). After a 4-min incubation with KRPH buffer containing [3H]-2DG, the reaction was stopped by adding phosphate-buffered saline (PBS) with 10 μM cytochalasin B (Sigma), and the cells were washed three times with ice-cold PBS. The cells were then lysed in PBS containing 0.2 M NaOH, and glucose uptake was assessed by scintillation counting. Cytochalasin B (20 μM) was added to the assay buffer for the measurement of nonspecific background. Results are specific uptake, i.e., the background subtracted from the total uptake, expressed as the mean ± SE of the indicated number of experiments. The protein content was determined in each experiment with a bicinchoninic acid protein assay kit (Pierce), and the count per milligram of protein was calculated (normalized 2-DG uptake). For each experiment, at least two assays of each condition were performed, and the average of two assays was presented as the percentage of the control. Briefly, the relative amounts of 2-DG uptake were calculated as 100 × (normalized 2-DG uptake of each treatment group)/(normalized 2-DG uptake of control group). The results obtained from at least three independent experiments were subjected to statistical analysis.

Anti-c-myc antibody uptake assay. GLUT4 recycling was analyzed as established previously (52). Briefly, the established C2C12 clones, which stably express myc-GLUT4 fused to enhanced cyan florescence proteins, were differentiated into myotubes and then serum starved for 4 h, washed three times with KRPH buffer, and then placed in a CO2 incubator with 2 ml of KRPH buffer. After 10 min of incubation, 4 μg/ml of the anti-c-myc antibody was added to the buffer, and the cells were stimulated with or without 100 nM insulin for 40 min. After a 1-h incubation with the anti-c-myc antibody, the cells were placed on ice to stop the reaction and washed five times with PBS (–). The cells were harvested using 1× Laemmli’s buffer and subjected to Western blot analysis using anti-mouse IgG antibody, anti-c-myc antibody, or anti-BD living color antibody (JL-8; BD Biosciences). The rate of antibody uptake is linear over the 60 min tested (data not shown).

Real-time PCR. Fluorescence real-time PCR analysis was performed using a Light Cycler instrument and SYBR Green detection kits (Roche Diagnostics, Indianapolis, IN). PCR primers for measuring each of the secreted protein factors were as follows: for CXCL1/KC, 5′-ATG AAC TCC CTG CTT TGA-3′ and 5′-ATG CAC CAA CAC TAT-3′; for CXCL5/LIX, 5′-GGT CAC CAA CAC TAT-3′ and 5′-GTC TCT CCT CTA TCG CAT-3′; for CCAAT/enhancer binding protein β (C/EBP-β), 5′-GCT CAA GAA GGA ACT TAC CAT-3′ and 5′-GCA TGG GAA GGA CAA TAC CCT TTA-3′; for c-Myc, 5′-CCC GAA TGA ACC TCA AAC TCT-3′ and 5′-ATG AAC TCC CTG CTT TGA-3′; for MAFG, 5′-GAA AAC TCC CTG CTT TGA-3′ and 5′-ATG AAC TCC CTG CTT TGA-3′; for Nfat-1, 5′-GCA TCA TTA GAA GAA GGA ACT TAC CAT-3′ and 5′-GTC TCT CCT CTA TCG CAT-3′; for a-Maf, 5′-GTC TCT CCT CTA TCG CAT-3′ and 5′-GCA TCA TTA GAA GAA GGA ACT TAC CAT-3′; for E1193, 5′-GCA TCA TTA GAA GAA GGA ACT TAC CAT-3′ and 5′-GTC TCT CCT CTA TCG CAT-3′; and for H11032, 5′-GTC TCT CCT CTA TCG CAT-3′ and 5′-GCA TCA TTA GAA GAA GGA ACT TAC CAT-3′.

ELISA. Levels of CXCL1/KC and CXCL5/LIX in culture supernatants were measured with commercially available ELISA kits (R&D Systems, Minneapolis, MN). The IL-6 concentration was measured using ELISA kits purchased from eBioscience (San Diego, CA).

Cytotoxicity assay. Cell death was quantified by measuring lactate dehydrogenase (LDH) release into cultured media. LDH measurements were performed with Cytotoxicity Detection Kit Plus (LDH; Roche Applied Science, Mannheim, Germany).

Measurement of intracellular ATP. Cells were washed three times with PBS (–) before assay. The ATP concentration in C2C12 myotubes was measured using a cellular ATP measurement kit (Toyo B-NET, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using Student’s paired t-test for independent samples. Data are expressed as means ± SE unless specified otherwise.

RESULTS

Establishment of in vitro contraction model. To establish our in vitro contraction model, we took advantage of mouse C2C12 cell lines, which show relatively good differentiation in terms of sarcomere development compared with other established cell lines, such as rat L6 (17). Wild-type C2C12 myoblasts were differentiated by culturing DMEM + 2% calf serum supplemented with 200% amino acids for 6 days to obtain multinuclear myotubes. Although we employed EPS conditions (40 V/60 mm, 24 ms, 1 Hz) in our previous study (22), which promoted de novo sarcomere assembly within just 2 h, we found these EPS conditions to be apparently too harsh for cultured myotubes used to study metabolic alterations in response to EPS-evoked contraction since EPS-evoked contractile activity diminished remarkably after 4 h of EPS. Thus, C2C12 myotubes, with or without EPS at 40 V/60 mm, 2-ms duration, and 1 Hz for 24 h, were used. As we reported previously (22), EPS evoked repetitive Ca2+ transients in C2C12 myotubes in a pulse-dependent manner (data not shown), leading to the acquisition of contractile activity (supplemental film; Supplemental Material for this article is available at the AJP-Endocrinology and Metabolism web site) as a consequence of de novo sarcomere assembly. The EPS conditions (40 V/60 mm, 2 ms, 1 Hz) utilized in the present study allowed vigorous contraction of C2C12 myotubes even after 24 h of EPS, and these contractile activities were evidenced by the index of movement (Fig. 1A) (22). Although EPS promoted the contraction of C2C12 myotubes, it did not appear to induce cell death since cytotoxicity, as assessed by LDH release, did not differ significantly between cell cultures with and without 24-h EPS treatment. (Fig. 1B). To address whether this EPS-evoked contractile activity produces exercise-like effects on C2C12 myotubes, we analyzed the phosphorylation state of AMP kinase as well as that of ACC, one of the major substrates for AMP kinase, after 24-h EPS. As shown in Fig. 1C, augmented phosphorylation of both AMP kinase and ACC was observed in C2C12 myotubes exposed to 24-h EPS (~4-fold). In addition, phosphorylation of Tbc1d1 (Ser231), an AMP kinase phosphorylation site, was also induced after 24-h EPS (Fig. 1C). Taken together, these data suggest that the AMP kinase cascade was activated in C2C12 myotubes, just as it is in skeletal muscle during exercise (23). We also found that EPS reduced intracellular ATP levels by ~30% (Fig. 1D), suggesting AMP kinase activation to be a result of changes in the intracellular AMP/ATP ratio. Similar to the AMP kinase cascade activation, MAP kinase cascades, including Erk1/2, Erk5, and...
JNK, were activated after 24-h EPS as assessed by the phosphorylation status of these kinases, suggesting that EPS-evoked contraction not only promotes ATP consumption but also produces stretch/contraction-induced cellular stress (Fig. 1E). Intriguingly, however, the other stress-activated MAP kinase, p38, tended to be suppressed in C2C12 myotubes after 24-h EPS, indicating EPS-dependent regulation of each MAPK to be differentially controlled (Fig. 1E). In addition, ATF2, a downstream transcription factor activated by JNK and p38 kinase, appeared to be phosphorylated in C2C12 myotubes exposed to 24-h EPS.

Effects of EPS on glucose uptake and GLUT4 recycling in contractile C2C12 myotubes. To elucidate whether the EPS-evoked contractile response affects the insulin-dependent and -independent GLUT4 recycling responsible for enhanced glucose uptake into C2C12 myotubes, we first performed a conventional 2-DG uptake assay in C2C12 myotubes after 24-h EPS. We found that 2-DG uptake was significantly increased in C2C12 myotubes exposed to 24-h EPS (1.5 fold; Table 1). Furthermore, EPS treatment also enhanced insulin-dependent glucose uptake (~1.6–1.7 fold; Table 1), indicating that EPS-evoked contraction provided an exercise-like effect on glucose metabolism in C2C12 myotubes. To confirm that this augmented glucose uptake is faithfully elicited by GLUT4 translocation, in a manner similar to that observed in vivo in skeletal muscle during/after exercise, GLUT4 recycling at the plasma membrane was analyzed by anti-myc Ab uptake assay using
the myc-GLUT4 that we established previously (52). Consistent with our previous report (52), insulin stimulation resulted in significant GLUT4 recycling, as evidenced by an increase in the amount of bound/incorporated anti-Myc Ab in naive C2C12 myotubes (Fig. 2A). C2C12 myotubes exposed to 24-h EPS displayed a higher level of GLUT4 recycling, even under basal conditions, which was further augmented by insulin stimulation. Densitometric analysis clearly illustrates amelioration of the effects of 24-h EPS on insulin-induced GLUT4 recycling calculated from the absolute value of Myc Ab-labeled myc-GLUT4 (Fig. 2B). It is noteworthy that the EPS-induced enhancement of GLUT4 recycling appeared to be prerequisite for a relatively long (24 h) EPS, since 2–4 h of EPS failed to affect GLUT4 recycling despite 2- to 4-h EPS being sufficient for inducing EPS-evoked contractile activity (data not shown).

To address which intracellular signals are involved in the EPS-dependent augmentation of GLUT4 recycling after 24-h EPS, we tested several inhibitors. Two inhibitors of MAP kinase cascades, PD-98059, which is a potent inhibitor of MEK1, an upstream kinase for Erk1/2, and SP-600129, which inhibits JNK, showed no effects on the augmentation of GLUT4 recycling after 24-h EPS (Fig. 2C). An AMP kinase inhibitor, compound C, and a Ca^{2+}/calmodulin (CaM)-dependent kinase kinase- (CaMKK-) inhibitor, STO-609, tended to reduce GLUT4 recycling, but the decreases did not reach statistical significance (Fig. 2C). Thus, these experiments suggest that EPS-dependent augmentation of GLUT4 recycling

**Table 1. Effect of EPS on 2-DG uptake in C2C12 myotubes**

<table>
<thead>
<tr>
<th>2-DG Uptake</th>
<th>No treatment</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>100.0±0.0a</td>
<td>123.7±4.1b</td>
</tr>
<tr>
<td>EP</td>
<td>136.4±1.4c</td>
<td>175.7±1.2c</td>
</tr>
</tbody>
</table>

Values are means ± SE. EPS, electric pulse stimulation; 2-DG, 2-deoxyglucose; EP, electric pulse. Fully differentiated C2C12 myotubes were stimulated with EPS (40 V, 1-Hz frequency, 2-ms duration) for 24 h, followed by 100 nM insulin treatment for 60 min. 2-DG uptake (%basal) was measured as described in MATERIALS AND METHODS. Means within columns with different superscripted letters are significantly different (P < 0.05).

AJP-Endocrinol Metab • VOL 295 • NOVEMBER 2008 • www.ajpendo.org

Fig. 2. Enhanced glucose transporter 4 (GLUT4) recycling in contractile C2C12 myotubes after EP stimulation. A–E: fully differentiated C2C12 myotubes were subjected to EP stimulation (40 V/60 mm, 1-Hz frequency, 2-ms duration) for 24 h. The medium was changed once at 12 h after EP stimulation was applied. Twenty hours after EP stimulation was applied, the medium was changed to DMEM without serum, and the myotubes were continuously cultured for 4 h. A: the cells were then treated with [insulin (I)] or without [no (N)] 100 nM insulin for 60 min in the presence of 4 µg/ml anti-myc antibody. The cells were washed 5 times with PBS and then analyzed by Western blotting using anti-mouse IgG horseradish peroxidase (HRP) conjugates or anti-BD living color. At least 3 independent experiments were performed, and representative results were obtained. B: the results from A, uptake of anti-c-myc antibody in response to EP stimulation and insulin, were subjected to densitometric analysis for quantification (+EP + insulin treatment = 100%). Different letters denote statistically significant differences (n = 3; P < 0.05). C: during EP stimulation, the indicated inhibitors (20 µM compound C, 5 µM STO-609, 20 µM SP-600125, or 50 µM PD-98059) were added in the medium, and GLUT4 recycling was then measured as described in MATERIALS AND METHODS (–EP = 100%). Different letters denote statistically significant differences (n = 3, P < 0.05). D and E: after EPS, total RNA was isolated, and the amounts of GLUT1 and GLUT4 mRNA were measured as described in MATERIALS AND METHODS (n = 4; NS). CFP, cyan fluorescence protein.
might be induced by other mechanisms or more likely results from a combination of these mechanisms. Moreover, GLUT1 and GLUT4 mRNA levels were unaffected by 24-h EPS (Fig. 2, D and E), indicating that enhancement of glucose uptake (Table 1) is not attributable to changes in the gene expressions of these glucose transporters. The augmented GLUT4 recycling appeared to last for ≥6 h but returned to baseline levels similar to those in naive C2C12 myotubes at 24 h after cessation of EPS treatment (Fig. 3, C and D). Importantly, once the C2C12 myotubes had been conditioned by 24-h EPS, resulting in their being endowed with contractility and metabolic properties (Figs. 1 and 2), the highly developed C2C12 myotubes after a rest period (6 h) displayed an ability to rapidly increase GLUT4 recycling in response to a second challenge with 45 min of EPS-induced contraction, which was augmented in a pulse duration-dependent manner (Fig. 3E). Phosphorylation of Tbc1d1 at Ser231 was also enhanced by short-term EPS treatment in contractile C2C12 myotubes (Fig. 3E).

A dose-responsive experiment also revealed potentiation of insulin-dependent GLUT4 recycling in C2C12 myotubes exposed to 24-h EPS. In addition to the increases in $V_{\text{max}}$ of insulin-stimulated GLUT4 recycling (~1.5-fold), the EC$_{50}$ of insulin for stimulation of GLUT4 recycling appeared to be reduced in C2C12 myotubes after 24-h EPS (naive: 60.14 nM vs. EPS: 14.31 nM; Fig. 3B). Overall, these results indicate that contractile C2C12 myotubes exposed to 24-h EPS not only have a higher glucose uptake, presumably resulting at least in part from GLUT4 translocation, but are also endowed with a potential improvement of the insulin-responsive GLUT4 recycling system, accounting for glucose uptake with better insulin sensitivity. Despite the potentiation of insulin action, insulin stimulation of the phosphatidylinositol 3-kinase signaling cascades as assessed by phosphorylation of PKB/Akt (Ser473 and Thr308) and GSK-3β (Ser9) did not differ between C2C12 myotubes exposed to vs. not exposed to 24-h EPS (Fig. 4). On the other hand, as described above (Fig. 1E), an obvious increase in the phosphorylation of Erk1/2 (Thr202/Tyr204) was observed, and this was further augmented by insulin stimulation (Fig. 4).

**Characteristics of the highly developed contractile C2C12 myotubes generated by applying EPS.** It has been well established that skeletal muscles are a highly plastic tissue and that...
established that exercise has a profound effect on the immune system, and recent evidence indicates various cytokines, such as IL-6, to be secreted from contracting skeletal muscles in vivo (55, 68). We therefore examined whether EPS-evoked contraction of C2C12 myotubes results in upregulation of cytokines and chemokines. As expected, IL-6 mRNA expression was remarkably upregulated in contractile C2C12 myotubes exposed to 24-h EPS as assessed by real-time PCR analysis (Fig. 6A). In addition to IL-6, we found expressions of CXCL1/KC and CXCL5/LIX to be remarkably upregulated in contractile C2C12 myotubes after 24-h EPS (Fig. 6, B and C). The EPS-induced upregulations of CXCL1/KC, CXCL5/LIX, and IL-6 were further confirmed by ELISA of the conditioned media, and massive production of these chemokines/cytokines was observed after 24-h EPS (CXCL1/KC, 648.5 ± 73.0 pg/ml; CXCL5/LIX, 1,998.0 ± 101.4 pg/ml; IL-6, 962.6 ± 29.5 pg/ml), whereas only small amounts of CXCL1/KC, CXCL5/LIX, and IL-6 were detected without EPS (141.5 ± 1.9, 88.0 ± 2.2, and 183.8 ± 2.8 pg/ml, respectively) (Fig. 6, D–F).

It has been well established that IL-6 is produced and released by contracting skeletal muscles in vivo in rodents and humans (54, 55, 60). However, whether productions of CXCL1/KC and CXCL5/LIX are elicited by exercise in vivo is unknown. Therefore, we finally performed mouse treadmill experiments to ascertain whether the increased productions of these chemokines in response to muscle contraction are physiologically relevant. Eleven-week-old C57BL/6J mice were divided into two groups (5 animals in each group), and one group received treadmill training (15 cm/s, 30 min, 8% slope, once) followed by immediate euthanization. Little difference was observed in body weight between the two groups (data not shown). The gene expressions of CXCL1/KC in both EDL and soleus muscles were significantly induced by 30 min of treadmill running, whereas the increases in IL-6 and CXCL5/LIX mRNA induced by exercise did not reach statistical significance (Fig. 7). Next, plasma samples were collected and subjected to ELISA to measure plasma chemokine concentrations (Fig. 8). The 30 min of treadmill running markedly increased the plasma CXCL1/KC concentration [from 60.26 ± 4.89 to 92.45 ± 11.98 pg/ml (P < 0.05, n = 5)] as well as the IL-6 concentration [from 0.75 ± 0.14 to 2.12 ± 0.67 pg/ml (P < 0.05, n = 5)]. CXCL5/LIX showed only a tendency to increase with treadmill exercise at a systemic level (from 26.70 ± 2.14 to 34.14 ± 5.32 pg/ml). These animal experiments demonstrate that treadmill exercise markedly alters plasma concentrations of these chemokines.

Finally, we analyzed the potential physiological role of chemokine production in contracting C2C12 myotubes in response to EPS by using SB-225002, a potent antagonist of CXCR2, a common receptor for CXCL1/KC and CXCL5/LIX (43). As shown in Fig. 9, insulin-responsive GLUT4 recycling was not enhanced when SB-225002 was present during 24-h EPS. This result suggests that these chemokines released from contracting C2C12 myotubes contribute to the development of insulin responsiveness through CXCR2 in an autocrine manner.

**DISCUSSION**

Establishment of advanced in vitro muscle contraction model using C2C12 myotubes. We herein established an advanced in vitro contraction model by employing EPS, which
allows us to generate highly developed C2C12 myotubes endowed with the capacity for vigorous contraction. This novel model is characterized by EPS-evoked contraction of C2C12 myotubes, followed by increases in glucose transport (Table 1) strongly associated with GLUT4 recycling at the plasma membrane (Figs. 2 and 3). Importantly, AMP kinase activation was significantly augmented in highly developed contractile C2C12 myotubes exposed to 24-h EPS (Fig. 1C). In addition, Tbc1d1, a Rab-GAP implicated in exercise-induced GLUT4 translocation in skeletal muscle, appeared to be phosphorylated on Ser231, an AMP kinase phosphorylation site, in the C2C12 myotubes after 24-h EPS. At the same time, phosphorylation of stress-responsive MAP kinases, including ERK5 and JNK, was also observed in these highly developed contractile C2C12 myotubes after EPS (Fig. 1E). These results suggest that EPS-evoked contractile activity not only induced higher energy expenditure but also sufficiently loaded mechanical stresses activating these stress-responsive signaling cascades. We further showed that the transcription factor ATF2, which is reportedly a specific substrate common to JNK and p38 (45, 62), was highly phosphorylated in contractile C2C12 myotubes after 24-h EPS (Fig. 1E). Although phosphorylation of p38 MAP kinase was apparently suppressed in C2C12 myotubes after 24-h EPS, the recent finding that ATF2 activates a negative feedback loop for suppressing p38 phosphorylation by upregulating dual specificity phosphatases for p38, the Dusp family (11), may at least partially explain this complexity of stress-activated MAP kinase regulation in response to EPS. ATF2 is also reportedly activated in response to acute exercise, and this activation is important for peroxisome proliferator-activated receptor-γ coactivator-1α activation, followed by an induction of mitochondrial gene expression, leading to the switch in muscle fiber types and improvement of glucose metabolism (2, 44). Although it is quite difficult to directly compare in vivo exercise and EPS-evoked contraction of cultured C2C12 myotubes, a culture condition that involves the process of muscle maturation in terms of contractility acquisition and subsequent alterations in gene expressions and meta-
physiologically, skeletal muscle contraction is induced by increases in the intracellular Ca\(^{2+}\) concentration triggered by motor neurons via the neuromuscular junction; on the other hand, in an advanced in vitro contraction model, the Ca\(^{2+}\) transient must be stimulated by applying EPS instead (22). Thus, although both of these stimuli differentially trigger a similar rise in the intracellular Ca\(^{2+}\) concentration responsible for the subsequent Ca\(^{2+}\)-evoked contraction and Ca\(^{2+}\)-dependent signaling activation, caution must be exercised in interpreting the results of such experiments. In addition, it is well known that the effects of exercise on skeletal muscle can vary according to the frequency, strength, and duration of the exercise (39); thus, more detailed experiments are needed to
clarify the extent to which EPS-evoked C2C12 contraction corresponds to various types of exercise.

Contraction-dependent GLUT4 recycling/glucose uptake in C2C12 myotubes. We showed that EPS-evoked contraction of C2C12 myotubes results in activation of both AMPK and stress-inducible MAPK cascades (Fig. 1) and that the induction of GLUT4 recycling is responsible for enhanced glucose uptake (Figs. 2 and 3 and Table 1), all of which have previously been reported as exercise-derived intracellular signals or biological responses (28, 67, 77). Since AMP kinase has been demonstrated to have a role in GLUT4 regulation in skeletal muscle (41, 56), the EPS-activated AMP kinase cascade in C2C12 myotubes is among the plausible explanations for the augmented glucose uptake and GLUT4 recycling observed in contractile C2C12 myotubes after EPS (Fig. 2). Additionally, there is growing evidence implicating Ca2+ in regulating GLUT4 translocation and glucose uptake in skeletal muscle (42, 76), which might be mediated through activation of Ca2+-dependent CaMKKβ, leading to the phosphorylation and activation of AMP kinase (29, 79). In addition, recent reports demonstrate the potential importance of Tbc1d1, a Rab-GAP structurally related to AS160 (also known as Tbc1d4), in the process of exercise-induced GLUT4 translocation in skeletal muscle (71). Consistent with these studies, we observed Tbc1d1 to be phosphorylated on Ser231, an AMP kinase phosphorylation site (15), in C2C12 myotubes exposed to 24-h EPS (Fig. 1C). Although we observed only a possible tendency for compound C (an AMP kinase inhibitor) and STO-609 (a CaMKKβ inhibitor) to exert inhibitory effects on the augmentation of GLUT4 recycling in the present study, EPS-induced repetitive Ca2+ transients (22) as well as energy expenditure may both contribute to triggering AMP kinase activation, resulting in augmented GLUT4 recycling and glucose uptake (Table 1) in the absence of insulin (Fig. 2). An interesting observation, made in the experiments depicted in Fig. 3E, was that the highly developed C2C12 myotubes exposed to 24-h EPS followed by a rest period appeared to become responsive to a second challenge with a short EPS-induced contraction.
This was validated using the myc-GLUT4 recycling assay under conditions that better mimic in vivo exercise, at least in terms of exercise duration. Since the naïve C2C12 myotubes did not possess the ability to induce GLUT4 translocation acutely in response to short-term EPS (data not shown), we speculate that 24-h EPS treatment apparently promotes an important maturation and/or adaptation process, including changes in gene expressions and structural alterations (Fig. 5) that also result in the acquisition of a contractile capability providing higher energy expenditure and mechanical stress (Fig. 1), as discussed in detail below, as well as improved insulin responsiveness (Figs. 2 and 3). Further experiments will be needed to clarify the mechanism(s) underlying this maturation/adaptation process. However, the enhanced GLUT4 recycling in our highly developed C2C12 myotubes associated with 45 min of EPS-induced contraction strongly suggests that this in vitro contraction model mimics in vivo exercise well and is thus physiologically relevant and useful for studying acute biological responses to contractile activity.

Another important observation in the present study is that insulin responsiveness in terms of insulin-induced GLUT4 recycling was also significantly improved in the highly developed contractile C2C12 myotubes exposed to 24-h EPS (Figs. 2 and 3). Remarkably, the insulin-dependent signaling, especially insulin-dependent phosphorylation of Akt/PKB, was not significantly influenced by 24-h EPS (Fig. 4). As described in the introduction, there is controversy as to whether or not exercise alters the insulin-dependent signaling responsible for improving insulin responsiveness (27, 73, 75, 78), but our present results indicate that the phosphatidylinositol 3-kinase CaM-dependent kinases (CaMK) (57, 81), as well as stress-related enzymes, including calcineurin and CaM-dependent kinases (CaMK) (57, 81), as well as stress-responsive MAP kinases, including p38 and Erk5 (38, 84). In addition, a recent report demonstrated AMP kinase involvement in mediating an exercise-dependent shift in muscle fiber type from type IIb (glycolytic) to type IIa (oxidative) (65). Given that slow/oxidative muscles such as the soleus represent higher insulin-responsive glucose uptake than fast/glycolytic muscles in vivo (8, 34), global alterations in myotube phenotype induced by EPS may be attributable to the acquisition of a better insulin-responsive GLUT4 recycling system in contractile C2C12 myotubes exposed to 24-h EPS. This is an important issue awaiting clarification and intensive studies that are presently underway.

Induction of chemokines in response to skeletal muscle contraction. Until recently, considerable effort was directed toward identifying factors derived from working skeletal muscle in response to exercise by using animal models as well as human subjects. To the best of our knowledge, only tumor necrosis factor-α, macrophage inflammatory protein-1, and interleukins (IL-1β, IL-1ra, IL-6, IL-8, IL-10, and IL-15) have been identified as exercise factors in humans (58). In the present study, using the advanced in vitro contraction model, we observed marked induction of IL-6 in contractile C2C12 myotubes after EPS (Fig. 6). In addition, we found that chemokines, mouse CXCL1/KC and mouse CXCL5/LIX, were also remarkably induced by EPS (Fig. 6), and this result was further confirmed by animal experiments showing treadmill running to induce these factors in vivo (Figs. 7 and 8). These results suggest expressions of IL-6 and CXCL1/KC in muscle cells to be adequately responsive to the contractile activity, although the contraction was evoked by EPS. Although CXCL1/KC mRNA levels in the EDL and soleus muscle adequately explained serum CXCL1/KC levels, further studies are needed to reveal which muscle plays the major role in raising serum exercise factor levels, since in our experiments IL-6 mRNA levels in neither the EDL nor the soleus muscle changed significantly with treadmill exercise and those serum IL-6 levels did (Fig. 7 and 8).

Mouse CXCL1/KC and CXCL5/LIX are categorized into the ELR-containing CXC chemokine family that also includes human IL-8, previously identified as an exercise factor in humans (14, 54), and all three display similar properties such as neutrophil activation and migration in mice (48, 74). However, the functional receptors they utilize are different, and human IL-8 is capable of binding to and activating both CXCR1 and CXCR2, whereas mouse CXCL1/KC and CXCL5/LIX trigger signals only through CXCR2 (10, 82). In this regard, although an IL-8 equivalent has not been identified in the mouse, Fan et al. (19) recently identified mouse CXCR1, which serves as a high-affinity receptor for the murine IL-8 equivalent that had long eluded investigators, and found that mouse CXCL6/GCP-6 functions as a specific ligand for mouse CXCR1. Taken as a whole, emerging evidence suggests that there may be a functional divergence between chemokines that can activate both CXCR1 and CXCR2, such as human IL-8 and mouse CXCL6/GCP-6, and that they bind only CXCR2, not CXCR1 (19, 82). Since similarity analysis revealed mouse CXCL1/KC and CXCL5/LIX to be equivalent to human growth-related oncogene factor-α (GROα) and epithelial neutrophil-activating peptide-78 (ENA-78), respectively (69), it is tempting to speculate that these human chemokines, in addition to IL-8, may also be upregulated in human muscles during exercise training.

AJP-Endocrinol Metab • VOL 295 • NOVEMBER 2008 • www.ajpendo.org
Moreover, Frydelund-Larsen et al. (20) recently reported that expression of CXCR2 in working muscle was increased in response to exercise in healthy individuals, suggesting the importance of CXCR2 agonists serving an autocrine function in contracting muscles. This possibility is presently being investigated in our laboratory using the above-described advanced in vitro contraction model, which is also a potentially powerful tool for analyzing the effects of these exercise factors. In addition, since ELR-CXC chemokines have been shown to be potent angiogenic factors (6, 70), mouse CXCL1/KC and CXCL5/LIX may also contribute to mediating the exercise-induced angiogenesis that occurs within active muscle (3). Indeed, CXCR2 is expressed in microvascular endothelial cells, which are responsible for angiogenesis induced by ELR-containing CXC chemokines, and the importance of CXCR2-mediated angiogenesis in vivo has been established with the utilization of CXCR2-deficient mice (1).

CXCL1/KC and CXCL5/LIX are reportedly secreted by cardiac myocytes in response to inflammatory stresses (47, 51) via transcriptional activation of NF-kB that can be mediated through the JNK MAP kinase pathway. Since JNK appeared to be strongly activated (phosphorylated) in C2C12 myotubes by EPS-evoked contraction (Fig. 1E), this signaling pathway may be involved in the upregulation of these chemokine expressions in response to contractile activity. In any case, taken together with our observation that exercise-dependent upregulation of these chemokines in contracting muscle is responsible for the increases in their systemic levels, it appears that, during exercise, these CXCR2-mediated signals contribute to exerting beneficial and/or detrimental effects of exercise on certain cells expressing this receptor in various other tissues/organs in addition to skeletal muscles. In fact, we identified a potential role of CXCL1/KC and CXCL5/LIX in the metabolic functions of C2C12 myotubes, i.e., the development of insulin resistance (Fig. 9). In this regard, several lines of evidence indicate that the systemic level of human IL-8, which is presumably released mainly from white adipose tissue, is associated with type 2 diabetes and obesity (12, 30). These results indicate that, although it remains unknown whether there are alterations in systemic levels of CXCL1/KC and CXCL5/LIX (as well as their human counterparts GROα and ENA-78), like human IL-8, under diabetic/obese conditions, these ELR-containing CXC chemokines activating CXCR2 may play a role in regulating metabolic status in multiple tissues/organs, including skeletal muscle itself. Future studies are anticipated to reveal the contribution of this exercise-dependent chemokine production to the enhancement of insulin-dependent GLUT4 recycling/glucose uptake demonstrated in the present study.

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