Characterization of contraction-inducible CXC chemokines and their roles in C$_2$C$_{12}$ myocytes

Taku Nedachi,$^{1,2}$ Hiroyasu Hatakeyama,$^1$ Tatsuyoshi Kono,$^2$ Masaaki Sato,$^3$ and Makoto Kanzaki$^{1,3,4}$

$^1$Center for Research Strategy and Support (CRESS), $^2$Division of Biomaterials, Biomedical Engineering Research Organization, and $^3$Graduate School of Biomedical Engineering, Tohoku University, and $^4$Japan Science and Technology Agency, Core Research for Evolutionary Science and Technology, Sendai, Japan

Submitted 17 February 2009; accepted in final form 18 July 2009

Nedachi T, Hatakeyama H, Kono T, Sato M, Kanzaki M. Characterization of contraction-inducible CXC chemokines and their roles in C$_2$C$_{12}$ myocytes. Am J Physiol Endocrinol Metab 297: E866–E878, 2009. First published July 21, 2009; doi:10.1152/ajpendo.00104.2009.—Physical exercise triggers the release of several cytokines/chemokines from working skeletal muscles, but the underlying mechanism(s) by which skeletal muscles decipher and respond to highly complex contractile stimuli remains largely unknown. In an effort to investigate the regulatory mechanisms of the expressions of two contraction-inducible CXC chemokines, CXCL1/KC and CXCL5/LIX, in contracting skeletal muscle cells, we took advantage of our in vitro exercise model using highly developed contractile C$_2$C$_{12}$ myotubes, which acquire properties similar to those of in vivo skeletal muscle via manipulation of Ca$^{2+}$ transients with electric pulse stimulation (EPS). Production of these CXC chemokines was immediately induced by EPS-evoked contractile activity in a manner dependent on the activities of JNK and NF-κB, but not p38, ERK1/2, or calcineurin. Intriguingly, exposure of myotubes to cyclic mechanical stretch also induced expression of these CXC chemokines; however, a much longer period of stimulation (~12 h) was required, despite rapid JNK phosphorylation. We also demonstrate herein that CXCL1/KC and CXCL5/LIX have the ability to raise intracellular Ca$^{2+}$ concentrations via CXCR2-mediated activation of pertussis toxin-sensitive Gi proteins in C$_2$C$_{12}$ myoblasts, an action at least partially responsible for their migration and differentiation. Although we revealed a possible negative feedback regulation of their own production in response to the contractile activity in differentiated myotubes, exogenous administration of these CXC chemokines did not acutely influence either insulin-induced Akt phosphorylation or GLUT4 translocation in C$_2$C$_{12}$ myotubes. Taken together, these data shed light on the fundamental characteristics of contraction-inducible CXC chemokine production and their potential roles in skeletal muscle cells.

Physical exercise exerts a highly complex physiological stimulus triggering multiple biochemical and biophysical aspects of cellular functions, leading to a broad array of metabolic and growth/differentiation effects in skeletal muscles, such as changes in gene transcription, hypertrophy induction, and maintenance of healthy insulin responsiveness (25, 46). In addition, a large body of evidence obtained from whole body exercise experiments now indicates that skeletal muscle can function as an endocrine organ that produces a wide variety of cytokines (myokines), including interleukin (IL)-1β, IL-6, IL-8, IL-10, and IL-15 and that physical activity (exercise) induces some of these muscle-derived myokines, referred to as “exercise factors” (46). In addition, several lines of evidence have demonstrated the potential involvement of contraction-inducible activation of neuregulin/ErbB signaling cascades in glucose transport in muscle cells and skeletal muscle (12, 34). Although the physiological significance of these muscle-derived soluble factors remains to be further elucidated, they could function not only in working skeletal muscles but also mediate some of the exercise-induced metabolic alterations in other tissues such as the liver and adipose (46).

A crucial issue in understanding these varied effects of exercise, including myokine expressions, is clarification of the intracellular signaling mechanisms by which muscle cells decipher and respond to the highly complex contractile/elongation stimulus, consisting of both mechanical stress and energy fluctuations evoked by Ca$^{2+}$ transients. Physical exercise has been shown to evoke several intracellular signaling cascades, including the AMP kinase and stress-activated MAP kinase signaling pathways, in contracting skeletal muscles (21, 53, 57). However, the precise molecular mechanism(s) by which exercise activity regulates intracellular signaling to the transcriptional machinery in the nucleus responsible for modulating gene expression is poorly understood, due at least in part to the lack of a muscle cell culture system that can reproduce muscle contraction.

In recent years, we have succeeded in establishing an advanced in vitro muscle contraction model using highly developed C$_2$C$_{12}$ myotubes displaying vigorous electric pulse stimulation (EPS)-evoked contractile activity associated with activation of the AMP kinase and stress-activated MAP kinase signaling pathways (20, 38). Importantly, the highly developed C$_2$C$_{12}$ myotubes generated by applying EPS possess some important aspects of the beneficial effects of exercise such as improved insulin sensitivity of GLUT4 translocation and fiber type switching. In addition, our contractile C$_2$C$_{12}$ myotubes appear to have the ability to secrete several chemokines/myokines, including CXCL1/KC, CXCL5/LIX, and IL-6, in response to EPS (38), approximating levels observed in working skeletal muscle in vivo.

Mouse CXCL1/KC and CXCL5/LIX are categorized as belonging to a Glu-Leu-Arg (ELR)-containing CXC chemokine family that also includes human IL-8, identified previously as an exercise factor in humans (2), and all of these chemokines are well known to be responsible for the chemotraction of neutrophils to sites of inflammation (58). In addition, several lines of evidence demonstrate that ELR-containing CXC chemokines exert potent angiogenic activities (51) as well as mitogenic activities on various cell types (14, 18). Both CXCL1/KC and CXCL5/LIX utilize CXCR2, a member of the superfamily of seven-transmembrane receptors, to trigger intracellular signals via heterotrimeric G proteins, whereas human

---

Address for reprint requests and other correspondence: M. Kanzaki, 2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan (E-mail: kanzaki@bme.tohoku.ac.jp).
IL-8 binds to and activates both CXCRL1 and CXCR2 (11, 59). Intriguingly, a recent study demonstrated that CXCR2 is expressed in skeletal muscle and that its expression levels were increased in response to exercise in humans (19). Together with our previous results (38), these observations allow us to speculate that contraction-inducible CXC chemokines linked to CXCR2 exert their influences on muscle in an autocrine/paracrine fashion. However, the intracellular signaling pathways responsible for the contraction-induced expressions of CCL1/KC and CXCL5/LIX as well as their potential roles in skeletal muscle cells remain unknown.

In the present study, we investigated the mechanistic details of the contraction-inducible regulation of CCL1/KC and CXCL5/LIX using an advanced in vitro contraction model consisting of contractile C2C12 myotubes. In addition, we explored the potential roles of these contraction-inducible CXC chemokines in skeletal muscle cells and obtained evidence that these chemokines not only contribute to inducing the migration of myoblasts but are also involved in myogenesis as autocrine/paracrine factors.

**MATERIALS AND METHODS**

**Materials.** The Western blot detection kit (West Super Femto Detection Reagents) was obtained from Pierce Biotechnology (Rockford, IL). Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and trypsin-EDTA were purchased from Sigma Chemicals (St. Louis, MO). Cell culture equipment was from BD Biosciences (San Jose, CA). Calf serum (CS) was used during the EPS treatments. The medium was changed every 12 h during EPS. (Sigma) was used during the EPS treatments. The growth medium was switched to DMEM 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 study, cells were grown on four-well plates (Nalgen Nunc International, Rochester, NY). Differentiation medium was changed every 24 h. Mouse skeletal muscle cell lines, C2C12 myoblasts (60), were maintained in DMEM 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 study, cells were grown on four-well plates (Nalgen Nunc International, Rochester, NY) at a density of 1 × 105 cells/well in 5 ml of growth medium or on six-well plates (BD Biosciences) at a density of 3 × 104 cells/well in 3 ml of growth medium. Three days after plating, the cells had reached ~80–90% confluence (day 0). Differentiation was induced by switching the growth medium to DMEM supplemented with 2% CS, 30 μg/ml penicillin, and 100 μg/ml streptomycin (differentiation medium). The differentiation medium was changed every 24 h.

**Electrical pulse stimulation.** Fully differentiated C2C12 myotubes in four-well or eight-well dishes (Nalgene 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% CS supplemented with 200% amino acids (Sigma) was used during the EPS treatments. The medium was changed every 12 h during EPS.

**Western blot analysis.** The expression and phosphorylation of each protein were analyzed by Western blotting. In brief, the harvested cell lysates were subjected to 7.5 or 12% SDS-polyacrylamide gel electrophoresis (1: 30, bis-acrylamide). Proteins were transferred to a PVDF membrane (Immobilon-P, Millipore), and the membranes were then blocked for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline (TBS) 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-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ATF2 (activating transcription factor-2), anti-ATF2, anti-phospho-IκB, anti-IκB, anti-mouse IgG, anti-BD living color, anti-myosin, anti-myogenin, anti-MEF2 antibody]. 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). Protein concentrations were determined using a bichinonic acid assay (Pierce). Three independent experiments were performed for each condition. Coomassie blue staining was also performed to assess the efficiency of protein transfer.

**Exposure to mechanical stretch.** C2C12 myoblasts were seeded onto an elastic silicone chamber (STB-CH-10; STREX, Osaka, Japan) coated with Matrigel (BD Bioscience) and were differentiated into myotubes by switching to differentiation medium. The silicone chamber was mounted onto a mechanical stretch device (STB140-04, STREX), and cyclic longitudinal mechanical stretch (15%) at 1-Hz frequency was applied. Control cells were maintained under quiescent culture conditions.

**Real-time reverse transcription-PCR.** Total RNA was prepared using TRIzol reagent according to the manufacturer’s instructions and was quantified using an ND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE). Real-time transcription was carried out using a first-strand cDNA synthesis kit for PCR (Roche Applied Science). Real-time PCR reactions were performed using the Roche LightCycler, utilizing Roche SYBR Green reagents. Amplification of PCR products was quantified during PCR by measuring fluorescence associated with the binding of double-stranded DNA to the SYBR Green dye incorporated into the reaction mixture. The sequences of the oligonucleotides used to PCR-amplify the cDNAs of interest were as follows: CCL1/KC (5′-GCT GCC TTC TGA CAA CAC TAT-3′ and 5′-CAA GCA GAA CTG AAC TAC CAT-3′), and CXCL5/LIX (5′-ATG AAC TCC CTG TTA G-3′ and 5′-TTC CCA TAG TGT GAC AGA TAG-3′). β-Actin was quantified as a housekeeping gene using 5′-CGT TGA CAT CCG TAA AGA CCT-3′ and 5′-AGC CAC CGA TCC ACA CAG A-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using 5′-GGGAAACCT-GCAGAATGTA-3′ and 5′-GATCGAGGTGTTGAGATG-3′. Following an initial denaturation step of 95°C for 1 min, 30–45 cycles of 95°C for 10 s, 60°C for 1 s, and 72°C for 10 s were used for CCL1/KC, CXCL5/LIX, and β-Actin. For GAPDH, an initial denaturation step of 95°C for 10 min, 45 cycles of 95°C for 10 s, 57.5°C for 15 s, and 72°C for 15 s were used.

**Measurement of the changes in intracellular Ca2+.** The acetoxyethyl (AM) ester form of fluo 4 (Invitrogen, Carlsbad, CA) was dissolved in dimethyl sulfoxide (DMSO) at 5 mM. C2C12 cells plated onto poly-1-lysine-coated glass-bottom dishes (no. 1S; Matsunami, Osaka, Japan) were serum starved for 3 h and incubated in serum-free DMEM-LG containing 2 μM fluo 4-AM, 0.008% Pluronic F-127 (Invitrogen), and 0.1% bovine serum albumin for 30 min at 37°C. Then, the cells were washed with Sol.A (150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES-NaOH, pH 7.4) containing 5.5 mM d-glucose. In the experiments shown in Fig. 7D, pertussis toxin (PTX, 200 μg/ml; Calbiochem) was added to the serum-depleted media, fluo 4 loading media and Sol.A. Images were acquired every 2 s with a confocal microscope (FV1000; Olympus, Tokyo, Japan) equipped with a argon laser and a ×20 oil immersion objective lens (Olympus, UPlanApo ×20 Oil; numerical aperture of 0.80) at room temperature (RT; 23–25°C). The filters used were a DM405/488 dichroic mirror and an emission filter (BA505IF, Olympus). Changes in intracellular Ca2+ ([Ca2+]i) were expressed by the ratio (F-F0)/F0, where F0 and F represent the fluorescence intensity of fluo 4 and the averaged fluorescence intensity of the dye during the 30 s (15 frames) before stimulation, respectively.

**Anti c-myc antibody uptake assay.** GLUT4 translocation was analyzed as described previously (39). Briefly, C2C12 myoblasts expressing myc-GLUT4-ECFP (enhanced cyan fluorescent protein) were serum-starved for 4 h, washed three times with Krebs-Ringer-Phosphate (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 30 min. After incubation for 30 min with the anti-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).

Migration assay. Chemotactic activities of C2C12 myoblasts were analyzed as previously described (6). In brief, the upper surfaces of cell culture inserts (6 mm diameter, 8 μM pore size; BD Falcon) were coated with Matrigel (250 μg/ml) for 60 min at 37°C and placed on 24-well plates (BD Falcon). C2C12 myoblasts were serum starved for 4 h, trypsinized, and resuspended in DMEM containing heat-inactivated BSA (250 μg/ml). Then, 1 × 10^4 cells were replated onto the membrane. The lower well contained serum-free media supplemented with the indicated amounts of recombinant mouse KC, mouse LIX, or insulin-like growth factor I (IGF-I; R&D Systems, Minneapolis, MN). The cell culture was incubated for 2 h at 37°C, and nonmigrating cells on the upper chamber were then removed with a cotton swab. The migrating C2C12 myoblasts located on the other side of the membrane were then fixed with 4% PFA in PBS, and stained with 0.4% crystal violet in 10% ethanol. Migrating cells in five different regions were counted in each experiment, and an average migration value was obtained. Five independent experiments were performed.

Short hairpin RNA-mediated reduction of JNK1/2 and CXCR2. We used a lentiviral mediated short hairpin (sh)RNA system from Santa Cruz Biotechnology (Santa Cruz, CA) and followed the manufacturer’s protocol. Lentiviral particles contain shRNA toward JNK1-, JNK2-, or CXCR2-specific sequences as well as a scrambled sequence that consists of nucleotides that do not match any known gene transcript in the murine CXCR2-specific sequences as well as a scrambled sequence that consists of nucleotides that do not match any known gene transcript in the murine genome. The target sequences of JNK1 were 5’-GGAGAAGCTATTTA-CAATTCAGAGATTTGAAATTACGCTTCC-3’, 5’-GGTTGAGCACTCAATTCAGAGATTTGAAATTACGCTTCC-3’, and 5’-GGAGGATTAGCTTTGTATCATATTCAAGAGATATGATATGGA-3’. The target sequences of JNK2 were 5’-GGAAAGAGCTAATTTA-CATTCAAGAGATTTGAAATTACGCTTCC-3’, 5’-GGATGCACTCAATTCAGAGATTTGAAATTACGCTTCC-3’, and 5’-GGATTAGCTTTGTATCATATTCAAGAGATATGATATGGA-3’. The target sequences of CXCR2 were 5’-GGATGTAGGTAA-TTATTACATTCAAGAGATTTGAAATTACGCTTCC-3’, 5’-GGCTAGGTAA-TTATTACATTCAAGAGATTTGAAATTACGCTTCC-3’, and 5’-GGAGGATTAGCTTTGTATCATATTCAAGAGATATGATATGGA-3’. The infected C2C12 myoblasts were selected on the basis of puromycin resistance and then assessed for their protein expression levels by western blotting.

ELISA. Levels of CXCL1/KC and CXCL5/LIX in culture supernatants were measured with commercially available ELISA kits (R&D Systems).

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

RESULTS

Characteristics of EPS-evoked chemokine production. To characterize EPS-induced production of CXCL1/KC and CXCL5/LIX in C2C12 myotubes, we first examined the time courses of CXCL1/KC and CXCL5/LIX accumulations in the medium during 24-h EPS at 1 Hz. Significant amounts of these chemokines were released immediately after 1 h of EPS, and their accumulations increased steadily during the 24-h EPS (Fig. 1, A and B). It should be noted that C2C12 myotubes started to show weak contraction even after just 1 h of EPS, and their contractility was gradually enhanced thereafter (20, 38). CXCL1/KC and CXCL5/LIX production were dependent on the frequency of EPS and were obviously lower when the cells were treated with 0.1 Hz EPS for 24 h (Fig. 1, C and D). EPS-induced releases of CXCL1/KC and CXCL5/LIX were associated with upregulation of their mRNA expressions (see Fig. 9, F and G) (38) and were almost completely suppressed in the presence of 10 μg/ml cycloheximide (data not shown), suggesting transcriptional regulation to be mainly responsible for the production of these chemokines by EPS-evoked contraction in C2C12 myotubes. It is also noteworthy that experimental alterations in amino acid levels in culture media did not influence the EPS-induced release of either CXCL1/KC or CXCL5/LIX (data not shown), indicating that nutritional availability does not play a major role in regulating the expressions of these CXC chemokines in response to EPS-evoked contraction.

Intriguingly, when the cells were exposed to 24-h EPS at 1 Hz in the presence of SB-225002, a potent receptor antagonist for CXCR2 serving as a common receptor for both CXCL1/KC and CXCL5/LIX (56), the production of these chemokines were further augmented (Fig. 1, E and F). This result strongly suggests that these CXC chemokines, released upon EPS-evoked contraction, serve as autocrine factors in a negative feedback loop blocking their excessive production, at least under the culture conditions employed in these experiments.

JNK is the primary MAP kinase responsible for EPS-induced CXCL1/KC and CXCL5/LIX production. In our previous study, we observed multiple MAP kinase families including ERK1/2, JNK, and ERK5 to be activated in contractile C2C12 myotubes after 24-h EPS (38). Since the production of CXCL1/KC and CXCL5/LIX were enhanced by EPS relatively quickly, i.e., within several hours, we examined the effects of short-term EPS (3 h) on the phosphorylation status of ERK1/2, JNK, and p38 MAP kinases. Western blot analysis demonstrated that phosphorylations of ERK1/2 (Fig. 2A, lanes 1–4) and JNK (Fig. 2A, lanes 5–8) were induced by 3-h EPS, whereas the phosphorylation status of p38 was unaffected (Fig. 2A, lanes 9–12).

To identify the intracellular signals involved in EPS-induced chemokine production, C2C12 myotubes were exposed to 3-h EPS in the absence or presence of MAP kinase inhibitors, the MEK (MAPK for Erk1/2) inhibitor PD-98059, the JNK inhibitor SP-600125, or the p38 MAP kinases. Western blot analysis demonstrated that phosphorylations of ERK1/2 (Fig. 2A, lanes 1–4) and JNK (Fig. 2A, lanes 5–8) were induced by 3-h EPS, whereas the phosphorylation status of p38 was unaffected (Fig. 2A, lanes 9–12).

In our previous study, we observed multiple MAP kinase families including ERK1/2, JNK, and ERK5 to be activated in contractile C2C12 myotubes after 24-h EPS (38). Since the production of CXCL1/KC and CXCL5/LIX were enhanced by EPS relatively quickly, i.e., within several hours, we examined the effects of short-term EPS (3 h) on the phosphorylation status of ERK1/2, JNK, and p38 MAP kinases. Western blot analysis demonstrated that phosphorylations of ERK1/2 (Fig. 2A, lanes 1–4) and JNK (Fig. 2A, lanes 5–8) were induced by 3-h EPS, whereas the phosphorylation status of p38 was unaffected (Fig. 2A, lanes 9–12).

To identify the intracellular signals involved in EPS-induced chemokine production, C2C12 myotubes were exposed to 3-h EPS in the absence or presence of MAP kinase inhibitors, the MEK (MAPK for Erk1/2) inhibitor PD-98059, the JNK inhibitor SP-600125, or the p38 inhibitor SB-203580. Among these MAP kinase inhibitors, only SP-600125, the JNK inhibitor, significantly blocked EPS-stimulated CXCL1/KC (P < 0.05, n = 3; Fig. 2B) and CXCL5/LIX (P < 0.05, n = 3; Fig. 2C) productions, whereas the other MAP kinase inhibitors, PD-98059 and SB-203580, exerted no such effects (Fig. 2, B and C). We also analyzed the phosphorylation status of JNK as well as that of ATF2, a common substrate for both JNK and p38 (7, 16, 23, 33, 44). As expected, SP-600125 blocked EPS-induced JNK phosphorylation as well as that of its substrate ATF2 (Fig. 2D, lanes 4 and 9). Involvement of JNK1/2 in these CXC chemokine production was further confirmed by shRNA-mediated knockdown experiments (Fig. 3, A–C). These results indicate that JNK plays a pivotal role in the upregulation of CXCL1/KC and CXCL5/LIX production elicited by 3-h EPS in contractile C2C12 myotubes. None of these inhibitors had any significant effect on development of the contractile activity endowed by 3-h EPS (data not shown).
We also confirmed removal of extracellular Ca\textsuperscript{2+} by EGTA to completely abolish the EPS-induced phosphorylations of both JNK and ATF2 (Fig. 4C, lanes 3 and 7), resulting in a significant suppression of both CXCL1/KC and CXCL5/LIX production (Fig. 4, A and B). Furthermore, under Ca\textsuperscript{2+}-chelating conditions, no contractile activity developed in response to EPS (data not shown), as we previously reported (20). Although calcineurin, a Ca\textsuperscript{2+}/calmodulin-activated protein phosphatase, is reportedly involved in a wide array of chemokine/interleukin expressions via regulation of nuclear factor of activated T cells (NFAT) family transcription factors in various cell types (54), cyclosporin A, a calcineurin inhibitor, had no effects on either the EPS-induced chemokine production or the phosphorylation status of JNK and ATF2 (Fig. 4C, lanes 4 and 8).

Taken together, these results demonstrate that, although multiple MAP kinase cascades are activated by EPS-induced contractile activity in C\textsubscript{2}C\textsubscript{12} myotubes, JNK is predominantly involved in the production of CXCL1/KC and CXCL5/LIX elicited by EPS in these contractile myotubes. In addition, our results indicate that a cyclosporine A (CsA)-sensitive intracellular signaling cascade(s) does not directly take a part in expressions of these CXC chemokines.

Involvement of NF-\textkappa B in EPS-induced CXCL1/KC and CXCL5/LIX production. It has been well established that NF-\textkappa B, a transcription factor directly involved in inflammatory and immunological responses, plays a role in the production of multiple chemokines in various cell types (3, 40). Therefore, we examined the possible involvement of NF-\textkappa B in the EPS-induced chemokine production in contractile C\textsubscript{2}C\textsubscript{12} myotubes. Parthenolide, a potent inhibitor of the NF-\textkappa B signaling pathway (61), significantly blocked EPS-dependent inductions of CXCL1/KC and CXCL5/LIX (Fig. 5, A and B). We also determined the phosphorylation status (Ser\textsuperscript{32/36}) and measured the amount of I\textkappa B protein, to ascertain whether the NF-\textkappa B signaling pathway is activated, via degradation of I\textkappa B (52), by 3-h EPS in contractile C\textsubscript{2}C\textsubscript{12} myotubes. Although there was no significant difference in I\textkappa B amounts between C\textsubscript{2}C\textsubscript{12} myotubes with vs. without 3-h EPS, phosphorylation of I\textkappa B (Ser\textsuperscript{32/36}) appeared to be induced by EPS treatment (Fig. 5C).
dinal mechanical stretch to C2C12 myotubes grown in an elastic silicone chamber and then measured mRNA levels of CXCL1/KC and CXCL5/LIX using the RT-PCR technique (Fig. 6, A and B). Although expressions of these chemokines were immediately detectable in myotubes with EPS-induced contractile activity (Fig. 1, A and B), cyclic stretch (15% at 1 Hz) did not significantly enhance CXCL1/KC and CXCL5/LIX expression compared to myotubes without stretch. These results suggest that EPS-induced mechanical stretch is a crucial factor in the induction of CXCL1/KC and CXCL5/LIX expression in C2C12 myotubes.

Fig. 2. Role of MAP kinase activation in EPS-dependent CXC chemokine production in C2C12 myotubes. Differentiated C2C12 myotubes were treated with or without EPS at 20V/25 mm, 1 Hz, 2 ms, for 3 h. (A) Total cell lysates were prepared as described in MATERIALS AND METHODS and subjected to Western blotting to analyze phosphorylation status and total amounts of ERK1/2 (lanes 1–4), JNK (lanes 5–8), or p38 MAP kinase (lanes 9–12). (B and C) EPS was applied in the presence or absence of PD-98059 (PD; 50 μM), SP-600129 (SP; 20 μM), or SB-203580 (SB; 5 μM) for 3 h, and concentrations of CXCL1/KC and CXCL5/LIX in conditioned media were measured by ELISA (*P < 0.05, n = 3). D: EPS was applied in the presence or absence of PD, SP, or SB for 3 h, and phosphorylation status and total amounts of JNK (lanes 1–5) and ATF2 (lanes 6–10) were assessed by Western blot analysis. At least 3 independent experiments were performed; representative results are shown.

Fig. 3. Effects of short hairpin (sh)RNA-mediated knockdown of JNK1/2 on EPS-dependent CXC chemokine production in C2C12 myotubes. C2C12 myoblasts were infected with lentiviral particles containing shRNAs toward JNK1- and JNK2-specific sequences as well as a scrambled sequence, and puromycin-resistant cells were differentiated into myotubes. Differentiated C2C12 myotubes were treated with or without EPS at 20V/25 mm, 1 Hz, 2 ms, for 3 h. (A) and (B): concentrations of CXCL1/KC and CXCL5/LIX in conditioned media were measured by ELISA (*P < 0.05, n = 3). (C) Total cell lysates were prepared and subjected to Western blotting to analyze phosphorylation status and total amounts of JNKs or ATF2 (activating transcription factor-2). Three independent experiments were performed; representative results are shown.

Fig. 4. Effects of MAP kinase inhibitors on EPS-dependent CXC chemokine production in C2C12 myotubes. Differentiated C2C12 myotubes were treated with or without EPS at 20V/25 mm, 1 Hz, 2 ms, for 3 h. A: Total cell lysates were prepared as described in MATERIALS AND METHODS and subjected to Western blotting to analyze phosphorylation status and total amounts of ERK1/2 (lanes 1–4), JNK (lanes 5–8), or p38 MAP kinase (lanes 9–12). B and C: EPS was applied in the presence or absence of PD-98059 (PD; 50 μM), SP-600129 (SP; 20 μM), or SB-203580 (SB; 5 μM) for 3 h, and concentrations of CXCL1/KC and CXCL5/LIX in conditioned media were measured by ELISA (*P < 0.05, n = 3). D: EPS was applied in the presence or absence of PD, SP, or SB for 3 h, and phosphorylation status and total amounts of JNK (lanes 1–5) and ATF2 (lanes 6–10) were assessed by Western blot analysis. At least 3 independent experiments were performed; representative results are shown.
Hz) required 12 h to upregulate these mRNA expression levels (Fig. 6A). This upregulation of these mRNA expression levels was not observed when myotubes were exposed to a sustained stretch (data not shown). As expected, the effect of cyclic stretch on the mRNA expressions of these chemokines was significantly inhibited by SP-600125, a JNK inhibitor. Despite the delayed expressions of these chemokines, the phosphorylation of JNK in response to cyclic stretch was apparent within just 10–30 min (Fig. 6C, lanes 1–4) and was similarly detected after 12 h of stimulation (Fig. 6C, lane 6).

**Potential role(s) of CXCL1/KC and CXCL5/LIX in C2C12 myotubes.** As shown in Fig. 1, E and F, the evidence that a CXCR2 antagonist, SB-225002, significantly influences the production of CXCL1/KC and CXCL5/LIX in response to EPS strongly suggests that these chemokines released from contracting muscle cells operate in an autocrine fashion. Consistent with this idea, we also previously demonstrated that SB-225002 interfered with the enhancement of insulin-responsive GLUT4 translocation, which occurs during EPS-evoked

**Fig. 4. Roles of Ca2+ and calcineurin cascades in EPS-dependent CXC chemokine production.** A and B: differentiated C2C12 myotubes were subjected to EPS at 20V/25 mm, 1 Hz, 2 ms, for 3 h in the presence or absence of 2 mM EGTA or 5 μM cyclosporine A (CsA), and concentrations of CXCL1/KC and CXCL5/LIX in conditioned media were measured by ELISA (*P < 0.05, n = 3). C: differentiated C2C12 myotubes were subjected to EPS for 3 h in the presence or absence of 2 mM EGTA or 5 μM CsA, and phosphorylation status and total amounts of JNK (lanes 1–4) or ATF2 (lanes 5–8) were measured by Western blot analysis. At least 3 independent experiments were performed; representative results are shown.

**Fig. 5. Role of NF-κB pathway in EPS-dependent CXC chemokine production.** A and B: differentiated C2C12 myotubes were subjected to EPS at 20V/25 mm, 1 Hz, 2 ms, for 3 h in the presence or absence of indicated concentration of parthenolide, and concentrations of CXCL1/KC and CXCL5/LIX in conditioned media were measured by ELISA (*P < 0.05, n = 6). C: differentiated C2C12 myotubes were subjected to EPS for 3 h, and phosphorylation status (lanes 1 and 2) and total amounts of IκB (lanes 3 and 4) were measured by Western blot analysis. Three independent experiments were performed; representative results are shown.
contraction in C2C12 myotubes (38). Since it has been reported that activation of G protein-coupled receptors modulates insulin responsiveness, presumably mediated via regulation of heterotrimeric G proteins including those of the Gαi family (13, 22, 27, 36), we examined whether insulin-dependent Akt phosphorylation (Ser473) and GLUT4 translocation are modified by treatment with CXCL1/KC and CXCL5/LIX in C2C12 myotubes. To address this possibility, we employed a myc-GLUT4 recycling assay using anti-myc antibody, as previously reported (39). However, neither CXCL1/KC nor CXCL5/LIX alone had any detectable effect on basal levels of GLUT4 translocation in naive C2C12 myotubes at least for 45 min, as assessed by the amount of incorporated/bound anti-myc antibody (Fig. 7, A–C). In addition, neither insulin-induced GLUT4 translocation nor Akt phosphorylation was significantly influenced even in the presence of CXCL1/KC or CXCL5/LIX.

Fig. 6. Cyclic mechanical stretch-induced mRNA expressions of CXC chemokines in differentiated C2C12 myotubes. A and B: cyclic longitudinal stretch (15% at 1 Hz) was applied to differentiated C2C12 myotubes for 6 or 12 h in the presence or absence of 20 μM SP-600125, and total RNA was isolated. Relative abundances of mRNAs for CXCL1/KC (A) and CXCL5/LIX (B) were evaluated by real-time PCR analysis. Data normalized using β-actin transcript were averaged over 3 independent experiments (*P < 0.05). C: cyclic longitudinal stretch (15% at 1 Hz) was applied to differentiated C2C12 myotubes for indicated times, and phosphorylation status and total amounts of JNK were measured by Western blot analysis. Three independent experiments were performed; representative results are shown.

Fig. 7. Effects of CXCL1/KC or CXCL5/LIX on insulin-induced Akt phosphorylation and GLUT4 translocation in differentiated C2C12 myotubes. A: differentiated C2C12 myotubes were serum starved and treated with 50 ng/ml CXCL1/KC or CXCL5/LIX for 15 min. Cells were treated with indicated concentration of insulin for 5 min in the presence or absence of these chemokines, and cell lysates were prepared. Western blotting analysis was performed using anti-phospho-Akt (Ser473) and anti-Akt antibodies. Data are shown as mean ± S.E.M. of three independent experiments. B: differentiated C2C12 myotubes expressing myc-GLUT4-ECFP (enhanced cyan fluorescent protein) were serum starved and incubated in 50 ng/ml CXCL1/KC or CXCL5/LIX for 15 min. Cells were treated with 100 nM insulin for 30 min in the presence of 4 μg/ml anti-myc antibody with or without these chemokines. Cells were then washed 5 times with PBS(−) and analyzed by Western blotting with anti-mouse IgG HRP conjugates or anti-BD living color antibodies. C: results from uptake of anti-myc antibody in response to insulin, were subjected to densitometric analysis for quantification (n = 6).
Potential role(s) of CXCL1/KC and CXCL5/LIX in C2C12 myoblasts. To further investigate the potential role(s) of CXCL1/KC and CXCL5/LIX in muscle, we examined whether these contraction-inducible CXC chemokines have any impact on undifferentiated C2C12 myoblasts (Fig. 8). Undifferentiated myoblasts displayed remarkable increases in $[\text{Ca}^{2+}]_i$ with administration of either CXCL5/LIX (Fig. 8A, a2 and a3; Fig. 8C, solid lines) or CXCL1/KC (Fig. 8B, solid lines). These two chemokines showed similar potencies (Fig. 8E) and latency periods (Fig. 8F) in C2C12 myoblasts. These effects were completely abolished by pretreatment with 200 ng/ml PTx (Fig. 8, B and C, dashed lines; Fig. 8D, b2, and Fig. 8E), indicating the $\text{G}_\alpha_i$ family to be predominantly responsible for the increases in $[\text{Ca}^{2+}]_i$ evoked by these CXC chemokines in C2C12 myoblasts. We confirmed that 1% FBS still increased $[\text{Ca}^{2+}]_i$ even in PTx-pretreated C2C12 myoblasts (Fig. 8D, b3), indicating $[\text{Ca}^{2+}]_i$ signaling systems other than those evoked via PTx-sensitive $\text{G}_\alpha_i$ family members, to be intact.

We then examined the possibility that these chemokines have an ability to induce migration of C2C12 myoblasts, since recent reports have revealed that CXCR4, a different family of CXC chemokine receptors, plays an important role in multiple steps of myogenesis including migration of myogenic progenitor/myoblast cells (5, 42). The chemotactic responses of C2C12 myoblasts to CXCL1/KC and CXCL5/LIX were assessed in a migration assay using an insert chamber, and we found that both CXCL1/KC and CXCL5/LIX induced slight but significant chemotactic activity on C2C12 myoblasts (Fig. 8, B and C). This chemotactic activity was potently inhibited by SB-225002 (Fig. 9A). In addition, shRNA-mediated knockdown of CCR2 in C2C12 myoblasts markedly suppressed their migration in response to CXCL1/KC and CXCL5/LIX but not that in response to IGF-I (Fig. 9, B and C).
We also measured amounts of CXCL1/KC and CXCL5/LIX accumulated in conditioned media (every 24 h) during 4 days of myogenesis and found that C2C12 myoblasts appeared to secrete considerable amounts of both CXC chemokines even under basal conditions, and these secretions were gradually downregulated upon myogenic differentiation (Fig. 9, D and E). These observations were further confirmed by mRNA expression levels of these CXC chemokines as assessed by real-time PCR analysis (Fig. 9, F and G). Fresh media containing 10% FBS (growth medium) or 2% CS (differentiation medium) did not contain measurable amounts of either chemokine. These results may explain the weak chemotactic activity observed in our experiments; i.e., C2C12 myoblasts might have spontaneously produced these CXC chemokines in an autocrine fashion that could affect their directional migration toward exogenously added chemokines.

Finally, we examined the potential myogenic effect(s) of these chemokines on C2C12 myoblasts (Fig. 9, H–J). We observed the effect of SB-225002, a CXCR2 antagonist, on myogenic differentiation of C2C12 cells. As expected, SB-
225002 significantly blocked low-serum-induced C2C12 differentiation as assessed by protein expressions of skeletal muscle-type myosin heavy chain and myogenin (Fig. 9H) as well as by C2C12 tubular formation (Fig. 9f). However, exogenous administration of either CXCL1/KC nor CXCL5/LIX further enhanced myogenic differentiation in C2C12 cells (Fig. 9f), presumably due to the high levels of these chemokines spontaneously released from C2C12 myoblasts under undifferentiated culture conditions (Fig. 9, D and E).

**DISCUSSION**

Since physical exercise provokes a highly complex series of interrelated stimuli, including mechanical force and energy expenditure, physiological responses to exercise have mainly been assessed using exercising animal models (32, 49). Muscle cell lines, such as L6 and C2C12, have proven to be useful for studying muscle functions, and indeed, various chemicals believed to mimic certain aspects of exercise are utilized to investigate molecular mechanisms underlying both beneficial and detrimental effects of exercise (37, 43, 50).

In an effort to elucidate the molecular details of biological responses of muscle to exercise, we took advantage of our in vitro contraction model using C2C12 myotubes to characterize contraction-inducible CXC chemokine production. We focused especially on intracellular signals directly responsible for the production of CXCL1/KC and CXCL5/LIX in response to EPS-evoked contractile activity. Our results clearly demonstrate main activation of JNK, but not of either ERK1/2 or p38 MAP kinases, to be involved in the immediate increases in CXCL1/KC and CXCL5/LIX production induced by EPS-evoked contractile activity (Figs. 1–3). These chemokine elevations require EPS-induced Ca2+-signaling, triggering the activation of Ca2+-dependent intracellular signaling cascades as well as the development of the contractile apparatus and its activity (Fig. 4). Our results also indicate that these CXC chemokine production require the activity of NF-κB (Fig. 5), the ubiquitous transcription factor capable of functionally interacting with ATF2 (28), phosphorylation of which appeared to be significantly induced by EPS in a JNK- and Ca2+-dependent manner in contracting C2C12 myotubes (Figs. 2–4). An interesting observation made in the experiments depicted in Fig. 6 was that, although cyclic mechanical stretch also resulted in significant increases in mRNA expressions of both CXC chemokines via the JNK-dependent mechanism, mechanical stretch alone required a much longer period of stimulation (~12 h) than (1 h) EPS-evoked contractile activity (Fig. 1), underscoring the complexity of stimuli elicited by actual exercise in skeletal muscle cells.

**Intracellular signaling intermediates responsible for CXCL1/KC and CXCL5/LIX expressions in response to EPS-evoked contraction.**

We (38) previously reported that systemic levels of CXCL1/KC were increased after just 30 min of treadmill running in an animal experiment. Consistent with this, significant increases in the secretions of both CXCL1/KC and CXCL5/LIX were rapidly, i.e., within 1 h, induced in C2C12 myotubes by EPS treatment (Fig. 1). These data indicate that differentiated C2C12 myotubes possess cellular machinery/systems sufficient to transduce the contractile/elongation signals for activating the JNK-ATF2 signaling cascades, leading to rapid induction of these CXC chemokines, a phenomenon similar to that observed in vivo in skeletal muscle. In excellent agreement with our findings, physical exercise has been reported to immediately induce phosphorylation/activation of JNK and subsequent increases in the transcriptions of target genes in working skeletal muscles in rodents (21) and human subjects (4, 9). Although other MAP kinase signaling cascades including ERK5 and ERK1/2 were obviously phosphorylated/activated in response to EPS-evoked contraction (Fig. 2), as observed in working skeletal muscles during treadmill running (21, 57), our present data strongly suggest mainly JNK involvement in transducing the contractile/elongation signals leading to phosphorylation/activation of ATF2, which perhaps functionally collaborates with NF-κB (31), resulting in rapid upregulation of CXCL1/KC and CXCL5/LIX. In addition, we have found that EPS stimulates phosphorylation of IkB (Ser32/36), a major inhibitory regulator of NF-κB, and that inhibition of NF-κB signaling cascades by parthenolide significantly suppresses CXC chemokine production. Although no obvious reduction of IkB after EPS was detected in the present study, these data strongly suggest the NF-κB signaling pathway to be directly involved in the CXC chemokine expressions in response to EPS-evoked contractility. In contrast, ERK1/2, p38 MAP kinase, and CsA-sensitive signaling cascades play no major roles in this contraction-inducible event.

In our in vitro contraction model, phosphorylation of p38 was not observed in contracting C2C12 myotubes, which is different from in vivo exercise experimental results (21, 57). Although the molecular details of this discrepancy remain to be clarified, a recent study revealed the existence of a negative feedback loop acting on the JNK-p38 activation system, which involves a DUSP family of dual-specificity phosphatases that dephosphorylates these MAP kinases (45). In fact, we observed inhibition of p38 MAP kinase by SB-203580 to significantly enhance JNK phosphorylation (Fig. 2D), possibly due to induction/activation of these phosphatases. Moreover, while we detected no inhibitory effect of SB-203580 on the acute CXC chemokine production during the first 3 h of EPS-evoked contraction (Fig. 2, B and C), treatment of the contracting myotubes with SB-203580 for 24 h resulted in a slight, but significant, reduction in CXC chemokine accumulations (data not shown). Thus, we cannot rule out the possibility that p38 may also play a role in contraction-inducible CXC chemokine production and that a complicated interplay among these MAP kinases and DUSPs might also be involved in regulating the productions of these CXC chemokines under contractile conditions, especially during the later period of EPS application (~3 h). The involvement of p38 as well as JNK in the regulation of these CXC chemokine production in response to inflammatory stimuli has also been reported in other cell types (8, 26).

**Inductions of CXCL1/KC and CXCL5/LIX expressions by mechanical cyclic stretch.** Our data presented herein also confirm the importance of actual exercise (contraction/elongation) in triggering the physiologically relevant CXC chemokine productions in muscle cells, in vitro, by showing that cyclic stretch alone failed to acutely induce expressions of these CXC chemokines, instead requiring much longer stimulation (Fig. 5) than those induced by the EPS-evoked contraction of C2C12 myotubes (Fig. 1) or by the treadmill running of model animals (38). Remarkably, the phosphorylation of JNK appeared to be acutely induced, paralleling the EPS-evoked responses, and to
be responsible for the CXC chemokine expressions induced by the cyclic stretch, although these mRNA expressions were significantly delayed (Fig. 5). These findings implicate the existence of further layers of complexity among intracellular signals, in addition to JNK, elicited by contractile activity (exercise), which cannot be mimicked by mechanical stress alone. At present, the underlying mechanism cooperatively functioning with JNK to produce rapid rises in CXC chemokine expressions with EPS-evoked contraction, but not mechanical stretch, remains unclear. However, given that EPS induces maximal increases in \([Ca^{2+}]_i\) (20) and that \([Ca^{2+}]_i\) has been implicated as an important regulator of CXC chemokine expressions in various cell types (30, 31, 62), the repetitive \([Ca^{2+}]_i\) transients induced by EPS may be involved in the rapid upregulation of CXC chemokines observed in contractile C2C12 myotubes. Consistent with this idea, depletion of extracellular \(Ca^{2+}\) completely abolished the phosphorylations of both JNK and ATF2 (Fig. 3C), resulting in significantly reduced CXC chemokine production in response to EPS (Fig. 3, A and B), although no contractile activity developed under this \(Ca^{2+}\)-chelated condition (20). Thus, it is difficult to reconcile the role of \([Ca^{2+}]_i\) in the immediate induction of these contraction-inducible CXC chemokine production. Another possible explanation is that, since naive C2C12 myotubes possessed none of the sarcomere structures implicated in an important mechanosensor function of striated muscles (24), whereas C2C12 myotubes exposed to EPS progressively developed these sarcomeres (20), and thereby started to display contractile activity, these structures might be required for the rapid signal transduction responsible for acute CXC chemokine expressions. In this regard, it is noteworthy that stretching (lengthening) rather than contraction reportedly may make the greater contribution to inducing increases in JNK activation in isolated rat skeletal muscles (10), suggesting the importance of mechanical force and its sensing system for the activation of JNK in skeletal muscle.

Caution must be exercised in interpreting our findings, since direct comparisons between in vivo muscle contraction and EPS-evoked myotube contraction are difficult to make. Overall, however, our present data indicate that our in vitro contraction model is a potentially valuable tool for investigating the mechanistic details of biological responses to contractile activity because it is literally capable of providing complicated stimuli consisting of mechanical stress as well as energy fluctuations without utilizing chemical exercise-mimetic agents (37). Instead of such mimetic agents, we employed rather more physiologically pertinent cell contraction triggered by \(Ca^{2+}\) transients.

**Potential roles of contraction-inducible CXC chemokines in skeletal muscle cells.** Our current data clearly demonstrate that CXCL1/KC and CXCL5/LIX not only have an ability to directly stimulate myoblast migration but are also involved in the cellular differentiation process (Fig. 9), which is mediated, at least in part, through a mechanism involving the PTx-sensitive heterotrimeric \(G_{\alpha}\)-dependent activation of intracellular \(Ca^{2+}\) signaling cascades (Fig. 8). Unexpectedly, we found that C2C12 myoblasts produce considerable amounts of both CXCL1/KC and CXCL5/LIX under basal conditions, followed by a gradual downregulation to negligible levels upon myogenic differentiation and that the expressions of these chemokines in differentiated myotubes appeared to be tightly controlled such that their production is rather quickly stimulated by EPS-evoked contractile activity (Fig. 1). Although whether satellite cells also possess a similar ability to involuntarily produce these CXC chemokines remains to be established, our present data strongly suggest that various functions of undifferentiated myocytes can be directly regulated by CXC chemokines through CXCR2 in both autocrine and paracrine fashions under physiological circumstances such as muscle regeneration and healing processes that are frequently observed in skeletal muscle tissues after physical exercise (35). In this regard, it has become increasingly apparent that several chemokines play important roles in skeletal muscle regeneration (15, 29, 41, 55), although the details of their sources and the induction mechanisms involved are still poorly understood. Skeletal muscle injury after exercise has been well documented (35), and the initial mechanical injuries induced by muscle contractions result in the death of the original myotubes followed by the proliferation of precursor satellite cells. Macrophages and neutrophils invade the site of muscle injury to trigger delayed secondary injuries, including an inflammatory response and the removal of damaged muscle fibers or cell debris, and the activated satellite cells are then able to differentiate into myoblasts followed by fusion into multinucleated myofibers. In addition to these behaviors of myocytes and phagocytic cells, muscle angiogenesis is also induced by exercise training (48). Thus, trafficking of these leukocytes as well as of satellite cells and myoblasts is crucial for the regeneration process of injured muscles, although excess accumulation of neutrophils is apparently deleterious (47). Overall, the characteristics of contraction-inducible CXCL1/KC and CXCL5/LIX released from working muscle and their involuntary production in myoblasts fit well with the entire process of muscle regeneration. In support of this notion, CXCR2-deficient mice display significant delays in wound healing with decreased neovascularization (17). Furthermore, the importance of CXCR2 for angiogenesis in mediating the activities of ELR+CXC chemokines, including CXCL1/KC and CXCL5/LIX, has also been reported (1). Unfortunately, no exercise experiments using CXCR2-null mice have yet been conducted; however, it is indeed possible that such CXCR2-null mice would show some defects in muscle regeneration after exercise.

We previously noted that CXCR2-mediated signals are involved in the improvement of insulin responsiveness, since in the presence of SB-225002, an antagonist for CXCR2, insulin-responsive GLUT4 translocation/recycling was not enhanced even after EPS-evoked repetitive contraction, a condition under which these CXC chemokines were remarkably induced and secreted into the culture medium (38). Intriguingly, however, exogenous administration of either CXCL1/KC or CXCL5/LIX had no acute influence on GLUT4 translocation/recycling or Akt phosphorylation under either basal or insulin-stimulated conditions in naive C2C12 myotubes (Fig. 7). These results indicate exogenous CXC chemokines alone to be insufficient and that other components, cooperatively functioning with CXCR2 activation, delivered by EPS-evoked contractile activity, are apparently required to improve insulin-responsive GLUT4 translocation/recycling in C2C12 myotubes. Further study is needed to determine the mechanism by which contraction-inducible CXC chemokines participate in the beneficial effects exerted on insulin responsiveness in working muscles.

**AJP-Endocrinol Metab • VOL 297 • OCTOBER 2009 • www.ajpendo.org**
ACKNOWLEDGMENTS
We thank Fumie Wagatsuma and Natsumi Emoto for technical assistance.

GRANTS
This work was supported by Special Coordination Funds for Promoting Science and Technology. This work was also supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, and the New Energy and Industrial Technology Development Organization.

REFERENCES
38. Ochoa O, Sun D, Reyes-Reyna SM, Waite LL, Michalek JE, McManus LM, Shireman PK. Delayed angiogenesis and VEGF production in...