Exposure to pressure stimulus enhances succinate dehydrogenase activity in L6 myoblasts

Noriteru Morita,¹ Kenji Iizuka,¹ Koichi Okita,² Takashi Oikawa,³ Kazuya Yonezawa,² Tatsuya Nagai,² Yukiko Tokumitsu,⁴ Takeshi Murakami,¹ Akira Kitabatake,² and Hideaki Kawaguchi¹

Departments of ¹Laboratory Medicine and ²Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Sapporo 060-8638; ³Health Sciences University of Hokkaido, Toutetsu 061-0293; and ⁴Faculty of Pharmaceutical Sciences, Aomori University, Aomori 030-0943, Japan

Submitted 13 August 2003; accepted in final form 27 July 2004

Morita, Noriteru, Kenji Iizuka, Koichi Okita, Takashi Oikawa, Kazuya Yonezawa, Tatsuya Nagai, Yukiko Tokumitsu, Takeshi Murakami, Akira Kitabatake, and Hideaki Kawaguchi. Exposure to pressure stimulus enhances succinate dehydrogenase activity in L6 myoblasts. Am J Physiol Endocrinol Metab 287: E1064–E1069, 2004.—Contraction of skeletal muscle generates pressure stimuli to intramuscular tissues. However, the effects of pressure stimuli, other than those created by electricity or nerve impulse, on physiological and biochemical responses in skeletal muscles are unknown. The purpose of this study is to examine the effects of a pure pressure stimulus on metabolic responses in a skeletal muscle cell line. Atmospheric pressure was applied to L6 myoblasts using an original apparatus. Succinate dehydrogenase (SDH) activity was evaluated by colorimetric assay using tetrazolium monosodium salt. The amounts of 2-deoxy-[3H]glucose uptake and lactate release were measured. SDH activity was 2.6- to 2.9-fold higher in pressurized L6 cells than in nonpressurized L6 cells (P < 0.01), and 2-deoxy-[3H]glucose uptake was 2.2-fold higher (P < 0.001). In addition, the amount of released lactate decreased from 6.8 to 3.7 μmol/dish when pressure was applied (P < 0.001). In contrast, the intracellular lactate contents of the pressurized cells were higher than those of nonpressurized cells (P < 0.01). However, the total amount of released lactate and intracellular lactate was lower in the pressurized cells than in nonpressurized cells. These findings demonstrate that a pure pressure stimulus enhances metabolic activity in L6 skeletal muscle cells and raise the possibility that elevated intramuscular pressure during muscle activity may be an important factor in stimulating oxidative metabolic responses in skeletal muscles.

METHODS

Materials. L6 myoblast cells were provided by the Cell Bank of the Japanese Collection of Research Bioresources. 2-Deoxy-d-[3H]glucose (2-[3H]DG) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). 2-Deoxy-d-glucose, lactate, succinate, 1-methoxy-5-methylphenazinium methylsulfate (mPMS), and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1) were purchased from Wako Pure Chemical (Osaka, Japan). Phloridzin and penicillin-streptomycin solutions were obtained from ICN Biomedicals (Aurora, OH). Gadolinium (III) chloride hexahydrate and cycloheximide were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma unless otherwise noted.

Cell culture. Rat L6 myoblasts were cultured in 10-cm dishes with high-glucose DMEM (4,500 mg glucose/l) containing 5% FBS and 1% penicillin-streptomycin solution. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: K. Iizuka, Dept. of Laboratory Medicine, Hokkaido Univ. Graduate School of Medicine, Kita-15 Nishi-7 Kita-ku, Sapporo 060-8638, Japan (E-mail: kizuka@med.hokudai.ac.jp).

The American Physiological Society
http://www.ajpendo.org
Pressure loading. An original pressure loading apparatus based on previous reported systems (7, 29) was used, as described in detail previously (10, 17). The plates were set up in the chamber of the apparatus in fresh normal-glucose DMEM containing 20 mM HEPES (pH 7.4) with 10 mM lactate. To raise internal atmospheric pressure of the chamber, room air was pumped in until the pressure reached 160 mmHg. The L6 cells were maintained at this pressure for 3 h at 37°C. The control cells were incubated with the same medium under normal pressure for 3 h in the presence of phloridzin and HgCl₂ were added to each well (final concentration 0.2 mM and 1 mM, respectively). Nonspecific uptake was determined in the presence of 0.2 mM phloridzin and 1 mM HgCl₂ and was subtracted from the total uptake. Then, the radioactive incubation medium was rapidly aspirated off, and the cells were rinsed three times with ice-cold buffer A and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The cells were subjected to three freeze-thaw cycles at −150°C. Samples were then centrifuged for 20 min at 10,000 g to obtain cell lysates. The protein content of each sample was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Twenty-five micrograms of protein per lane were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked for 1 h at room temperature with 4% nonfat dry milk in PBS containing 0.2% Tween 20. Hypoxia-inducible factor-1α (HIF-1α) was detected by use of rabbit anti-HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody and peroxidase-conjugated donkey anti-rabbit IgG (Amersham) as the secondary antibody, in concentrations recommended by the manufacturer. Antibody-bound protein was detected by chemiluminescence detection system (Amersham) and light emission exposed to X-ray film (model RX-U; Fujifilm, Kanagawa, Japan) for 3 min to 2 h. To verify the reactivity of the anti-HIF-1α antibody, a fusion protein corresponding to amino acids 575–780 of HIF-1α (Santa Cruz Biotechnology) was applied at the same time.

Statistical analysis. All results are expressed as means ± SD. Statistical significance was tested with the Student’s two-tailed t-test or one-way ANOVA; in the latter case, the test was combined with Scheffé’s test for post hoc analysis. Values of P < 0.05 were considered statistically significant.

Lactate release and intracellular lactate concentration. Concentrations of lactate, an anaerobic metabolic end product, were measured to determine whether the pressure stimulus had caused any changes in metabolic products. Using a lactate analyzer (YSI 1500 SPORT Lactate Analyzer; Yellow Springs Instruments, Yellow Springs, OH), we measured the lactate contents in fresh medium and then in the medium after pressure loading; the difference was the lactate release. To evaluate intracellular lactate concentration, the cells treated with pressure stimulation or with normal pressure incubation were harvested in a constant amount (500 μl) of medium. All samples were sonicated and then boiled for 3 min to inactivate enzymes. The lactate content in a sample aliquot was measured, and the value was corrected according to the original amount of the medium in the well.

RT-PCR. Total RNA was extracted from L6 cells after treatment with pressure stimulation or normal pressure incubation by use of an RNeasy Protect Mini Kit (Qiagen, Valencia, CA), following the procedures recommended by the manufacturer. A Perkin-Elmer DNA thermal cycler (Norwalk, CT) was utilized for the assay with a OneStep RT-PCR kit (Qiagen). Total RNA (8 ng) was reverse transcribed and PCR amplified in a 50-μl volume with the use of an equal amount of the RNA sample. 5× Qiagen OneStep RT-PCR buffer, each primer at 2 μM, each dNTP at 400 μM, and 2 μl of Qiagen OneStep RT-PCR Enzyme Mix in the thermal cycler. Samples were heated for 30 min at 50°C for reverse transcription and further heated for 15 min at 95°C for activation of HotStarTag DNA polymerase and inactivation of reverse transcriptases, and then the following conditions: 35 cycles of 45-s denaturation at 94°C, 50-s annealing at 55°C, and 1-min extension at 72°C. Samples were then kept for 10 min at 72°C and cooled at 4°C. The following oligonucleotide primers were used: inducible nitrate oxide synthase (iNOS; Ref. 5) forward (sense), 5′-TGG-ATC-ATG-ATG-GTT-AAG-CCC-GAA-GTT-GTT-CAT-GTG-GTC-ATG-G-3′, and reverse (antisense), 5′-TTG-CCC-TTT- TTC-CTT-ACA-CTA-3′; and α-skeletal actin (22) forward (sense), 5′-GTT-CAC-CAG-GGT-GTC-ATG-G-3′, and reverse (antisense), 5′-TGT-AGG-AGG-AGG-CCG-GCC-AGA-TA-3′. Amplified samples (15 μl) were electrophoresed on 1.5% agarose gel in Tris-acetate-EDTA buffer containing 10 μg of ethidium bromide. As a positive control, we used lipopolysaccharide (LPS)-treated L6 cells, which have been reported to express iNOS mRNA (2).

Immunoblot analysis. After treatment with pressure stimulation or normal pressure incubation, the cells were washed twice with ice-cold PBS and harvested in PBS containing protease inhibitors (100 μM benzamidine, 2 μM leupeptin, 0.15 μM aprotinin, 1.5 μM pepstatin A, and 100 μM phenylmethylsulfonyl fluoride), and 1% Phosphatase Inhibitor Cocktail I and II (Sigma). The cells were subjected to three freeze-thaw cycles at −150°C. Samples were then centrifuged for 20 min at 10,000 g to obtain cell lysates. The protein content of each sample was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Twenty-five micrograms of protein per lane were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked for 1 h at room temperature with 4% nonfat dry milk in PBS containing 0.2% Tween 20. Hypoxia-inducible factor-1α (HIF-1α) was detected by use of rabbit anti-HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody and peroxidase-conjugated donkey anti-rabbit IgG (Amersham) as the secondary antibody, in concentrations recommended by the manufacturer. Antibody-bound protein was detected by chemiluminescence detection system (Amersham) and light emission exposed to X-ray film (model RX-U; Fujifilm, Kanagawa, Japan) for 3 min to 2 h. To verify the reactivity of the anti-HIF-1α antibody, a fusion protein corresponding to amino acids 575–780 of HIF-1α (Santa Cruz Biotechnology) was applied at the same time.

Statistical analysis. All results are expressed as means ± SD. Statistical significance was tested with the Student’s two-tailed t-test or one-way ANOVA; in the latter case, the test was combined with Scheffé’s test for post hoc analysis. Values of P < 0.05 were considered statistically significant.
RESULTS

Pressure stimulus enhanced SDH activity and glucose uptake. In nonpressurized L6 cells, only subtle color differences were apparent between presence and absence of succinate. In contrast, the pressurized cells showed significantly larger differences in the degree of coloring (2.6-fold increase; Fig. 1). As shown in Fig. 2, the amount of 2-[3H]DG was greater in pressurized than in nonpressurized L6 cells (2.2-fold increase). These findings suggest that the pressure stimulus enhanced SDH activity and resulted in enhancement of glucose uptake in L6 skeletal muscle cells.

Effects of Gd3+ and cycloheximide on pressure-induced SDH activation. We tested whether stretching stimuli were involved in pressure-induced SDH activation. It has been reported that stretch-activated channels are critically involved in stretch-induced cellular responses and that Gd3+ is a potent blocker of stretch-activated channels (11). When pressure was applied to the cells in the presence of 30 μM Gd3+, SDH activity was enhanced to a similar degree as when pressure was given alone (pressurized cells, 2.9-fold increase; Gd3+ treatment with pressure, 3.1-fold increase; Fig. 3A). This suggested that stretch stimuli were not involved in the pressure-induced SDH activation.

To examine the effects of protein synthesis on SDH activation by pressure stimulus, we determined whether cycloheximide, a protein synthesis inhibitor, suppressed the pressure-induced SDH activation. At the concentration of 20 μg/ml, used by Hatfaludy et al. (6), absorption indicating SDH activity was reduced under both the nonpressurized and the pressurized condition compared with that in untreated controls (data not shown). Because this concentration of cycloheximide may cause a degree of damage to the cells, we used the inhibitor at a concentration of 10 μg/ml in the medium. As shown in Fig. 3B, although pressure stimulus significantly enhanced SDH activity in the presence of cycloheximide (1.6-fold increase compared with that in nonpressurized controls), the degree of the pressure-induced activation of SDH was reduced when compared with the pressurized cells in the absence of cycloheximide. These results suggested that protein synthesis was at least partly involved in the pressure-induced SDH activation.

Pressure reduced lactate release. Under nonpressure conditions, 6.8 ± 0.7 μmol/dish lactate were released, whereas the amount of released lactate in the pressure-applied cells was 3.7 ± 0.5 μmol/dish (Fig. 4A). On this point, we investigated whether enhanced lactate utilization caused the reduction of lactate in the pressure-applied cells. The contents of intracellular lactate in nonpressurized cells and pressurized cells were
0.23 and 0.32 μmol/dish, respectively (Fig. 4B). Although significantly more intracellular lactate was found in the pressurized cells, the amounts and difference in intracellular lactate between pressurized and nonpressurized cells were an order of magnitude smaller than that of released lactate. Consequently, the total amount of released lactate and intracellular lactate was significantly lower in the pressurized cells than in the nonpressurized cells (P < 0.001).

Effects of oxygen content on L6 cells. We tested whether both pressurized and nonpressurized cells were exposed to hypoxic, normoxic, or hyperoxic conditions, respectively. It has been reported that iNOS and HIF-1α are detected under hypoxic conditions (12, 18, 19, 26). As shown in Fig. 5A, iNOS mRNA of L6 cells, in contrast to that of LPS-treated cells, was weakly exhibited in both pressurized and nonpressurized conditions. In immunoblot analysis, HIF-1α protein was hardly detected in either group (Fig. 5B).

DISCUSSION

Intramuscular pressure is elevated in the contraction phase of active muscles during physical activity. However, the effects of elevated intramuscular pressure per se on the physiological and biochemical responses of skeletal muscles are unknown. In this study, we have attempted to approach these questions by investigating the effects of an artificial pressure stimulus on metabolic responses in cultured skeletal muscle cells. The present study demonstrates for the first time that a pressure stimulus activated SDH activity and glucose uptake and reduced lactate release in L6 myoblasts. It is thought that enhancement of SDH, which is an indicator of TCA cycle activity, increases the capacity to degrade substrates (e.g., lactate and glucose). Thus the pressurization of L6 cells stimulated lactate utilization and glucose uptake. These findings suggest that mechanical pressure enhanced aerobic metabolism in skeletal muscle cells and may provide valuable clues toward elucidating the nervous system-independent mechanism(s) for metabolic activation and/or adaptation by skeletal muscle contraction.

The mechanisms by which exercise and/or contractile activity stimulates skeletal muscle metabolism are of great interest and importance. Until now, adaptive and metabolic responses to contractile activity of skeletal muscle have been investigated only by electrical stimulation and stretch stimuli in ex vivo studies. The effects of pressure stimuli, which are produced by muscle contraction, on adaptation and metabolic activity of skeletal muscle are not yet well recognized. In the present study, we examined the contribution of stretch stimuli in pressure-activated aerobic metabolism by treatment with a stretch-activated channel inhibitor, Gd^{3+}. Although Caldwell et al. (4) demonstrated a possibility that treatment with Gd^{3+} to identify the involvement of stretch-activated channels could lead to false-negative conclusions, the present experiments did not use the phosphate, carbonate, and sulfate buffers that were shown to react with Gd^{3+} in the study (4). In addition, a decrease of lactate release induced by pressure stimulus in the present study was opposite to the result of stretch-applied cells reported by Hatfaludy et al. (6). Thus we could exclude electricity and stretching as factors in demonstrating the enhancing effects of a pressure stimulus on aerobic metabolism of L6 skeletal muscle cells. However, we do not yet understand the mechanism(s) underlying the pressure-activated aerobic metabolism in L6 cells.
On the basis of our results and the fact that both SDH activity and TCA cycle activity are regulated mainly by the ADP-to-ATP ratio, we could consider the following explanations as possibly responsible for pressure-activated metabolism. The activation of SDH was at least partially attributable to enhancement of protein synthesis, because cycloheximide partly inhibited the pressure-activated metabolic shift. Protein synthesis is a metabolically costly process requiring a large amount of ATP, possibly driving an increase in SDH activity. The suppression of the pressure-induced SDH activation by cycloheximide could be the result of an inhibition of the synthesis of enzymes of mitochondrial metabolism and/or an inhibition of general protein synthesis. Also, L6 cells can differentiate into myofibers from myotubes and myoblasts (27). This process requires many proteins as well as ATP. Therefore, the pressure stimulus may accelerate protein synthesis, cell growth, and/or the process of differentiation, resulting in an increase in aerobic metabolism. As for the protein synthesis-independent effects of the pressure on aerobic metabolism, we observed, however, small but significant increases of intracellular lactate in the pressurized cells in the presence of cycloheximide (27). This process requires many proteins as well as ATP. Therefore, the pressure stimulus may accelerate protein synthesis, cell growth, and/or the process of differentiation, resulting in an increase in aerobic metabolism. As for the protein synthesis-independent effects of the pressure on aerobic metabolism, the results of the present study may provide a new perspective in the study of the mechanisms by which muscle contraction induces metabolic activation and/or adaptations.

Another interesting finding of the present study was that glucose and lactate may be used as a substrate in the pressure-activated aerobic metabolism of L6 cells. For a long time, lactate was thought to be a metabolic end product of glycolysis. Recently, however, lactate was shown to be incorporated into intracellular lactate and was utilized during skeletal muscle contraction and/or exercise (23–25). In the present study, we found that the pressure stimulus reduced lactate release. Considering that the pressure stimulus enhanced the aerobic metabolic capacity and glucose uptake, the reduction of the release of lactate from the L6 cells raises the following two possibilities: a decrease in lactate production and enhancement of lactate utilization. The first possibility (i.e., less conversion of pyruvate to lactate) is consistent with enhanced aerobic metabolism. We observed, however, small but significant increases of intracellular lactate in the pressurized cells in the presence of enhanced glucose uptake and aerobic metabolism (i.e., no decrease in lactate production). Thus we considered that the latter possibility was largely responsible for the reduction in released lactate in the cells.

During pressure loading experiments, increased oxygen partial pressure (Po2) may occur in the culture medium because of the use of room air (theoretically 21% O2; normobaric condition, 150 mmHg Po2; hyperbaric condition, 180 mmHg Po2). It may be possible for increased Po2 to affect metabolic responses in insufficient Po2 conditions. However, our pressure loading experiments were performed in an ambient Po2 condition, which is much higher than tissue Po2 (10–20 mmHg Po2; Ref. 8). In fact, HIF-1α and iNOS, which are induced by hypoxia (12, 18, 19, 26), were not expressed or altered under our pressurized and nonpressurized conditions, indicating that the cells were not exposed to an insufficient Po2 condition. Additionally, with respect to effects of increasing Po2, an in vitro study showed that oxygen concentration had no influence on enzyme activity and metabolite production involved in TCA cycle turnover and glycolysis (20). Taken together, these previous findings and our results support our view that atmospheric pressure-increased Po2 did not affect the activation of aerobic metabolism by the pressure stimulus.

In conclusion, we indicate for the first time that a pressure stimulus induced the activation of SDH and increased glucose transport and lactate utilization in L6 myoblasts, and we suggest that elevated intramuscular pressure during muscle contraction may be an important stimuli inducing metabolic adaptive responses in skeletal muscles.

ACKNOWLEDGMENTS

We thank Fuyuko Kanda for technical assistance.

GRANTS

This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan (no. H14-trans-013).

REFERENCES


