The microRNA miR-696 regulates PGC-1α in mouse skeletal muscle in response to physical activity

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1Laboratory of Health Science, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University; 2Department of Gastroenterology and Hepatology and 3Department of Preventive Medicine for Health Science, Kyoto Prefectural University of Medicine; and 4Department of Medical Life Systems, Doshisha University, Kyoto, Japan

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Aoi W, Naito Y, Mizushima K, Takanami Y, Kawai Y, Ichikawa H, Yoshikawa T. The microRNA miR-696 regulates PGC-1α in mouse skeletal muscle in response to physical activity. Am J Physiol Endocrinol Metab 298: E799–E806, 2010. First published January 19, 2010; doi:10.1152/ajpendo.00448.2009.—MicroRNAs (miRNAs) are small noncoding RNAs involved in posttranscriptional gene regulation that act by degrading mRNA molecules or, in some cases, repressing translation of protein-coding genes by binding to the 3′ untranslated regions. Many studies have shown that miRNA expression is dynamically regulated by inactivity compared with the effect of exercise. Although considerable progress has been made in understanding the functions of muscles that are associated with physical activity, the underlying molecular pathways remain obscure, especially those related to translational regulation. In fact, the level of expression of a particular mRNA does not necessarily reflect the abundance of the corresponding protein because the complex mechanisms that regulate protein expression are not dependent on mRNA alone (7, 30, 37, 43).

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by degrading mRNA molecules or, more frequently in mammalian cells, by inhibiting their translation (3, 8). It has been suggested that miRNA-mediated gene regulation is part of the fundamental mechanism of posttranscriptional regulation and may have diverse functional effects. In fact, 30% of the protein-coding genes may be regulated by miRNAs (27), and several of these miRNAs have been suggested to have a role in a wide range of biological processes, including development, cell death, carcinogenesis, and the response to stress (1, 26, 29, 45). Some miRNAs have also been suggested to act as modulators of skeletal muscle function. It has been reported that miR-1 and miR-133, as well as miR-181 and miR-206, have distinct roles in modulating the proliferation and differentiation of cultured skeletal myoblasts (12, 22, 32). Furthermore, various miRNAs have been reported to show abnormalities in patients with myopathy (13). However, the changes of miRNAs in skeletal muscle associated with physical activity remain unclear, although miRNAs may be an important part of the molecular network that regulates muscle phenotypic changes and adapts to the daily level of muscle activity. Therefore, we investigated the response of miRNAs in skeletal muscle to physical activity by using animal models of exercise and immobilization, along with functional analysis of the miRNAs in a myocyte cell line. Here we report that the response of miR-696 to physical activity could be involved in regulating energy metabolism via translation of a key metabolic modulator in murine skeletal muscle.

PHYSICAL ACTIVITY IMPROVES VARIOUS BODILY FUNCTIONS, including metabolism, cardiovascular function, and immune function. The skeletal muscles comprise the major organ supporting physical activity, and muscle function is altered dramatically by contractile activity. Numerous studies have shown that daily exercise improves muscle energy metabolism and strength (9, 21, 23), along with changes of the expression or activity of muscle enzymes and proteins, as well as alterations of their mRNA transcription. Conversely, low levels of physical activity and immobilization induce the atrophy of skeletal muscle and decrease its metabolic capacity (9, 15, 20, 52), with the expression/activity of many proteins and miRNAs being negatively regulated by inactivity compared with the effect of exercise. Although considerable progress has been made in understanding the functions of muscles that are associated with physical activity, the underlying molecular pathways remain obscure, especially those related to translational regulation. In fact, the level of expression of a particular mRNA does not necessarily reflect the abundance of the corresponding protein because the complex mechanisms that regulate protein expression are not dependent on mRNA alone (7, 30, 37, 43).

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EXPERIMENTAL PROCEDURES

Animals and experimental design. The present study complied with the principles and guidelines of the Japanese Council on Animal Care, and it was also approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine (permission no. M19-42). C57BL/6 mice (8 wk old) were obtained from Shimizu Laboratory Supplies, (Kyoto, Japan) and were acclimatized for 2 wk in an air-conditioned (22 ± 2°C) room with a 12:12-h light-dark cycle (lights on from 0730 to 1930). The mice were divided into two groups of eight animals each, consisting of a sedentary control group and an exercise training group. The training group was allowed to run on a

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motorized treadmill 5 times/wk for 4 wk. During this period, the level of exercise was gradually increased from running for 20 min with the treadmill set at 18 m/min to running for 60 min with it set at 32 m/min. On the other hand, the sedentary mice were handled identically to the trained mice. At the same time of day, the sedentary mice were placed on a stationary treadmill 5 times/wk for the duration of the treadmill training session. The diet was identical for the sedentary and the training groups. The mice were euthanized at 24 h after the last training session, and the gastrocnemius muscle was removed, immediately frozen in liquid nitrogen, and stored at −80°C. In addition, the effect of inactivity was examined in an immobilization model by fixing one hindlimb in a cast. In C57BL/6 mice (14 wk old), the knee and ankle joints of one hindlimb were fixed. After 5 days, the gastrocnemius muscles were harvested from the fixed and unfixed hindlimbs and stored for investigation.

Microarray analysis. Total RNA was extracted from the frozen muscle tissues with Isogen (Nippongene, Tokyo, Japan). Equal amounts of total RNA were obtained from eight samples in each group and pooled. miRNA was extracted from 5 μg of total RNA with a Purelink miRNA Isolation Kit (Invitrogen, Carlsbad, CA), and its integrity was confirmed by agarose gel electrophoresis using a 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA). After labeling with Cy5 dye (Invitrogen), the miRNAs were hybridized on 3D-Genes miRNA Oligo chip (Toray, Kanagawa, Japan), which contained 610 mature miRNA probes, for every group by incubation with each target solution for 16 h at 32°C. Microarrays were scanned with a ScanArray HT (PerkinElmer, Boston, MA) after washing and processing of the chip, and signal quantification was carried out. Differences of total fluorescence intensity between arrays were adjusted by global normalization. The mean values for duplicate microarrays were calculated and used for comparison between groups. When the difference in relative miRNA expression between the two groups was >2.0-fold, this was defined as a change of expression.

Cell culture. C2C12 myoblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with high glucose and 10% fetal bovine serum on six-well plates. To induce differentiation of cells, the medium was changed to DMEM with low glucose and 2% horse serum for 96 h. Then miRNA precursor (prec-mmu-miR-696) from Applied Biosystems (Foster City, CA) were transiently transfected into C2C12 cells with the sipORT Lipid transfection reagent (Applied Biosystems) according to the manufacturer’s instructions. Optimal transfection efficiency was determined empirically at 5 μl of sipORT Lipid and 20 nM precursor or inhibitor. These transfection reagents were dissolved in 200 μl Opti-MEM (Invitrogen) and added to 2 ml of serum-free medium. For negative control, random oligonucleotides (Applied Biosystems) were transfected at the same concentration. Total RNA, DNA, and protein were isolated before and at 24 h after transfection and were used for the polymerase chain reaction (PCR) and Western blotting.

Real-time PCR. Reverse transcription (RT)-PCR was performed using total RNA samples obtained from muscle tissues and cultured myocytes. We used ready-made solutions containing the primers and probes for miR-696 and miR-let7 (Applied Biosystems) and performed real-time RT-PCR with an ABI 7300 system (Applied Biosystems). The ratio of the signal for miR-696 to that for miR-let7 was calculated for each sample. On the other hand, real-time PCR using the DNA-binding dye SYBR Green was employed for the detection of PCR products for RNA sample obtained from cells after transfection of cDNA. The following PCR primers (Sigma-Aldrich Japan, Hokkaido, Japan) were used: peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), 5’-CATTCTTGAGCTGCTTCCATGCAG-3’ (forward) and 5’-CCGTCGAGAGCTTCTCAGG-3’ (reverse); pyruvate dehydrogenase kinase-4 (PDK4), 5’-AGAGTTACTGACGCCTGTGG-3’ (forward) and 5’-TTCCGGGATGTGTTTCCG-3’ (reverse); cytochrome c oxidase subunit II (COX II), 5’-ATCCCGAGGCCAGCTTAACTA-3’ (forward) and 5’-TTTCCAGGATTGGCCGCTAG-3’ (reverse); 18S ribosomal RNA (rRNA), 5’-GCGCGTAGGGTTTGTGCTTTCG (forward) and 5’-CATTCTTGAGGCTTCTCAG-3’ (reverse); β-actin, 5’-TATGACCCTTCCAGGATGT (forward) and 5’-AGGCTAGAACAGTCCGCTTA-3’ (reverse). The ratio of the other signals to that of 18S RNA was calculated for every sample. Additionally, to determine mitochondria DNA contents in myocytes, PCR was performed using DNA obtained from cells. Total DNA was extracted using DNeasy (Qiagen, Hataoworth, CA) according to standard procedures and digested with 100 μg/ml RNase A for 30 min at 37°C. The relative copy numbers of mitochondrial to nuclear DNA were determined by real-time PCR with above primers specific to the COX II (mitochondrial) and β-actin (nuclear) genes.

Western blotting. Protein was extracted from muscle tissues of the experimental animals and from cultured myocytes using lysis buffer (Sigma, St. Louis, MO). Equal amounts (20 μg) of protein in the lysates were separated by 10% SDS-PAGE, and then the proteins were transferred onto polyvinyldene difluoride membranes. The blots were incubated with a primary antibody directed against PGC-1α (Chemicon International, Temecula, CA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and reaction products were visualized by using a horseradish peroxidase-conjugated secondary antibody (GE Healthcare BioSciences, Buckinghamshire, UK) and enhanced chemiluminescence (Westpico; Pierce, Rockford, IL). Band densities were measured with Scion image software (National Institutes of Health, Research Service Branch).

Fatty acid oxidation assay. Fatty acid oxidation assays were carried out as described previously (11). In brief, differentiated C2C12 myotubes were incubated in medium containing 0.1 mM palmitate ([9,10-3H]palmitate, 5 μCi/ml) and 2% BSA at 48 h after transfection. After 2 h incubation at 37°C, the mixture was removed and added to a tube containing cold 10% trichloroacetic acid. The tubes were centrifuged for 10 min at 2,000 g at 4°C. Aliquots of the supernatants were removed, mixed with 6 N NaOH, and applied to ion exchange resin. The columns were washed twice with water, and 1H2O in the eluates was measured in a scintillation counter.

Fluorescence microscopy. Mitochondrial biogenesis was measured by using Mitotracker Orange (Invitrogen), a dye with selective mitochondrial permeability. This dye accumulates in active mitochondria and reacts with the accessible thiol groups of proteins and peptides to form fluorescent aldehyde conjugates. C2C12 cells were plated on chamber slides and induced differentiation. At 48 h after transfection with the miR-696 precursor, cells were incubated for 30 min at 37°C with 100 nM Mitotracker Orange (Invitrogen). Then the cells were washed twice with phosphate-buffered saline, fixed in serum-free medium containing 3.7% paraformaldehyde for 15 min at 37°C, and then washed twice more with phosphate-buffered saline. Next, the cells were embedded in Mowiol and examined under a laser-scanning confocal microscope (FV1000; Olympus, Kyoto, Japan).

Statistics. Results are reported as the mean ± SE. Differences between groups were evaluated by one-way ANOVA or Student’s t-test. If ANOVA indicated a significance difference, Fisher’s protected least significant difference test was used to determine the significance of differences between mean values. In all analyses, P < 0.05 was considered to indicate statistical significance.

RESULTS

Changes of miRNAs with physical activity. To search for miRNAs associated with the adaptation of muscles to increased activity, muscle tissues obtained from mice trained for 4 wk or immobilized for 5 days were used for analysis. We examined the profile of miRNA expression in harvested gastrocnemius muscles by hybridization to miRNA microarrays. This microarray analysis revealed that one miRNA (miR-21) was increased and three miRNAs (miR-696, miR-709, and miR-720) were decreased by exercise training compared with their expression in sedentary control mice (Table 1). On the other
Table 1. miRNAs associated with muscle activity detected by microarray

<table>
<thead>
<tr>
<th></th>
<th>Increase</th>
<th>Decrease</th>
<th>Fold Change</th>
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<tbody>
<tr>
<td>Exercise</td>
<td>miR-21</td>
<td>miR-696</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-709</td>
<td>2.90</td>
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<td></td>
<td></td>
<td>miR-720</td>
<td>2.32</td>
</tr>
<tr>
<td>Immobilization</td>
<td>miR-680</td>
<td>miR-696</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>miR-705</td>
<td>miR-762</td>
<td>2.13</td>
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miRNA, microRNA.

hand, four miRNAs (miR-680, miR-696, miR-705, and miR-762) were upregulated by immobilization compared with the normal control, whereas downregulation of miRNAs was not found. When miRNAs showing opposite changes with exercise and immobilization were investigated, we found that miR-696 was decreased by exercise and increased by immobilization. To confirm these changes of miR-696, we examined its expression in muscle tissue by RT-PCR. As shown in Fig. 1A, the expression of miR-696 in gastrocnemius muscle was decreased significantly by exercise training compared with that in the muscles of sedentary control mice. On the other hand, immobilized muscles showed a significant increase of miR-696 expression compared with control muscles (Fig. 1B). We also confirmed the identical changes of miR-696 with snoRNA202 (Applied Biosystems) as the internal control.

Target of miR-696. To investigate the biological role of miR-696, we predicted its protein-coding gene targets by using the miRanda algorithm, which is commonly employed to predict miRNA gene targets (18). We focused on PGC-1α from among 827 possible genes because miR-696 had a high complementarity and binding stability among the predicted miRNAs to regulate PGC-1α and because PGC-1α is well known as a key metabolic modulator in skeletal muscle. We found that the level of PGC-1α protein was significantly increased in muscle tissue by exercise training compared with that in sedentary control muscles (Fig. 2B). Conversely, PGC-1α protein was decreased by hindlimb immobilization compared with the level in control muscle tissue (Fig. 2C). PGC-1α protein negatively correlates \((r = 0.64)\) with miR-696 expression in the muscle \((P < 0.001)\) (Fig. 3). Although mRNA level of PGC-1α was also changed by either training or immobilization (Fig. 2, D and E), these changes of the mRNA level were more marked for the protein level, suggesting that translational regulation occurred in addition to transcriptional regulation.

PGC-1α regulation via miR-696 in myocytes. To confirm the relationship between PGC-1α and miR-696, experiments using cultured myocytes were carried out. We examined the miR-696 function using its precursor and an inhibitor. Elevation of miR-696 expression was seen after transfection of its precursor into C2C12 cells, and PGC-1α protein was suppressed compared with the level in control cells at 24 h after transfection (Fig. 4A) along with marked elevation of the miR-696 content (Fig. 4C). On the other hand, inhibition of miR-696 activity elevated the PGC-1α protein level without miR-696 expression being changed because the inhibitor was designed from complimentary oligomers to miR-696 and thus blocked its binding to target mRNAs but did not affect its expression. Content of miR-696 did not significantly affect the expression of PGC-1α mRNA (Fig. 4B). We also measured the expression of miRNAs for PDK4 and COX II, which are located downstream and are regulated by PGC-1α. We found that the expression of two genes was downregulated significantly compared with control levels after transfection of the miR-696 precursor and upregulated by treatment with the miR-696 inhibitor (Fig. 5).

Fatty acid oxidation and mitochondrial biogenesis. PGC-1α upregulates aerobic metabolism and mitochondrial biogenesis in skeletal muscle (38, 50, 51). To assess phenotypic change via PGC-1α by miR-696, we further investigated fatty acid oxidation capacity and mitochondrial biogenesis. We found that fatty acid oxidation was reduced by the miR-696 precursor, but was increased by its inhibitor (Fig. 6). Also, the mtDNA content (COX II-β-actin) was decreased significantly by transfection of the precursor along with marked elevation of the miR-696 content, whereas it was elevated by transfection of the inhibitor (Fig. 7A). Furthermore, when miR-696-transfected cells were incubated with the dye Mitotracker Orange, miR-696-transfected cells showed weaker fluorescence than negative control C2C12 cells at 48 h after transfection of the miR-696 precursor (Fig. 7B). These observations indicate that miR-696 negatively regulates mitochondrial biogenesis.

DISCUSSION

In the present study, we demonstrated that miR-696 expression in murine skeletal muscle is negatively regulated by physical activity and that miR-696 downregulates events related to aerobic metabolism and mitochondrial biogenesis by

\[ \begin{align*}
A & \quad \text{miR-696 expression} \\
\text{Sedentary} & \quad 120 \\
\text{Exercise} & \quad 140 \\
B & \quad \text{miR-696 expression} \\
\text{Control} & \quad 150 \\
\text{Immobilization} & \quad 170
\end{align*} \]

\( \star \text{Significant difference from the sedentary and the control at the level of } P < 0.05 \)}
inhibiting the translation of PGC-1α in myocytes. miRNAs are small noncoding transcripts that regulate protein expression by promoting the degradation or inhibiting the translation of mRNAs. Although bioinformatic studies have suggested that miRNAs may regulate the expression of a large part of the genome (27), the detection of miRNA targets has been comparatively limited so far. Several studies have indicated that miR-1 and miR-133a, together with miR-206 and miR-181, could be muscle-specific microRNAs expressed in skeletal muscle, which have an important role in regulating gene expression during muscle differentiation (12, 22, 32). However, it has been unclear whether miRNAs are regulated by changes of physical activity such as exercise or immobilization. Recently, Safdar et al. (41) suggested that miR-23 may regulate PGC-1α expression in skeletal muscle after acute exercise because of a negative correlation between them, but an effect of the miRNA on PGC-1α expression was not demonstrated directly by their in vitro study. Thus, the present study is the first to demonstrate that a miRNA responds to physical activity and directly modulates energy metabolism in skeletal muscle by regulating metabolic proteins.

Numerous studies have demonstrated that functional adaptation of muscles, such as increased metabolic activity associated with enzyme activation, occurs after 4 wk of exercise training in mice and humans (14, 25, 36). On the other hand, immobilization due to denervation or fixing in a plaster cast causes metabolic suppression along with muscle atrophy from 3 to 8 days (32, 44, 49). In the present study, to identify miRNAs associated with the process of muscle adaptation, muscle tissues obtained from mice performing gradually increasing exercise for 4 wk or mice with limbs immobilized for 5 days were used for analysis. As a result, we found that miR-696 was markedly altered by both exercise training and...
immobilization, being regulated in opposite directions by the two interventions. Habitual aerobic exercise improves capacity of energy metabolism in skeletal muscle, whereas hypertrophy hardly occurs (2, 4). On the other hand, inactivity decreases muscle metabolic capacity along with muscle loss (15, 20, 52). Thus, it was supposed that miR-696, which showed opposite changes with aerobic exercise and inactivity, was associated with metabolic regulation.

According to bioinformatic prediction of the target genes for miR-696, one possibility was PGC-1α/H9251. In fact, we found that PGC-1α/H9251 protein was increased by exercise and decreased by immobilization along with changes of miR-696 expression. Additionally, an increase of miR-696 expression in myocytes led to downregulation of PGC-1α/H9251 protein along with reduced expression of mRNAs for its downstream genes. These observations strongly suggest that miR-696 regulates the translation of PGC-1α in skeletal muscle. Because the level of PGC-1α protein frequently does not correspond to its mRNA level (17, 46), this also suggests that translational regulation by a miRNA may influence the expression of this protein. PGC-1α has been shown to have the central role in a family of transcriptional coactivators involved in aerobic metabolism (38, 50, 51), and it is activated by exercise (39). Activation of PGC-1α alters the
metabolic phenotype through interaction with nuclear respiratory factor and PPARα (6, 35). Improved understanding of the activation of PGC-1α protein by exercise has implications beyond better athletic performance (10, 19), including the possibility of providing targets for the treatment of muscle weakness in the elderly, obesity, and various diseases such as the mitochondrial myopathies and diabetes (5, 47, 48). miR-696 may modulate the metabolic activity of skeletal muscle through translational regulation of PGC-1α. A major function of PGC-1α is the promotion of mitochondrial biogenesis and activation of respiratory enzymes (38, 50, 51). We found the inhibition of mitochondrial biogenesis, as well as a decrease of COX II, in miR-696-overexpressing myocytes compared with normal control myocytes. In addition, fatty acid oxidation was decreased by the increment of miR-696 expression, supporting a role of miR-696 in metabolism along with PGC-1α. These findings suggest that translational regulation of PGC-1α via miR-696 may have an important role in metabolic adaptation to physical activity in addition to the role of transcriptional regulation shown by previous studies (34, 39).

miRNA can bind to various mRNAs that contain complementary sequence and inhibit the translation activity. Therefore, we should also consider that the PGC-1α regulation may be indirectly caused via other proteins that are regulated by miR-696. Additionally, miR-696 may have other functions in addition to regulating energy metabolism in skeletal muscle. The algorithm for prediction of miRNA gene targets also identified several genes associated with the maintenance of homeostasis, including glutathione peroxidase, thioredoxin, heat shock protein 70, tumor-suppressing protein, and defensin. Some of these proteins have been reported to show changes due to exercise training or immobilization, and the levels of these proteins often do not correspond to their mRNA levels (16, 24, 28, 31, 33), suggesting that translational regulation by miRNAs may influence the protein content, too. Other genes, such as insulin-like growth factor receptor and aquaporins, were also identified as possible targets of miR-696. Thus, further studies will be needed to clarify the additional functions of miR-696 in detail.

In conclusion, we found that the expression of miR-696 in skeletal muscle was affected markedly by physical activity in mice. Increased expression of miR-696 in skeletal myocytes led to negative regulation of PGC-1α protein along with reduced expression of mRNAs for its downstream genes. Mitochondrial biogenesis was also inhibited along with a reduction of fatty acid oxidation in miR-696-overexpressing myocytes compared with normal control myocytes. These observations demonstrate that miR-696 is a physical activity-dependent miRNA that is involved in the translational regulation of PGC-1α and the modulation of skeletal muscle metabolism.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the author(s).
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