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Mitochondrial oxidative phosphorylation complexes exist in the sarcolemma of skeletal muscle

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Although proteomic analyses have revealed the presence of mitochondrial oxidative phosphorylation (OXPHOS) proteins in the plasma membrane, there have been no in-depth evaluations of the presence or function of OXPHOS I-V in the plasma membrane. Here, we demonstrate the *in situ* localization of OXPHOS I-V complexes to the sarcolemma of skeletal muscle by immunofluorescence and immunohistochemistry. A portion of the OXPHOS I-V complex proteins was not co-stained with MitoTracker but co-localized with caveolin-3 in the sarcolemma of mouse gastrocnemius. Mitochondrial matrix-facing OXPHOS complex subunits were ectopically expressed in the sarcolemma of the non-permeabilized muscle fibers and C2C12 myotubes. The sarcolemmal localization of cytochrome *c* was also observed from mouse gastrocnemius muscles and C2C12 myotubes, as determined by confocal and total internal resonance fluorescence (TIRF) microscopy. Based on these data, we conclude that a portion of OXPHOS complexes is localized in the sarcolemma of skeletal muscle and may have non-canonical functions. [BMB Reports 2016; 49(2): 116-121]

INTRODUCTION

Mitochondrial oxidative phosphorylation (OXPHOS) is a major source of ATP production in eukaryotic cells. OXPHOS takes place in the inner mitochondrial membrane *via* five OXPHOS complexes including NADH dehydrogenase (OXPHOS I), succinate dehydrogenase (OXPHOS II), ubiquinone cytochrome *c* oxidoreductase (OXPHOS III), cytochrome *c*, cytochrome oxidase (OXPHOS IV) and ATP synthase (OXPHOS V). The energy obtained by electron transport from NADH or succinate to oxygen *via* the OXPHOS I-III-IV or II-III-IV supercomplex is utilized to pump protons across the inner mitochondrial membrane, creating a proton gradient. The proton gradient is ultimately used by ATP synthase (OXPHOS V) to generate ATP from ADP and P_i. In addition to ATP production, the mitochondria also contribute to cellular stress responses such as autophagy and apoptosis (1). For example, cytochrome *c*, a key participant in ATP synthesis, is translocated from the mitochondria to the cytosol, to trigger programmed cell death after cellular exposure to different apoptotic stimuli (2).

Lipid rafts are plasma membrane subdomains that consist of cholesterol and glycosphingolipids (3). Due to the long and saturated fatty acids of glycosphingolipids and the stiff four-ring structure of cholesterol, lipid raft subdomains have more rigid property than other phospholipid compartments with short and unsaturated fatty acids in the plasma membrane (4). Thus, the lipid rafts are easily isolated from other cellular organelles based on their detergent insolubility (3, 5). The detergent-resistant lipid rafts contain many different receptors and their downstream signaling molecules, making the lipid rafts serve as the center for signal transduction activity (6-9).

Surprisingly, various proteomic analyses reveal that the lipid rafts also concentrate mitochondrial OXPHOS proteins in different cell lines such as HepG2 hepatocytes (10), C2C12 myotubes (11), human adipocytes (6), THP-1 monocytes (12, 13), RNK (natural killer) cells (14) and human umbilical vein endothelial cells (HUVEC) (15, 16). The observation of mitochondrial OXPHOS proteins in the plasma membrane lipid rafts has been biochemically demonstrated by different experimental approaches such as proteomics, subcellular fractionation and surface biotin-labeling in different cell lines and tissues (10, 11, 17). However, the finding of mitochondrial proteins in the lipid rafts has also raised the possibility of mitochondrial contamination during lipid raft isolation or the possible existence of mitochondrial lipid rafts (6).

To resolve the controversy, the *in situ* localization of mitochondrial OXPHOS proteins in the plasma membrane must be investigated. In this study, we show that OXPHOS I-V complexes are localized in the sarcolemmal cell membrane of skeletal muscle tissue and C2C12 myotubes. Cytochrome *c* is also

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localized in the sarcolemma based on total internal resonance fluorescence (TIRF) microscopy. The sarcolemmal localization of OXPHOS complexes may provide novel insights into the non-canonical functions of OXPHOS in the sarcolemma of skeletal muscle.

RESULTS

A portion of OXPHOS complexes is not localized in the mitochondria

The existence of mitochondrial proteins in the plasma membrane has been demonstrated by different experimental approaches such as proteomics, lipid raft and plasma membrane isolation and surface biotin-labeling in different cell lines and tissues. However, there is no report describing *in situ* localization of the mitochondrial OXPHOS complexes in the plasma membrane. To visualize the localization of the OXPHOS complexes in the sarcolemmal cell membrane, we used different anti-OXPHOS antibodies against the NDUFB6 subunit of OXPHOS I, SDHA subunit of OXPHOS II, core I subunit of OXPHOS III, COX4I1 subunit of OXPHOS IV and ATP synthases α and β for immunofluorescence. Each of these antibodies detected a single band corresponding to each OXPHOS subunit in the immunoblotting of C2C12 myoblasts and myotubes (Fig. S1), suggesting that the immunofluorescent signals for OXPHOS subunits may be specific.

We carried out immunofluorescence in mouse gastrocnemius. In cross-sections and longitudinal sections (Fig. 1 and Fig. S2), a portion of immunofluorescent signals of OXPHOS subunits in the sarcolemma was not stained with MitoTracker. This indicates that OXPHOS complexes in the sarcolemma were not conjugated to the mitochondria. It should be noted that there was no immunofluorescent signal in gastrocnemius tissues treated with mock IgG and secondary antibody (Fig. 1), showing that the immunofluorescent signals were specific for the indicated OXPHOS subunit.

A portion of OXPHOS complexes is co-localized with caveolin-3 in the sarcolemma.

Next, we investigated the co-localization of OXPHOS subunits with caveolin-3 in mouse gastrocnemius muscle tissue by immunofluorescence. It should be noted that caveolin-3 is specifically expressed in the sarcolemmal caveolae, which are plasma membrane invaginations formed from lipid rafts (18, 19). As shown in Fig. 2, OXPHOS subunits were co-localized with caveolin-3 in the sarcolemma, indicating that OXPHOS complexes are expressed in the sarcolemma. These fluorescent signals corresponding to the OXPHOS subunits and caveolin-3 were specific because there were no background signals in samples incubated with mock IgG.

A portion of OXPHOS complexes is exposed to the cell surface

ATP synthase is ectopically expressed on the cell surface and a portion of each OXPHOS complex is localized in the sarcolemma of skeletal muscle as shown in Figs. 1 and 2. Therefore, it is tempting to speculate that the mitochondrial matrix-facing OXPHOS subunits may also be exposed to the cell surface if the OXPHOS complexes are localized in the sarcolemma as proposed in Fig. S3. In order to address this issue, we de-

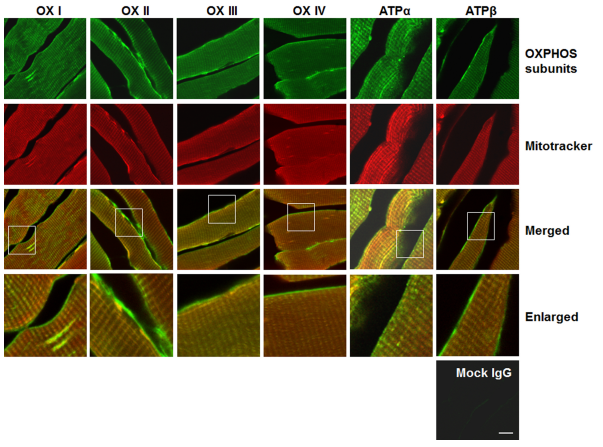

Fig. 1. A portion of OXPHOS complexes is expressed in the sarcolemma. Gastrocnemii were isolated from 8-week-old male mice. The longitudinally sectioned muscles were co-stained with MitoTracker and an anti-NDUFB6 (OX I), SDHA (OX II), core I subunit of OXPHOS III (OX III), COX4I1 (OX IV) or ATP synthase α or β (ATPα or β) antibody. The boxed areas are enlarged in merged images. Scale bar = 10 μm.

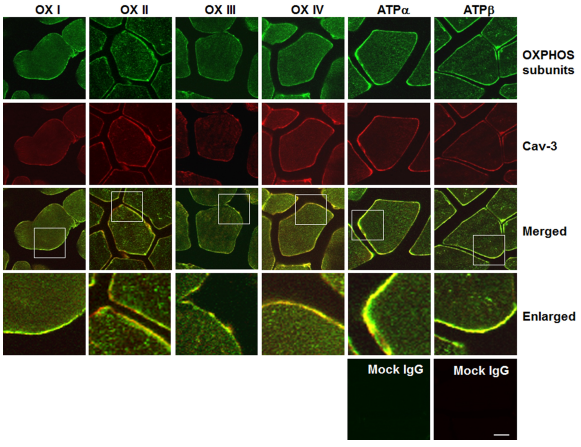

Fig. 2. A portion of OXPHOS complexes is co-localized with caveolin-3 in the sarcolemma. Gastrocnemii were isolated from 8-week-old male mice. The co-localization of caveolin-3 with each OXPHOS subunit [NDUFB6 (OX I), SDHA (OX II), core I subunit of OXPHOS III (OX III), COX4I1 (OX IV) or ATP synthase α or β (ATPα or β)] was determined from the cross-sectioned muscle. The boxed areas are enlarged in merged images. Scale bar = 10 μm.

termined the surface expression of matrix-facing OXPHOS subunits in non-permeabilized C2C12 myotubes by immunofluorescence. For these experiments, we selected antibodies against NDUFV2 (a subunit of OXPHOS I), SDHA (a subunit of OXPHOS II), UQCR2 (a subunit of OXPHOS III), COX_IV (a subunit of OXPHOS IV), and ATP_β (a subunit of OXPHOS V) because all of these antigens are exposed to the mitochondrial matrix. As shown in Fig. 3A, immunofluorescent signals for OXPHOS subunits were found along with the sarcolemma in non-permeabilized multinuclear myotubes. These data indicate that the matrix-facing OXPHOS subunits are also expressed on the cell surface. It should be noted that there was no fluorescent signal for cytochrome *c* or mock IgG (Fig. 3A). The lack of a surface signal for cytochrome *c* suggests that cytochrome *c* faces toward the cytosol if it localizes in the sarcolemma.

To further confirm the surface expression of each OXPHOS complex, we carried out immunofluorescence using intact muscle fibers obtained from mouse extensor digitorum longus (EDL). After collagenase treatment, the isolated muscle fibers were immediately stained with anti-OXPHOS antibodies without a permeabilization step. Fig. 3B shows strong surface expression of the OXPHOS subunits in the plasma membrane of EDL fiber. Because there was no immunofluorescent signal for cytochrome *c* and mock IgG, the surface signals for each OXPHOS are specific.

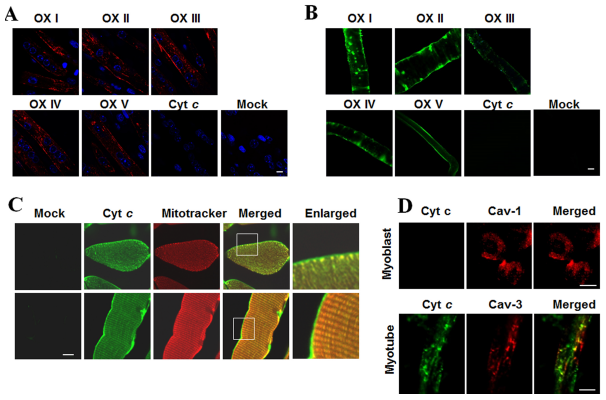

Fig. 3. OXPHOS complexes are ectopically expressed in the sarcolemma. (A) Immunofluorescence for OXPHOS proteins [NDUFV2 (OX I), SDHA (OX II), UQCR2 (OX III), COXIV (OX IV), ATP β (OX V) and Cytochrome c (Cyt c)] was carried out using 4-day-differentiated C2C12 myotubes without a permeabilization step. The nuclei were counterstained with DAPI. Scale bar = 10 μm. (B) Muscle fibers were isolated from extensor digitorum longus (EDL) muscles of 8-week-old mice. Immunofluorescence for OXPHOS subunits was carried out without a permeabilization step. Scale bar = 10 μm. (C) Cross-sectioned and longitudinally sectioned mouse gastrocnemius muscles were analyzed by immunofluorescence with an anti-cytochrome *c* antibody and MitoTracker. Scale bar = 10 μm. (D) Cytochrome *c* and caveolin-3 are co-localized in the sarcolemma of myotubes. Immunofluorescence of cytochrome *c* and caveolin proteins (caveolin-1 for myoblasts and caveolin-3 for myotubes) was observed in C2C12 myoblasts and 3-day-differentiated myotubes using TIRF microscopy. Scale bar = 10 μm.

Cytochrome c is localized in the sarcolemma as well as in the mitochondria

Because all of the OXPHOS complexes were localized in the sarcolemma of skeletal muscle, as shown in Figs. 1-3, cytochrome *c* may also be localized with other OXPHOS complexes in the sarcolemma of skeletal muscle. Therefore, we monitored the cellular localization of cytochrome *c* in mouse gastrocnemius skeletal muscles by immunohistochemistry with an anti-cytochrome *c* antibody. As shown in Fig. S4, cytochrome *c* was highly expressed in the sarcolemma and the mitochondria isolated from the cross-sectioned and longitudinally sectioned mouse gastrocnemii. We further confirmed the sarcolemmal localization of cytochrome *c* by immunofluorescence in mouse gastrocnemius. As shown in Fig. 3C, cytochrome *c* was found in the sarcolemma that was not co-stained with MitoTracker and in the sub-sarcolemmal mitochondria that were co-stained with MitoTracker. It should be noted that incubation with mock IgG did not result in any positive fluorescent signals, indicating that the immunofluorescent signals for cytochrome *c* are specific.

To further confirm the sarcolemmal localization of cytochrome *c*, the fluorescent signals for cytochrome *c* and caveolin-1 or -3 (sarcolemmal marker protein of myoblasts or myotubes) were observed in C2C12 myoblasts and myotubes under total internal reflection fluorescence (TIRF) microscopy which generates a high spatial resolution of fluorescent probes that are localized in the plasma membrane or just beneath the plasma membrane. As shown in Fig. 3D, the co-localization of cytochrome *c* and caveolin-3 was observed in C2C12 myotubes by TIRF microscopy, but cytochrome *c* was not co-localized with caveolin-1 in myoblasts. Thus, all these data indicate that cytochrome *c* is localized with all of the OXPHOS complexes in the sarcolemma of skeletal muscles.

Sarcolemmal OXPHOS is not involved in extracellular NADH-dependent respiration

Based on the immunofluorescence experiments examining the OXPHOS complexes in non-permeabilized C2C12 myotubes and EDL muscle fibers, we propose the function of sarcolemmal OXPHOS complexes as shown in Fig. 4A. According to this model, electrons may transfer from extracellular NADH or succinate to oxygen via sarcolemmal OXPHOS I-III-IV or II-III-IV, generating a proton gradient across the sarcolemma. Finally, the proton gradient may be utilized to generate extracellular ATP from extracellular ADP and P_i via sarcolemmal OXPHOS V. Based on this topology of sarcolemmal OXPHOS complexes, extracellular NADH may induce oxygen consumption in the sarcolemma. In order to address this hypothesis, we measured the oxygen consumption rate (OCR) of C2C12 myotubes in the presence of NADH. As shown in Fig. 4B, the OCR did not change after cellular exposure to NADH, suggesting that sarcolemmal OXPHOS complexes are not involved in extracellular NADH-dependent respiration.

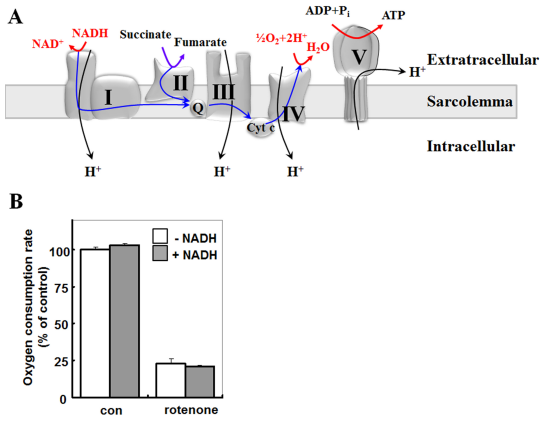

Fig. 4. Sarcolemmal OXPHOS complexes do not have the ability to consume oxygen. (A) The proposed function of the sarcolemmal OXPHOS complexes. In this model, electrons are transferred from extracellular NADH to oxygen via the sarcolemmal OXPHOS complexes, generating a proton gradient. The sarcolemmal ATP synthase generates extracellular ATP from extracellular ADP and P_i using the proton gradient. The proton gradient is utilized by OXPHOS V to generate extracellular ATP from extracellular ADP and P_i. Blue lines indicate the flow of electrons and red lines indicate the flow of protons. (B) The oxygen consumption rate (OCR) was measured in 4-day-differentiated C2C12 myotubes treated with different combinations of NADH (4 μM) and rotenone (10 μM). To inhibit mitochondrial respiration, myotubes were treated with rotenone, which is an inhibitor of OXPHOS I complex.

DISCUSSION

The plasma membrane has two separate phases, the liquid-ordered phase (L_o) and the liquid-disordered phase (L_d). The L_o phase, also known as lipid rafts, shows tight packing of phospholipids with longer and saturated acyl chains because of rigid cholesterol, whereas the L_d phase contains loose packing of phospholipids with shorter and unsaturated acyl chains (6). Thus, lipid rafts of the L_o phase cannot be solubilized by non-ionic detergents such as Triton X-100, Brij or NP40, and can be easily isolated from the L_d phase, based on their detergent insolubility and low density in sucrose gradient ultracentrifugation. The detergent-resistant lipid rafts contain many mitochondrial and microsomal proteins as well as plasma membrane proteins based on proteomic analyses of various mammalian cells and tissues (5, 6, 10-15, 17, 20, 21). These results spark a controversy that rafts contain many contaminants originating from mitochondria and microsomes because of detergent usage (6).

However, many mitochondrial proteins, such as ATP synthase, voltage-dependent anion channels (VDAC) and prohibitin, have been unambiguously demonstrated to be localized in the plasma membrane by using various experimental approaches such as cellular fractionation, immunofluorescence, and cell surface biotinylation (10, 11, 22-26), indicating that mitochondrial proteins isolated from the detergent-resistant lipid rafts are not simple contaminants. Thus, we investigated the possibility that OXPHOS complexes are localized in the sarcolemma of skeletal muscle because proteomic analysis has revealed that the detergent-resistant lipid rafts isolated from C2C12 myotubes have many mitochondrial proteins, particularly, many subunits of the OXPHOS complexes (11).

All of the OXPHOS complexes and cytochrome c were undoubtedly localized in the sarcolemma of skeletal muscles as determined by immunofluorescence (Figs. 1-3). The immunofluorescent analysis of skeletal muscle revealed that OXPHOS proteins are highly concentrated in the sarcolemma and the mitochondria. Because many mitochondria are also localized in sub-sarcolemmal regions based on electron microscopy (27), it is difficult to imagine that these OXPHOS complexes are localized in the subdomains of the sarcolemma that do not contain mitochondria. However, we observed that some immunofluorescent signals of OXPHOS subunits in the sarcolemma did not overlap with MitoTracker signals that have been known to specifically accumulate in the mitochondrial matrix (28), indicating that OXPHOS complexes are indeed localized in the sarcolemma of skeletal muscle.

By performing immunofluorescence experiments for OXPHOS complexes in non-permeabilized muscle fibers (Fig. 3A, B), we determined the topology of sarcolemmal OXPHOS complexes (Fig. 4A). According to this topology, mitochondrial matrix-facing OXPHOS subunits are exposed to the extracellular space, while mitochondrial intermembrane space-facing OXPHOS subunits are exposed to the intracellular space. Thus, a proton gradient may be generated across the sarcolemma by transfer of electrons from extracellular NADH to oxygen *via* the sarcolemmal OXPHOS I, III and IV complexes. Finally, the sarcolemmal OXPHOS V may utilize the electron gradient for extracellular ATP production. Indeed, purified sarcolemma show NADH-dependent respiration activity after addition of cytochrome c (11). Calzia *et al*. observed the OXPHOS complexes from the rod outer segment (OS), which is a continuum of the plasma membrane in the rod cells of the vertebrate retina and reported the observation of respiratory activity from the purified OS after addition of pyruvate and malate (29). However, our results show that intact C2C12 myotubes did not have NADH-dependent sarcolemmal respiration activity (Fig. 4B). In addition, the sarcolemmal ATP synthase is not involved in generating extracellular ATP because extracellular ATP generation is not disrupted in C2C12 myotubes even after the knockdown of ATP synthase β (30).

These data indicate that the sarcolemmal OXPHOS complexes are not involved in oxidative phosphorylation. Just as ectopic ATP synthase has the ability to bind to many different ligands (apolipoprotein A-I, angiostatin, β amyloid oligomers, etc.) (22, 23, 31, 32) and to transfer HIV-1 from antigen-presenting cells to $CD4^+$ cells (33), the sarcolemmal OXPHOS complexes may have as yet unknown non-canonical functions. Thus, a direction for future research on the sarcolemmal OXPHOS proteins is to identify their novel non-canonical functions.

MATERIALS AND METHODS

Animals and cell line

C57/BL6 male mice were housed in plastic cages on a 12:12 h light-dark photoperiod with free access to water and food. Animals were handled according to the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1985). The mouse myoblast cell line C2C12 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, US) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% penicillin/streptomycin (WelGene, Daegu, Korea) and 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37°C according to the previously described method (34). In brief, C2C12 myoblasts at approximately 90-100% confluence were differentiated into myotubes by using the growth medium with differentiation medium (DMEM supplemented with 2% penicillin/streptomycin and 2% horse serum). Every 48 h, the differentiation medium of the myotubes was replaced with fresh medium.

Antibodies and reagents

Different anti-OXPHOS antibodies were obtained from Molecular Probes (Carlsbad, California, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cytochrome *c*, anti-caveolin-1 and anti-caveolin-3 antibodies were obtained from BD Biosciences (Franklin Lakes, New Jersey, USA). MitoTracker Red FM dye and Alexa Fluor 488-conjugated anti-mouse IgG were purchased from Invitrogen (Carlsbad, California, USA). FITC-conjugated anti-mouse IgG and Rhodamine-conjugated anti-rabbit IgG were obtained from Abcam (Cambridge, UK). Collagenase type I and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, Missouri, USA).

Immunoblotting, immunofluorescence and immunohistochemistry

The proteins in the whole cell lysates were separated on SDS-polyacrylamide gels and transferred to a PVDF membrane. The antigens were visualized by sequential treatment with specific primary antibodies, HRP-conjugated secondary antibodies, and an enhanced chemiluminescence substrate kit.

Intact muscle fibers of mouse extensor digitorum longus (EDL) muscle were isolated with 0.2% collagenase type I in DMEM and analyzed by immunofluorescence according to the previously described method (35). C2C12 myotubes were fixed with 3.7% paraformaldehyde for 10 min, left non-permeabilized and blocked for 1 h with blocking buffer (5% BSA in PBS). Subsequent incubations with primary antibodies and fluorescent-conjugated secondary antibodies, and DAPI staining were conducted at room temperature. Immunofluorescence images were captured by a laser scanning confocal microscope (ZEISS LSM 510 META, Oberkochen, Germany) or a Laser TIRF-2 system (Olympus, Tokyo, Japan).

Isolated mouse gastrocnemii were fixed with 4% paraformaldehyde, snap-frozen in chilled 2-methylbutane and sectioned with a cryomicrotome. The muscle sections were subsequently incubated with blocking buffer, primary antibodies and fluorescent-conjugated secondary antibodies. Immunofluorescence images were captured by confocal microscopy.

For immunohistochemistry, mouse gastrocnemii were washed with saline and embedded in an optimal cutting temperature compound to generate frozen cross sections and longitudinal sections. Standard 6-μm sections were stained using the Labeled Streptavidin Biotin kit (Dako, Produktionsvej, Glostrup, Denmark) according to the manufacturer's instructions.

Measurement of oxygen consumption

The oxygen consumption rate (OCR) was determined in C2C12 myoblasts and 4-day-differentiated myotubes using a Seahorse XF24 Extracellular Flux analyzer (Seahorse Bioscience, North Billerica, MA, USA). For OCR measurement, culture media were exchanged with Dulbecco's-modified PBS (Hyclone) 1 h before the assay. After three baseline measurements of OCR were taken, NADH (4 μM) and rotenone (10 μM) were separately injected into each experimental group in different combinations.

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